Fruit maturity and post-harvest environmental conditions influence the pre-penetration stages of *Monilinia* infections in peaches

C. Garcia-Benitez, P. Melgarejo, A. De Cal

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A B S T R A C T

Brown rot caused by the fungi *Monilinia laxa* (Aderhold and Ruhland) Honey, *M. fructicola* (Winter) Honey, or *M. fructigena* (Aderhold and Ruhland) is a serious fungal disease of peaches. The fungal infection process begins when fungal conidia germinate on the fruit surface to produce germ tubes and/or appressoria, and the incidence of brown rot increases as fruit approaches maturity. The interaction between the fungal infection process, peach maturity, and the environmental conditions is not well understood. Accordingly, the objectives of this investigation were to investigate germ tube and appressorial formation by *M. laxa* and *M. fructicola* when they were exposed to peach skin from mature and immature fruit at various temperatures and relative humidities (RHs). The greatest number of germ tubes was found when *M. laxa* or *M. fructicola* was incubated in culture medium which contained a skin extract of mature peaches. In contrast, the greatest number of appressoria was found when *M. laxa* or *M. fructicola* was incubated in culture medium which contained a skin extract of immature peaches. Although *M. fructicola* produced the same number of germ tubes and appressoria at 4 °C, *M. fructicola* produced more germ tubes than appressoria at temperatures higher than 10 °C. *M. laxa* produced more germ tubes than appressoria at any temperature, except when it was incubated for 48 h on culture medium which contained a skin extract of immature peaches at 10 °C at 80% or 100% RH, or at 25 °C at 60% RH. *M. laxa* conidia germinated better than *M. fructicola* conidia at low temperatures. Germ tube and appressorial formation by *Monilinia* spp. were influenced by fruit postharvest handling. The number of germ tubes that were formed by *M. laxa* conidia was significantly greater than that for *M. fructicola* when the conidia were incubated at 100% RH, and this number increased after 3 days of refrigeration. The number of appressoria that were formed by both *Monilinia* spp. also increased after 3 consecutive days of refrigeration. Negligible or no germination of *M. fructicola* and *M. laxa* conidia occurred when the RH was 60%. We concluded that the dissimilar abilities of *M. laxa* and *M. fructicola* to germinate and form appressoria at low temperatures conferred a competitive advantage to *M. laxa* to survive during fruit postharvest refrigeration and cold storage at 4 °C.

1. Introduction

Brown rot caused by *Monilinia laxa* (Aderhold and Ruhland) Honey, *M. fructicola* (Winter) Honey, or *M. fructigena* (Aderhold and Ruhland) is a serious fungal disease of peaches (Hong et al., 1997; Mari et al., 2008; Ogawa et al., 1995). Postharvest losses due to brown rot are typically greater than preharvest losses, and routinely occur during handling, storage, and transport (Hong et al., 1997). Since its detection in 2006 in Spain, *M. fructicola* has supplanted *M. fructigena* as the main cause of brown rot and now exists at the same frequency of occurrence as *M. laxa* (Villarino et al., 2013).

The fungal infection process can be divided into three phases: pre-penetration or adhesion, penetration, and colonization. The infection process begins when *Monilinia* conidia germinate on the fruit surface to produce germ tubes and/or appressoria, which then penetrate the fruit surface depending on the prevailing environmental conditions (Lee and Bostock, 2006). Most studies on appressorial formation by *M. fructicola* have focused on the surface properties of the fruit, the stage of fruit maturity, and the signaling pathways that are required for surface attachment and appressorial formation (Cruickshank and Wade, 1992; Lee and Bostock, 2006). The incidence of brown rot increases as fruit approaches maturity (Gell et al., 2008; Lee and Bostock, 2007; Villarino et al., 2011): immature fruit are more resistant to infection than mature fruit (Gell et al., 2008; Lee and Bostock, 2006, 2007; Xu et al., 2007).

