Role of gluconic acid and pH modulation in virulence of *Monilinia fructicola* on peach fruit

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A R T I C L E   I N F O

A B S T R A C T

Colonization of nectarines and peaches fruit by *Monilinia fructicola* was accompanied by local acidification of the host tissue. The fungus acidified the host tissue in peaches and nectarines from pH 4.5 and 4.45, to pH 3.75 and 3.9, respectively. Analysis of the acidification process in colonized fruit and secondary inducing media showed that gluconic acid was the main organic acid accumulated at the infection site and under liquid-culture conditions. When comparing a nectarine cv. Big Top and peach cv. Plácido with differing sensitivities to *M. fruticola*, a 250% higher accumulation of gluconic acid was observed in the susceptible peach cultivar than in the less susceptible nectarine cultivar. Under liquid conditions, at pH 3.6–3.7, the relative expression of transcripts of *mfpg 2* and *mfpg3*, encoding for two polygalacturonase genes of *M. fructicola*, increased 12-fold and 6-fold, respectively, suggesting the importance of acidification for the secretion of pathogenicity factors by *M. fructicola*. Our results indicate that ambient pH is a regulatory cue for processes linked to pathogenicity of postharvest pathogens, and that specific genes contributing to pathogenicity are expressed as a result of the environmental pH created by the pathogen.

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1. Introduction

Brown rot caused by *Monilinia* spp. is an economically important fungal disease of stone fruit; it causes substantial preharvest and postharvest losses (Byrde and Willetts, 1977). Brown rot occurs in peach (*Prunus persica* (L.) Batsch) and nectarine (*Prunus persica* var. *nectarina* Maxim) and can be caused by the three *Monilinia* spp., *M. laxa*, *M. fructicola*, and *M. fructigena* (Byrde and Willetts, 1977). Direct yield losses result from infection of flowers, as flower and twig blight, and from fruit rot at preharvest, harvest, and postharvest stages (Villarino et al., 2012). Postharvest losses are typically more severe than preharvest losses, and routinely occur during storage and transport (Hong et al., 1997).

Susceptibility to brown rot infection is strongly dependent on the developmental stage of the fruit when infection occurs (Lee and Bostock, 2007; Gell et al., 2008); immature fruit at the pit-hardening stage are more resistant to infection by *Monilinia* spp.

than mature ones (Lee and Bostock, 2006, 2007; Xu et al., 2007; Gell et al., 2008). Villarino et al. (2011) found that susceptibility of peach fruit to *M. laxa* infection was greatest after the 32nd and 34th weeks of growth, when the endocarp and pericarp were completely formed, and they suggested that at this stage the low concentrations of chlorogenic and neochlorogenic acids in the peach peel and flesh of mature fruit might contribute to their susceptibility to brown rot infection by not interfering with fungal melanin production to *Monilinia* penetration.

Monilinia spp. develop appressoria to penetrate intact tissues, thus providing adhesion of the pathogen to the surface of the host during infection (Lee and Bostock, 2006). Penetration of *Monilinia* spp. into a host can occur through wounds, directly through stomata or the cuticle of the intact surface, or via surface-hair bases (Curtis, 1928; Smith, 1936; Wade and Cruickshank, 1992), but disease incidence is always greater if the fruit have small cracks or wounds (Byrde and Willetts, 1977; Hong and Michailides, 1998). Direct penetration of *Monilinia* is enhanced by its production of cutinases (Wang et al., 2000, 2002), and their over-expression increased fungal virulence in stone fruit (Lee et al., 2010).

After penetration of the exocarp, *Monilinia* colonizes the fruit mesocarp by development through intercellular and intracellular spaces, thereby causing the collapse of the host cell (Pring et al., 1981; Lee and Bostock, 2006). Simultaneously, the fungus secretes cell wall degrading enzymes and/or generates low molecular weight metabolites associated with tissue degradation (Byrde and Willetts, 1977; Pring et al., 1981). The initial attack of *M. fructicola* is accompanied by secretion of endopolygalacturonases and pectin esterases (Hall, 1971; Wade and Cruickshank, 1992) causing cell wall maceration and death of affected host cells (Paynter and Jen, 1975).

Prusky et al. (2001) have suggested that pathogens may enhance their virulence by locally modulating the host's ambient pH. This mechanism ensures that genes encoding cell wall degrading enzymes are expressed, and that their products are secreted under the optimal pH conditions for their functioning (Prusky et al., 2001; Eshel et al., 2002). This indicates that, although several genes encode cell wall degrading enzymes, only specific ones are activated during pathogenicity in vivo (Prusky et al., 2001). Two types of postharvest pathogens have been described: those that alkalinize the environment, and those that acidify it. *Colletotrichum* and *Alternaria* were described as pathogens that alkalinize the tissue during decay development in several subtropical fruit (Prusky and Yakoby, 2003; Alkan et al., 2008), whereas *Penicillium* sp., *Sclerotinia*, *Phomopsis*, and *Botrytis* have been described as acidifiers of the decayed tissue: *S. sclerotiorum* (Magro et al., 1984) and *B. cinerea* (Verhoeff et al., 1988) acidify their host tissues by producing oxalic acid, whereas *P. expansum*, and *P. mangiferae*, produce gluconic acid as the main organic acid (Hadas et al., 2007; Davidzon et al., 2009).

Our objectives in the present study were: (1) to determine the possibility of pH modification of peach fruit tissue during infection and colonization by *M. fructicola*; (2) to identify the pH modulating factor induced by *M. fructicola* during pathogenic attack; (3) to study the role of this pH regulation in the transcriptional regulation of *M. fructicola* genes that contribute to fungal colonization.

