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

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Abstract

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

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

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

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

M. fructigena, instead, is more prevalent in pome fruits and it has a low incidence in stone fruits (Martini and Mari 2014). *M. polystroma,* morphologically similar to *M. fructigena*, was initially identified in Japan (Côté et al. 2004) and has been introduced in Europe with reports in apples in Hungary (Petróczy and Palkovics 2009) and Croatia (Di Francesco et al. 2015), in apricots in 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 *Monilina* 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

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

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

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

Materials and Methods

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

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

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

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

LAMP primer design. Six LAMP primers including two external primers, F3 and B3; two internal primers, FIP (F1c+ F2) and BIP (B1c+B2); and two loop primers, Floop and Bloop, were designed from *M. fructicola* and *M. laxa* sequences according to the method described by Notomi et al. (2000). The LAMP primers for *M. fructicola* were designed on an intron in the cytochrome b, associated with the Qol fungicides resistance, present in *M. fructicola* (GenBank accession number GQ304941.1), but absent in other *Monilinia* species (Luo et al. 2010). The primers for *M. laxa* were designed on a genomic sequence identified as a SCAR marker by Gell et al. (2007) (GenBank accession number: EF207417.1). All the primers were manually designed taking into account the annealing temperature. Possible hairpin and secondary structures were checked using the OligoCalc program (http://biotools.nubic.northwestern.edu/OligoCalc.html), while possible interactions between primers was controlled using the Multiple Primer Analyzer (Thermo Scientific) (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

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

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

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

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

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

Real-Time PCR. The primers designed by Hughes et al. (2000), Mcf-F1 and Mfc-R1 specific for *M. fructicola* and MI-Mfg-F2 and MI-Mfc-R1 specific for *M. laxa*, were used to quantify the number of cells present in the samples. The 25 µl reactions were carried out using 1x Power SYBR Green PCR Master Mix (Applied Biosystems), 120 nM of each primer and 1 µl of the peach genomic DNA extracted using the commercial kit. The amplification was carried out using the following 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 Plus Real-Time PCR system (Applied Biosystems). A standard curve was performed using *M. fructicola* MSR2 and *M. laxa* 1506 ranging from 9.4 ng/µl to 9.4 fg/µl. A positive control with DNA extracted from cultured pathogen and a negative control of water were included in each run. The quantity of DNA present in the sample was calculated according to the standard curve measures, while the number of cells was estimated using the *Monilinia fructicola* genome weight (0.000048 ng;

https://www.ncbi.nlm.nih.gov/Traces/wgs/?val=NGKE01#contigs) using the formula: number of cells /µl = DNA quantity/0.000048 (Amaral Carneiro et al. 2017). The amount of fruit used (ranging from 1 g to 2 g) and the elution volume of the extracted DNA were used to calculate the total number of cells.

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

Results

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

One of the biggest drawbacks of the PCR-based methods is the inhibition of the reaction by some components of plant tissue which results in false negative results (Wilson 1997), therefore most PCR-based methods involve long and complex DNA extraction methods. As an alternative, the LAMP assays for the detection of *M. fructicola* and *M. laxa* were validated using a crude extraction method, which is simpler, and showed to be reliable and effective in the detection of both pathogen species.

The validation of the assays was carried out according to the international standard published by EPPO (PM7/98). A significant number of isolates from the monitoring were used to validate the specificity of both LAMP assays. To verify the parameters of both LAMP assays *in vivo*, we focused on fully ripe nectarines, which were stored at two temperatures reflecting the storage and shelf life conditions of stone fruit.

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

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

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

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592

593 ## Tables

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

595

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

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

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

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

FIGURE LEGENDS

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).

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

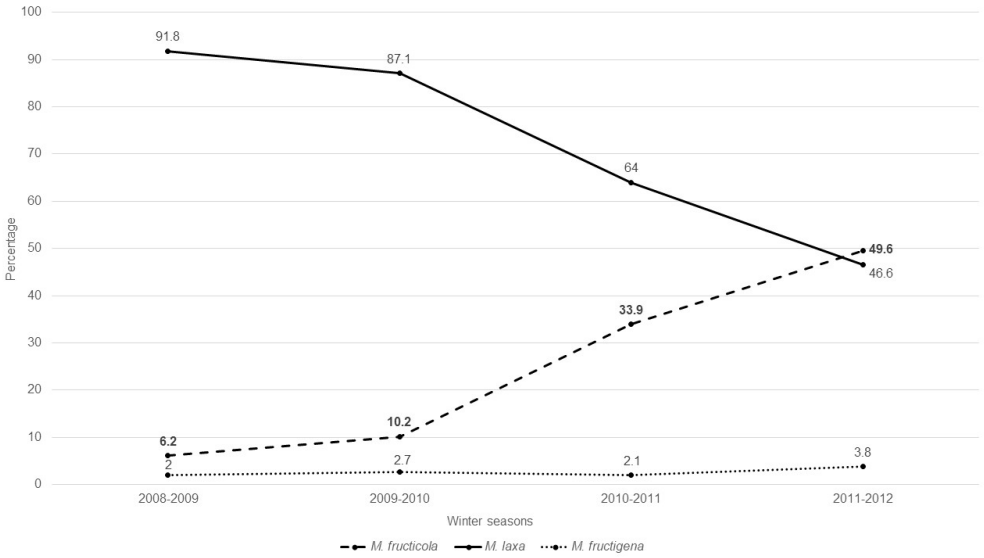

Figure 1. Incidence of Monilinia spp. (%) during the monitoring of four winter seasons (2008-2009 to 2011-2012). Monilinia laxa (solid line), Monilinia fructicola (dashed line), Monilinia fructigena (dotted line).

338x190mm (96 x 96 DPI)

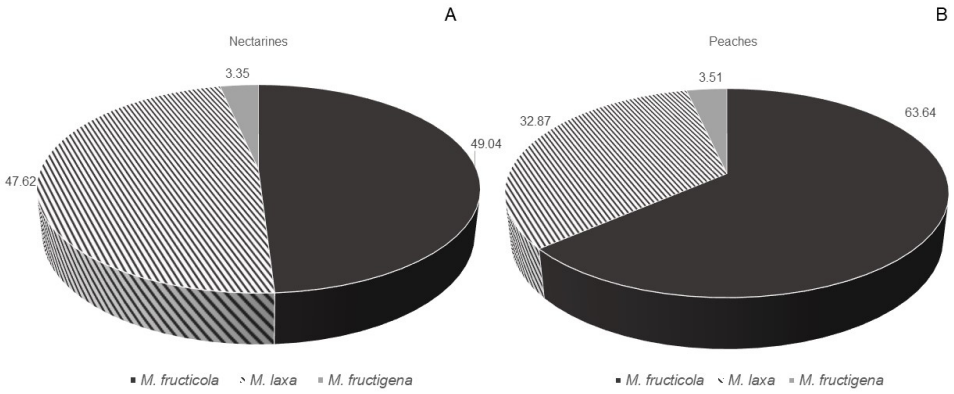

Figure 2. Percentage of Monilinia spp. isolated from A) nectarine and B) peaches during the winter season 2011-2012.

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 *M. fructicola* during			Percentage of different species of *Monilinia* during 2011-2012		
				2008-2009	2009-2010	2010-2011	*M. fructicola (%)*	*M. laxa (%)*	*M. fructigena (%)*
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.

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