The chemical content of fruit and its epidermis can also influence the first step of the infection process. For example, the content of volatile and phenolic compounds and organic acids in fruit and/or the fruit epidermis changes substantially as fruit approaches maturity. The matrix glycans in the walls of peach mesocarp cells are gradually depolymerized and the polymers are deglycosylated during

maturation, and the chelator-soluble polyuronides are solubilized and depolymerized after the initiation of ripening (Brummell et al., 2004). Villarino et al. (2011) reported that the susceptibility of peaches to *M. laxa* infection was greatest when the pericarp was completely formed, and the concentrations of chlorogenic and neochlorogenic acid in the pericarp are low. Melanin production by *M. laxa* is inhibited when the concentrations of chlorogenic and neochlorogenic acid in the pericarp are high and melanin is essential for penetration of the pericarp by *Monilinia* spp. (Villarino et al., 2011). The presence of phenolic acids, such as chlorogenic and caffeic acids can inhibit the cutinase and polygalacturonase activity of *M. fructicola* in immature nectarines and peaches. Furthermore, this inhibition is related to changes in the electrochemical redox potentials of *M. fructicola* cultures and alters the intracellular levels of the cellular antioxidant, glutathione (Bostock et al., 1999; Lee and Bostock, 2006). The malic/citric acid ratio in the mesocarp has two maxima during peach maturation. The first maximum occurs in very immature fruit and the second maximum occurs in mature fruit when sucrose levels are at their highest and quinic acid levels are at their lowest (Chapman et al., 1991).

Conidial germination, growth, and sporulation of *Monilinia* spp. are also dependent on ambient temperature, relative humidity (RH), and water availability (water activity: a_w) (Luo et al., 2001; Tamm and Flückiger, 1993; Xu et al., 2001). Casals et al. (2010) reported that more than 80% of viable conidia of *M. fructicola* and *M. fructigena* can germinate within 2 h and those of *M. laxa* can germinate within 4 h at 25 °C and 0.99 a_w on culture media. They also reported that (a) no germination of *M. laxa*, *M. fructicola*, and *M. fructigena* conidia occurred at 38 °C, (b) the conidia of *M. laxa*, *M. fructicola*, and *M. fructigena* can germinate at 0 °C, and (c) the conidia of the three *Monilinia* spp. do not germinate at 0.87 a_w. It has also been reported that (a) the mycelial growth of *M. laxa* is better than that of *M. fructicola* isolates at low temperatures on blossoms and fruit (Papavasileiou et al., 2015; Tamm et al., 1995), (b) *M. laxa* conidia can germinate at temperatures as low as −4 °C on fruit (Tian and Bertolini, 1999), and (c) mycelial growth of *M. laxa* can still occur at 0 °C (Tamm and Flückiger, 1993).

The interaction of fruit maturity and environmental conditions on germ tube and appressorial formation in a *Monilinia* infection is not well understood. Accordingly, the objectives in this investigation were to (i) investigate the effect of mature and immature peach skin extract components, T, and RH on germ tube and appressorial formation by *M. laxa* and *M. fructicola*, and (ii) study the effects of postharvest environmental conditions on germ tube and appressorial formation by *M. laxa* and *M. fructicola*.

2. Materials and methods

2.1. Fungal culture and conidial production

Six isolates from the culture collection of Plant Protection Department of Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain were used in the investigation (Table 1). All isolates were originally collected from several commercial peach and nectarine orchards in the Ebro Valley, Lleida, Spain. All isolates were stored as conidial suspensions in 20% glycerol at −80 °C for long-term storage or as cultures on potato dextrose agar (PDA) (Difco Laboratories, Detroit, MI, U.S.A.) at 4 °C for short-term storage. For conidial production, (a) the three *M. fructicola* isolates were grown on PDA plates at 20 to 25 °C for 7 days in the dark, and (b) the three *M. laxa* isolates were first grown on PDA plates at 20 to 25 °C for 10 days and then for 5 days at 4 °C in the dark.

Table 1
The host, isolation tissue and year of isolation of the six *Monilinia laxa* and *M. fructicola* isolates that were used to study the effects of fruit maturity and post-harvest environmental conditions on the pre-penetration stages of *Monilinia* infection in peaches.

Isolate name	Collection name	*Monilinia* species	Host	Isolation tissue	Year of isolation
Ml1	FLOR2009SUD5.1	*M. laxa*	Nectarine	Flower	2009
Ml2	2014COSFANTASIA8	*M. laxa*	Nectarine	Fruit	2014
Ml3	2014COSFANTASIA9	*M. laxa*	Nectarine	Fruit	2014
Mf1	ALF2009COS5R7	*M. fructicola*	Peach	Fruit	2009
Mf2	ALF2009MOA4	*M. fructicola*	Peach	Mummified fruit on trees	2009
Mf3	SUD2009COS2R6	*M. fructicola*	Nectarine	Fruit	2009

Conidial suspensions of all isolates were prepared using conidia that were harvested from the PDA plates and then transferring the harvested conidia into Czapek-Dox broth (Difco Laboratories, Detroit, MI, U.S.A.) or sterile distilled water (SDW). After a 30 s sonication of the conidia in an ultrasonic bath (J.P. Selecta s.a., Barcelona, Spain), the conidial concentration of the suspensions was first determined using a hemocytometer and a light microscope, and then adjusted to a final concentration of 10^4 conidia/mL.