2. Materials and methods

2.1. Isolates

Three isolates of *M. fructicola*, Mfc-1, Mfc-2, and Mfc-3, that had been recovered from three different commercial peach orchards in the Ebro Valley (Spain) in 2009 and 2010, were used. Conidial suspensions of each isolate in 20% glycerol were stored at $-80\,°C$ and grown on potato dextrose agar (PDA; Difco, Detroit, MI, USA) for 7 d at $20–25\,°C$ in darkness, to produce mycelia and conidia.

2.2. Fruit samples

Two nectarine cultivars, 'Big Top' and 'Venus', and one peach cultivar, 'Plácido', were used. 'Big Top' was early harvest, whereas 'Venus' and 'Plácido' were late harvest cultivars.

2.3. pH determination

To determine pH in infected peaches, the fruit were first sterilized according to Sauer and Burroughs (1986), after which their surfaces were dried with sterile air in a flow-cabinet for 2 h. Cultivars 'Big Top', and 'Venus' were inoculated with two blocks of agar inoculum, approximately 8 mm in diameter, obtained from a 7 d old PDA colony of *M. fructicola* (isolate Mfc-1), were placed 2 cm apart on each of two sides of each fruit, to inoculate seven fruit of each cultivar. Cultivar 'Plácido' was inoculated as described above but with isolate Mfc-2. The inoculated fruit were then incubated for 7 d at $20–22\,°C$ and 80% RH. Lesion growth (cm/day) was recorded on each fruit throughout the incubation period. At the end of this assay,

the juice was obtained from the fruit by squeezing the flesh tissue from decayed (infected tissues) and healthy fruit though miracloth. The pH of juice of each fruit, both infected and uninfected, was measured with a Micro pH 2000 instrument (Crison Instruments, SA, Barcelona, Spain).

2.4. pH determination in liquid medium

In order to analyze the effect of *M. fructicola* on pH modulation in a liquid medium, flasks containing 50 mL of potato dextrose broth (PDB) (Difco, Detroit, MI, USA) at pH 5.3 were inoculated with 1 mL conidial suspension of *M. fructicola* (isolates Mfc-1 and Mfc-3) (5×10^6 conidia mL^{-1}). The conidia were obtained from a 7 d old colony on PDA. All of these flasks were incubated for 7 d at room temperature with shaking at 150 rpm. The entire culture of each flask was harvested by vacuum filtration onto sterile filter paper, and washed twice under vacuum with 50 mL of sterile distilled water. Approximately 3.0 g of washed mycelia from each culture were resuspended and incubated for an additional 48 h, in 50 mL of fresh inducing medium (secondary medium, SM) containing, per liter: 1 g of K_2HPO_4, 0.5 g of $MgSO_4·7H_2O$, 7 g of $NaNO_3$, 3 g of peptone, 0.5 g of KCl, and 10 g of sucrose. Uninoculated flasks were used as controls. Secondary cultures of the different isolates were harvested by vacuum filtration after different periods after inoculation up to 48 h. Five flasks of each isolate were filtered at each incubation time. The supernatants were saved for pH determination and the hyphae for dry weight determination. The experiment was performed twice.

2.5. Determination of organic acid concentrations in Monilinia-colonized peach fruit and liquid media

Fruit juices from cvs. Big Top and Plácido, uninfected, and infected with Mfc-1 and Mfc-2, respectively, and the supernatant of the Mfc-3-inoculated secondary medium described above, were subjected to extraction. The juice had been obtained by squeezing the decayed fruit flesh though miracloth, after inoculation and 7 d of storage at $20–22\,°C$ and 80% RH. Juice samples were kept at $-80\,°C$ until required for organic acid analysis. The supernatants of *M. fructicola*-inoculated secondary medium and peach juice samples were filtered through 0.2 μm polyester filters with 15 mm of diameter (Droquima, Madrid, Spain). The extracts were directly analyzed by means of liquid chromatography–mass spectrometry (LCMS). The analysis was carried out immediately after the samples were defrosted because of the instability of organic acids in the aqueous media.

Twenty microliters of each extract were analyzed with an HPLC system coupled to a mass spectrometer detector (MS) with a Series 1200 quadrupole analyzer (Agilent Technologies, Palo Alto, CA, USA), operated by Chemstation software (Agilent Technologies, Palo Alto, CA, USA). The MS detection was performed by electrospray ionization in negative mode. Mass spectrometer conditions were optimized by ramping the fragmentor value to determine the maximum ionic efficiency for each molecular ion. Mass spectrometer chromatograms were obtained over mass ranges of 50–2000 atomic mass units (AMU) and a scan rate of 1.65 s/cycle.

A reversed-phase C18 column (4.6 mm × 150 mm; 3 μm) (Waters, Dublin, Ireland) with a column guard with the same phase was used to separate organic acids. The flow rate was 0.5 mL/min and the mobile phase was water, acidified with 0.1% of formic acid. Retention times of organic acids were: oxalic acid, 2 min; gluconic acid, 3.9 min; malic acid, 6.2 min; citric acid, 12.5 min; and fumaric acid, 13.5 min. Concentrations of organic acids generated by *M. fructicola* in liquid media were quantified by measuring peak areas and compared with a fortified liquid-medium calibration curve ($n = 5$, $R^2 = 0.98$). Organic acid standards (malic, citric, gluconic, fumaric,