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Pectin methyl esterases and rhamnogalacturonan hydrolases: weapons for successful *Monilinia laxa* infection in stone fruit?

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Abstract

The secretion of cell wall-degrading enzymes is one of the mechanisms used by necrotrophic fungi to colonise host tissues. However, information about virulence factors of *Monilinia* spp., the causal agents of brown rot in stone fruit, is scarce. Plant cell walls have three main components that are broken down by fungal enzymes: cellulose, hemicellulose and pectin. In order to identify *Monilinia laxa* candidate proteins involved in pectin hydrolysis, two *in vitro* approaches were conducted: a) phenotypic and ecophysiological characterisation of growth of the pathogen at different pHs, in glucose- and pectin-containing solid media for 7 days of

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incubation, and b) expression analysis of genes encoding *M. laxa* pectin methyl esterases (*MlPMEs*) and rhamnogalacturonan hydrolases (*MlRG-HYDs*) after incubation for 0.5, 2, 6, 24 and 48 h in glucose- and pectin-containing liquid media. Phenotypic tests evidenced the role of carbon source on *M. laxa* growth rate and aggressiveness, and indicated that pectinases were greatly affected by pH. Gene expression analyses uncovered differences among members of each family of pectinases and between the two families, defining sets of genes expressed at earlier (0.5-6 h) and later (48 h) phases. Notably, the up- or down-regulation of these target genes was carbon source-dependent. Finally, an *in vivo* study confirmed the synergistic and complementary role that these genes play in the *M. laxa*-stone fruit pathosystem. Based on these results, we hypothesise that *MlPME2*, *MlRG-HYD1* and *MlRG-HYD2* may be potential virulence factors of *M. laxa* in the process from infection to colonization.

Keywords

Brown rot, carbon sources, cell wall-degrading enzymes, gene expression analysis, host-pathogen interaction, pH.

Introduction

Monilinia spp. are one of the main pathogens responsible for brown rot, a disease that causes important losses worldwide, both in the field and in postharvest. There are three main species that have been described as pathogenic: *M. fructicola* and *M. laxa* to stone fruit, and *M. fructigena* to pome fruit. However, we still have very little information about the pathogenic mechanisms of these fungi. This is particularly true for *M. laxa*, the most important causal agent of brown rot in Europe (Rungjindamai *et al.*, 2014). In this context, knowledge

regarding pathogenic mechanisms involved in the development and spread of this disease is fundamental to controlling the pathogen in a more specific way.

It is known that while some pathogens require the presence of wounds to infect, others such as *Monilinia* spp. can overcome this need and penetrate the plant cell wall by altering its composition (Nakajima & Akutsu, 2014). In this latter case, secretion of cell wall degrading enzymes (CWDEs) and, in particular, polysaccharide-specific enzymes constitute one of the virulence factors by which necrotrophic fungi colonize host tissues (Cantu *et al.*, 2008b). Moreover, it has been shown that necrotrophic pathogens can selectively attack host wall polysaccharide substrates depending on the host tissue (Blanco-Ulate *et al.*, 2014) due both to the specific plant pectin-structure and to the profile of pectinolytic enzymes secreted by each fungus, which is sometimes strain-specific (Reignault *et al.*, 2008; Benoit *et al.*, 2012). Thus, multiple factors including host tissues and consequently, carbon source and pH changes occurring during the different pathogenic stages (Wubben *et al.*, 2000; Chou *et al.*, 2015), can influence the complex regulation of CWDEs' genes and hence, their biochemical specialization (Akimitsu *et al.*, 2004).

The plant cell wall comprises polysaccharides such as pectin, celluloses and hemicelluloses that represent above 90% of the primary cell wall (Lagaert *et al.*, 2009), and which maintain the cell and tissue integrity due to their structural and water binding capacity (Prasanna *et al.*, 2007). Physiological processes such as ripening in peach lead to restructuring of the cell wall that implies a reduction in arabinan content and depolymerisation of both hemicellulose and pectin from a mid-ripe to full-ripe stage (Brummell *et al.*, 2004; Fruk *et al.*, 2014), a process that, among others, involves an increased susceptibility to fungal infections (Cantu *et al.*, 2008a; Garcia-Benitez *et al.*, 2017).