2.2. Bioassays

Germ tube and appressorial formation were measured using previously described protocols (De Cal et al., 1988) with some modifications. Briefly, three sterile glass slides and three moistened filter papers were placed inside 150 mm diameter sterile Petri dishes. A 15 μL-aliquot of an *M. fructicola* or *M. laxa* conidial suspension was mixed with a 30 μL-aliquot of culture medium on each glass slide. Germinated *Monilinia* conidia can form either germ tubes or appressoria. A germ tube was considered to be formed when a slender hypha with pointed ends, which was longer than the minor diameter of the conidium, was formed. An appressorium was considered to be formed when swollen structures, that were mostly oval-shaped, were formed instead of slender hyphae with pointed ends (Cruickshank and Wade, 1992; Lee and Bostock, 2006). For the bioassays which are described below, three replicates of each condition were done on 50 randomly selected conidia in each replicate and each bioassay was repeated at least twice with each studied isolate.

To determine the effects of temperature, RH, and fruit maturity on germ tube and appressorial formation, conidial suspensions in Czapeck-Dox broth were incubated with a skin extract from immature or mature peaches at 4, 10, 25, and 35 °C for 24, 48 and 72 h in the dark at an RH of 60%, 80%, and 100%. For preparing the peach skin extracts, the skins of fungicide-free 'Rojo Albesa' peaches were collected using a previously described protocol (Tomas-Barberan et al., 2001). The skins were first manually peeled with a razor blade, and then ground into powder in liquid nitrogen with a sterilized pestle and mortar. The resultant powders were transferred to a 50 mL Falcon tube, weighed, and mixed with ethanol, MilliQ water, and beach sand (3.6 g of powder was mixed with 5.76 mL 100% ethanol, 1.44 mL MilliQ water, and 2 g of beach sand). The mixture was then homogenized twice at a speed of 6.0 m/s for 60 s using a high-speed benchtop tissue homogenizer (FastPrep®-24 Instrument, MP Biomedicals, Solon, Ohio, U.S.A.). The homogenates were then transferred to sterile tubes which were centrifuged at 10,000 rpm for 20 min at 4 °C in a Sorvall RC-5C Plus centrifuge (Kendro Laboratory Products, Newton, Connecticut, U.S.A.). When the centrifugation was complete, the supernatant was carefully collected, transferred to a new sterile 50 mL Falcon tube, and stored at −20 °C until required. Immature peaches were collected at BBCH = 79, 76 days before harvest (4 July) and mature peaches at BBCH = 89 harvest date (18 September) (BBCH, general scale from Biologische Bundesantalt, Bundessortenamt and Chemische Industrie, Germany; Meier et al., 1994). The three RHs were created by preparing different mixtures of distilled water (DW) and glycerol using a previously described protocol (Forney and Brandl, 1992). Briefly, DW without glycerol was used to create the 100% RH environment and 104.3 mL or 209.3 mL of glycerol were mixed with 1 L DW (final volume) to create the 80% RH and 60% RH environments, respectively.

The environmental conditions under which harvested fruit are processed and stored vary in different fruit processing and storage centers. To determine the effects of those environmental conditions on germ tube and appressorial formation, a mixture of Czapek-Dox broth and the *M. fructicola* (Mf1) or *M. laxa* (Ml1) (Table 1) conidial suspensions in SDW was first incubated at 4 °C for 0, 1, or 3 days in the dark at an RH of either 60% or 100% to simulate refrigeration before fruit processing. The mixtures were then incubated for 20 min at 15 °C to simulate fruit processing conditions at an RH of either 60% or 100%. At the end of the incubation, the mixtures were incubated at 4 °C for 0, 3, or 10 days in the dark at an RH of either 60% or 100% to simulate the cold storage conditions until distribution to the wholesalers and markets.

2.3. Data analysis

Data on the effects of temperature, RH, fruit maturity, and the duration of refrigeration and cold storage of harvested fruit at 4 °C on germ tube and appressorial formation were analyzed by a multiway analysis of variance (Snedecor and Cochram, 1980). Data were arcsine transformed before the analysis in order to improve the homogeneity of variance. When F-test was significant at $P \leq 0.05$, the means were compared using the Student-Newman-Keuls multiple range test.

The linear regression models, f (T, RH, it), germ tube formation (%), and appressorial formation (%) were used to investigate the relationship between germ tube or appressorial formation and temperature (T), RH, and incubation period (it) for each culture medium which contained the skin extracts of mature and immature peaches and fungal species. In these models, f (T, RH, it) is a linear function of T, RH, it, TRH, Tit, T^2, $RHit$, RH^2, it^2, $TRHit$, T^3, T^2RH, T^2it, RH^3, TRH^2, RH^2it, it^3, Tit^2, and $RHit^2$, whose values were estimated by the regression analysis. Data were fitted to the model using the Regression Model Selection procedure of Statgraphics Centurion XVI for Windows, Version 16.1.03 (StatPoint Technologies, Inc., Herndon, VA, U.S.A.). Selection of the final model was performed according to the significance of the estimated parameters and the adjusted coefficient of determination.

3. Results

3.1. The effects of temperature, RH, and fruit maturity on germ tube and appressorial formation

M. fructicola and *M. laxa* conidia emitted germ tubes and/or produced appressoria at different temperatures and RHs. The greatest effects of temperature, RH, and fruit maturity on germ tube and appressorial formation by *M. fructicola* and *M. laxa* isolates were seen after a 48 h incubation period (Fig. 1).

The number of germ tubes and appressoria were the same when *M. fructicola* conidia were incubated at 4 °C for 48 h at 60% and 80% RH (Fig. 1a and b). The number of germ tubes was greater than the number of appressoria when *M. fructicola* conidia were incubated at 10 or 25 °C for 48 h at all tested RHs (Fig. 1a and b). For *M. laxa* the number of germ tubes was greater than the number of appressoria at all tested temperatures, except when the conidia were incubated in culture medium which contained a skin extract of immature peaches at 10 °C for 48 h at 80% or 100% RH, and at 25 °C for 48 h at 60% RH (Fig. 1c and d). Maximum germ tube formation occurred for both species at 25 °C and 100% RH when the conidia were incubated in culture medium that contained a skin extract from either mature or immature peaches (Fig. 1a and c). Little or no germ tube formation by *M. fructicola* conidia was recorded for 48 h of incubation at (a) 4 °C and 60% or 80% RH, (b) 10 °C and 60% RH, and (c) 35 °C and 60% RH (Fig. 1a). Little or no germ tube formation by *M. laxa conidia* was recorded at (a) 4 °C and 60% RH, and (b) 35 °C at all tested RHs (Fig. 1c).

When *M. fructicola* and *M. laxa* conidia were incubated in culture medium that contained a skin extract of mature peaches, the number of germ tubes was greater than that formed when the conidia of both species were incubated in culture medium that contained a skin extract of immature peaches (Fig. 1a and c). Specifically, statistically significant differences in the number of germ tubes were found after a 48 h incubation at (a) 4 °C and 100% RH, (b) 10 °C and 80% RH, and (c) 25 °C and 80% RH for *M. fructicola* (Fig. 1a), and at (a) 4 °C and 80% or 100% RH, (b)10 °C and 80% or 100% RH, and (c) 25 °C and 60% RH for *M. laxa* (Fig. 1c).

We found that the numbers of appressoria that were produced by *M. fructicola* and *M. laxa* conidia (Fig. 1b and d) were lower than the numbers of germ tubes that were emitted by *M. fructicola* and *M. laxa* conidia (Fig. 1a and c). We also found that the number of appressoria that were produced by *M. laxa* was greater than that for *M. fructicola* conidia at (a) 10 °C for 48 h at 80% and 100% RH, and (b) 25 °C for 48 h at 60% RH (Fig. 1b and d). The maximum number of appressoria that were produced by *M. fructicola* and *M. laxa* occurred when the temperature and RH were not optimal for germ tube formation, namely at 10 °C for 48 h at 60% RH (Fig. 1). The number of appressoria that were produced by *M. fructicola* conidia was significantly greater when the conidia were incubated in culture medium which contained a skin extract of immature peaches than one containing a skin extract of mature peaches. Specifically, this difference was found after a 48 h incubation at (a) 10 °C and 100% RH and (b) 25 °C and 60% RH (Fig. 1b). The number of appressoria that were produced by *M. laxa* conidia when the conidia were incubated in culture medium which contained a skin extract of immature peaches was significantly greater than when the conidia were incubated in culture medium which contained a skin extract of mature peaches. Specifically, this difference was found at (a) 10 °C and 100% RH and (b) 35 °C and 80% RH (Fig. 1d).

3.2. Linear regression models of germ tube formation by M. fructicola and M. laxa

The linear regression models for germ tube formation by *Monilinia* spp. at different temperatures (T), RHs, and incubation periods (it) for the two types of skin extracts and the two fungal species are shown in Table 2. Linear regression models for appressorial formation were not generated.

3.3. Effects of the environmental conditions of harvested fruit on germ tube and appressorial formation by the M. fructicola (Mf1) and M. laxa (Ml1) isolates

Tables 3 and 4 summarize the effects of the environmental conditions of harvested fruit on germ tube and appressorial formation by the Mf1 and Ml1 isolates. Although Mf1 and Ml1 conidia were able to germinate in Czapek-Dox broth under all simulated refrigerated conditions, the number of germ tubes that were formed by Ml1 conidia was significantly bigger than that for Mf1 conidia when the conidia were incubated at 100% RH (Table 3). Negligible or no germination of Mf1 and Ml1 conidia occurred when the RH was 60% (Table 3). We also found that (a) the number of germ tubes that were formed by Ml1 conidia increased after 3 days of refrigeration when the RH was 100% and (b) reached its maximum after 10 days of cold storage (Table 3). The numbers of appressoria produced by Mf1 and Ml1 at an RH of 100% were greater than the number that were produced by these two isolates at an RH of 60%. The number of appressoria that were formed by Mf1 increased after 3 consecutive days of refrigeration for each cold storage duration (Table 4), and the numbers of appressoria were always greater than those for Ml1.

4. Discussion

In this investigation, we found that germ tube and appressorial formation by *Monilinia* spp. are influenced by temperature, RH, and the maturity stage of the fruit. The greatest number of germ tubes was

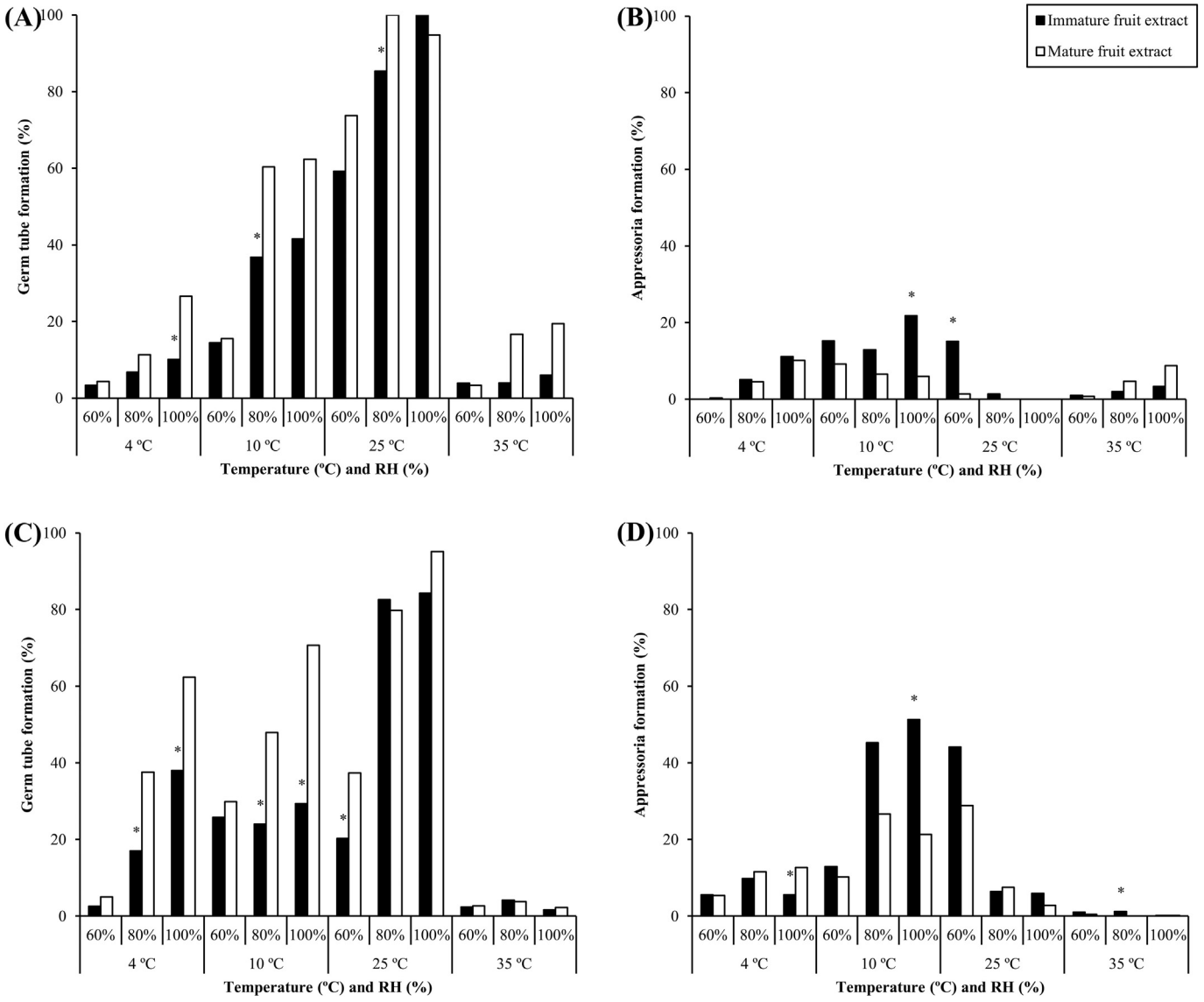

Fig. 1. Germ tube and appressorial formation by *Monilinia* spp. which were incubated for 48 h at different temperatures and relative humidities in culture medium which contained a skin extract of either immature or mature peaches. Germ tube (a and c) and appressorial (b and d) formation by *M. fructicola* (a and b) and *M. laxa* (c and d) in culture medium which contained a skin extract of immature (■) or mature (□) peaches after a 48-hour incubation at different temperatures (4, 10, 25, and 35 °C) and relative humidities (60%, 80%, and 100%). Data on the number of germ tubes and appressoria are displayed as a mean percentage of 50 randomly selected conidia of each of the three *M. fructicola* and *M. laxa* isolates. Groups of columns with an asterisk (*) represent statistically significant differences at a 95% confidence level according to the results of the Student-Newman-Keuls multiple range test.

found when *M. fructicola* and *M. laxa* conidia were incubated in culture medium that contained a skin extract of mature peaches. This finding is different from that for appressorial formation: the greatest number of appressoria was found when *M. fructicola* and *M. laxa* conidia were incubated in culture medium that contained a skin extract of immature peaches. Although the optimal environmental conditions for germ tube formation (25 °C and 80 to 100% HR) by *M. fructicola* and *M. laxa* are similar, we found that the number of germ tubes formed by *M. fructicola* conidia was greater than that for *M. laxa* conidia under these conditions. Casals et al. (2010) reported that the maximum germination of *M. laxa* and *M. fructicola* conidia occurred when temperature was 25 °C and RH was 99%. However, no conidial germination was recorded by these authors when RH was lower than 87%. Our results are in agreement with those of Papavasileiou et al. (2015) who found that maximum germination of *M. laxa* and *M. fructicola* conidia occurred when the isolates were grown on PDA and the temperature was

Table 2
Linear regression models which describe the relationship between temperature (T), relative humidity (RH) and incubation period (it) and germ tube formation (y) by *M. fructicola* and *M. laxa* conidia that were incubated in culture medium which contained a skin extract of either immature or mature peaches.

Monilinia specie	Type of peach skin extract	Equation	R^2 values
M. fructicola	Immature peach	$y = -45.213 + 0.381\,RH + 0.099\,Tit + 0.211\,T^2 - 0.003\,T^2it - 0.006\,T^3$	0.62
	Mature peach	$y = -50.206 + 0.490\,RH + 0.119\,Tit + 0.202\,T^2 - 0.003\,T^2it - 0.005\,T^3$	0.64
M. laxa	Immature peach	$y = -39.638 + 0.407\,RH + 0.109\,Tit + 0.139\,T^2 - 0.003\,T^2it - 0.004\,T^3$	0.55
	Mature peach	$y = 5.340 - 2.110\,it + 0.213\,Tit + 0.025\,RHit - 0.005\,T^2it - 0.0006\,TRHit$	0.61

The regression models were obtained from the number of germ tubes that were formed by 50 randomly selected conidia of each of the three *M. fructicola* and *M. laxa* isolates. The parameters of the equations are given in the following units: temperature (°C), RH (%), and incubation period (hours).

Table 3
The effect of refrigeration and cold storage on germ tube formation by *Monilinia fructicola* and *M. laxa* isolates[y].

Specie	RH (%)	Duration of refrigeration before processing (days)	Duration of cold storage after processing (days)		
			0	3	10
M. fructicola	60	0	2.0 a	2.0 a	1.3 ab
		1	2.0 a	2.7 a	0.0 a
		3	0.7 a	1.3 a	0.0 a
	100	0	2.0 a	0.7 a	3.3 ab
		1	0.0 a	2.0 a	0.0 a
		3	2.0 a	1.3 a	1.3 ab
M. laxa	60	0	0.0 a	0.7 a	6.7 ab
		1	0.7 a	0.7 a	9.3 b
		3	0.7 a	3.3 a	2.0 ab
	100	0	0.0 a	53.3 b	72.0 c
		1	0.7 a	64.0 bc	87.3 d
		3	60.0 b	73.3 c	87.3 d
MSE[z]			(27.5)	(31.5)	(28.2)

The two post-harvest environmental conditions that were tested are refrigeration for 0, 1, or 3 days before fruit processing and cold storage for 0, 3, or 10 days at 4 °C in the dark at a relative humidity (RH) of either 60% or 100%.
Data on the number of germ tubes are expressed as a percentage of 50 randomly selected conidia of one *Monilinia fructicola* isolate (Mf1) or one *Monilinia laxa* isolate (Ml1) (Table 1) which formed a germ tube and are the mean of three replicates. Experiments were repeated at least twice.
[y] Means with the same letter in each column are not significantly different from each other according to the results of the Student-Newman-Keuls multiple range test ($P < 0.05$). Data were arcsine transformed before analysis.
[z] MSE = mean square error.

between 10 and 25 °C. Tamm and Flückiger (1993) have also reported that maximum germination of *M. laxa* conidia occurred when temperature was between 15 and 25 °C and RH was 100%.

We found that (a) *M. laxa* conidia emit germ tubes when the temperature is 4 °C and the RH is between 80% and 100%, and (b) *M. fructicola* conidia are not able to emit germ tubes under these conditions. These results are in accordance with those that were reported by Tamm and Flückiger (1993) and Tian and Bertolini (1999), who found that *M. laxa* conidia can germinate when the temperature is as low as −4 °C. The ability of *M. laxa* conidia to germinate at low temperatures has also been reported by Watson et al. (2002), Casals et al. (2010) and Papavasileiou et al. (2015) who reported that *M. fructicola* conidia can germinate at low temperatures, namely 0 and 5 °C. Papavasileiou et al. (2015) measured conidial germination of five different *M. fructicola* and *M. laxa* isolates 6 days after plating on PDA. They found that (a) germination of the conidia of the two *Monilinia* species was greater than 70%, and (b) mycelial growth of the *M. laxa* isolates was greater than that of the *M. fructicola* isolates after a 6 day incubation period at 5 °C. Accordingly, we surmise that our short incubation periods could account for higher levels of germination of *M. laxa* conidia than *M. fructicola* conidia at low temperatures.

Table 4
The effect of refrigeration and cold storage on appressorial formation by *Monilinia fructicola* and *M. laxa* isolates[y].

Specie	RH (%)	Duration of refrigeration before processing (days)	Duration of cold storage after processing (days)		
			0	3	10
M. fructicola	60	0	0.0 a	0.0 a	1.3 a
		1	0.7 a	2.0 a	4.0 a
		3	0.0 a	4.7 ab	5.3 a
	100	0	0.0 a	2.0 a	38.0 c
		1	2.0 a	10.7 ab	33.0 c
		3	13.3 c	30.0 c	70.0 d
M. laxa	60	0	0.0 a	0.0 a	0.0 a
		1	0.0 a	0.0 a	0.0 a
		3	0.0 a	0.0 a	0.0 a
	100	0	0.0 a	9.3 ab	24.0 b
		1	0.0 a	4.0 ab	11.3 a
		3	8.0 b	18.0 b	11.3 a
MSE[z]			(1.8)	(11.8)	(8.6)

The two post-harvest environmental conditions that were tested are refrigeration for 0, 1, or 3 days before fruit processing and cold storage for 0, 3, or 10 days at 4 °C in the dark at a relative humidity (RH) of either 60% or 100%.
Data on the number of appressoria are expressed as a mean percentage of 50 randomly selected conidia of one *Monilinia fructicola* isolate (Mf1) or one *Monilinia laxa* isolate (Ml1) (Table 1) which formed an appressorium and are the mean of three replicates. Experiments were repeated at least twice.
[y] Means with the same letter in each column are not significantly different from each other according to the results of the Student-Newman-Keuls multiple range test ($P < 0.05$). Data were arcsine transformed before analysis.
[z] MSE = mean square error.

We found that little or no germ tube and appressorial formation occurred at 35 °C for both *Monilinia* spp. These results were similar to those of Papavasileiou et al. (2015) who reported that the conidia of five *M. laxa* isolates and five *M. fructicola* isolates did not germinate on PDA at 35 °C. However, Casals et al. (2010) reported that the germination of conidia of three different *Monilinia* isolates, one *M. laxa* isolate, one *M. fructigena* isolate, and one *M. fructicola* isolate, occurred at 35 °C, but not at 38 °C. A possible reason for the difference between these three sets of results could be due to the use of only one *Monilinia* isolate of each species.

We found that the environmental conditions of harvested fruit before, during, and after their processing did not prevent germ tube and appressorial formation by *M. fructicola* and *M. laxa*. High RH in the refrigeration chambers is required to maintain fruit quality. We found that a high RH increased the germ tube formation by *M. laxa* and *M. fructicola* conidia, and that this increase in germ tube formation at a high RH was very evident for *M. laxa*. The ambient temperature in washing and sorting units is between 15 and 25 °C and the washing and sorting of harvested fruit takes between 1 and 6 h. We found that a cold stop in fruit refrigeration could reduce appressorial formation by *M. fructicola* conidia, which needs 3 consecutive days of refrigeration or cold storage.

Germ tube formation by *M. laxa* and appressorial formation by *M. fructicola* occurs during the first stage of the infection process. We found that appressorial formation by *M. fructicola* increased when the temperatures and/or RHs for conidial germination were not optimal. These results differ from those on appressorial formation by other phytopathogenic ascomycetes, such as *Colletotrichum acutatum*. In this species, the formation of melanised appressoria decreased when temperatures and/or RHs for conidial germination were sub-optimal (Miles et al., 2013). This difference between the results of our study and those from Miles et al.'s study suggests that pathogenic *C. acutatum* requires an appressorium to penetrate its hosts, and that *M. fructicola* need an appressorium to penetrate fruit only when the environmental conditions are unfavorable. Lee and Bostock (2006) proposed that the appressoria of *M. fructicola* facilitates its penetration into immature fruit and could even be a resting or a non-pathogenic phase of the pathogen when unfavorable environmental conditions for infection were present. We also found that the rate of appressorial formation by *M. laxa* was higher than that of *M. fructicola* when we investigated the effects of temperature, RH, and fruit maturity on appressorial formation. Although we did not detect such a difference when we investigated the effects of the environmental conditions of harvested fruit on *M. laxa* appressorial formation before, during, and after fruit processing, we attribute this difference in the rates of appressorial formation to the utilization of the nutrients in the Czapeck-Dox broth by *M. laxa* and *M. fructicola* conidia. Lee and Bostock (2006) reported that the composition of nutrient media, such as CV8 medium, can induce the germination of already-formed appressoria.

When our results are appraised together with those of Papavasileiou et al. (2015), we concluded that *M. laxa* and *M. fructicola* have their own competitive edge for conidial germination and mycelial growth in different environmental conditions. We also surmise that the competitive edge of each species in different environmental conditions accounts for the coexistence of *M. laxa* and *M. fructicola* in Spanish peach and

nectarine orchards. This competitive edge becomes advantageous for *M. fructicola* when temperature is between 10 and to 25 °C and when temperature is between 4 and 10 °C for *M. laxa*. These individual competitive advantages could explain (a) the incomplete and slow supplanting of *M. laxa* by *M. fructicola*, even though *M. fructicola* is more aggressive and grows faster than *M. laxa* under optimum growing conditions (25 °C and 100% RH) (Villarino et al., 2016), and (b) the different adaptabilities to postharvest conditions of *M. fructicola* and *M. laxa*.

Accordingly, we suggest that the incidence of brown rot in harvested peaches could be reduced by shortening the period between fruit arrival times and their processing at fruit processing units, reducing RH in the refrigeration chambers, and shortening the duration of cold storage. Incorporating these measures and practices into the modus operandi of the handling of harvested peaches should create the environmental conditions that constrain conidial germination of *Monilinia* spp. Reducing RH in the refrigeration chambers could potentially reduce peach quality by causing fruit dehydration. Hence, further investigations are needed to determine the optimum RH for reducing conidial germination of *Monilinia* spp. without adversely affecting fruit quality.

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