Pectin, the major component of the primary cell wall and middle lamella, is the name for a series of polymers rich in D-galacturonic acid, including homogalacturonan (HG) which constitute the most abundant type of pectin (over 60% of total pectin in plant cell walls), rhamnogalacturonan I (RGI), rhamnogalacturonan II (RGII) and xylogalacturonan (XGA). For complete degradation of pectin, a synergistic action of all the pectic enzymes is required (Martens-Uzunova & Schaap, 2009). Pectin degradation is facilitated by a battery of enzymes that belong to different families of Carbohydrate-Active enZymes (CAZymes). These families are: i) polysaccharide lyases (PLs) such as pectate and pectin lyases, ii) glycoside hydrolases (GHs) such as polygalacturonases (PGs) and rhamnogalacturonan hydrolases (RG-HYDs), as well as iii) carbohydrate esterases (CEs) such as pectin methyl esterases (PMEs) and rhamnogalacturonan acetylesterases (Hugouvieux-Cotte-Pattat *et al.*, 1996; http://www.cazy.org/). Some of these common CAZymes are known virulence factors in *Sclerotinia sclerotiorum* and *Botrytis cinerea* (Heard *et al.*, 2015). Similarly to these closely related necrotrophic ascomycetes, extracellular enzymes in *Monilinia* spp. play an important role during fruit surface penetration, invasion and colonisation of the fruit (Byrde & Willets, 1977). Recently, Garcia-Benitez *et al.* (in press) detected pectinolytic, proteolytic, cellulolytic and xylanolytic enzyme activities in *M. fructicola*, *M. fructigena* and *M. laxa* isolates when cultured on media amended with different substrates similar in chemical composition to fruit.

To date, many studies have focused on PGs, positioning these CWDEs as required for full virulence in many pathosystems (Bravo Ruiz *et al.*, 2016; Liu *et al.*, 2018; Vilanova *et al.*, 2018). However, the pivotal role of *MfPG1*, which diminishes fungal virulence when overexpressed, has also been highlighted (Chou *et al.*, 2015). Among other CWDEs, PMEs and RG-HYDs may be involved in *M. laxa* pathogenicity, according to recently published transcriptomic data (De Miccolis Angelini *et al.*, 2018). Nevertheless, no other studies have

tried to elucidate the putative role that these CAZymes may play in the *M. laxa*-stone fruit interaction, and thus little evidence is available for their role as virulence determinants in pathogenesis. Hence, considering both the scarce information available for *M. laxa*, and previous results emphasizing the different roles that CWDEs can play in the pathogenicity processes regardless the taxonomy of the fungus (Isshiki *et al.*, 2001), further studies are desirable. Accordingly, the aim of this study was to characterise *M. laxa* PMEs and RG-HYDs using the recently published *M. laxa* genome (Naranjo-Ortiz *et al.*, 2018). First, a preliminary *in vitro* approach was performed to sift some genes involved in pectin degradation. Then, emphasis was given to an *in vivo* analysis aimed at providing insight into the putative role of these genes as virulence factors in strain ML8L of *M. laxa*. Unravelling the *M. laxa*-stone fruit interaction process would allow for a better understanding of the disease and hence, to more specific and effective crop protection strategies.

Materials and methods

Fungal strain and culture conditions

The *Monilinia laxa* single-spore strain ML8L used in this study was isolated from a mummified 'Sungold' plum fruit from a commercial orchard in Lagunilla (Salamanca, Spain) in 2015, and deposited in the Spanish Culture Type Collection (CECT 21100). The strain was cultured on potato dextrose agar medium (PDA; Biokar Diagnostics, 39 g L^{-1}) supplemented with 25% tomato pulp and incubated under a 12-h light and 12-h dark photoperiod at 25 ºC / 18 ºC, respectively, for 7 days.

Conidial suspensions were prepared by rubbing the surface of a 7-day-old culture grown on PDA supplemented with 25% tomato pulp with sterile water containing 0.01% (*w/v*) Tween-80. The inoculum was filtered through two layers of sterile cheesecloth to minimize the

presence of mycelium fragments. Then, conidia were counted in a haemocytometer and diluted to the desired concentration.

Identification of two major family members involved in pectin degradation

B. cinerea was used as a model organism because it is closely related to *Monilinia*. Thus, the well-known proteins of *B. cinerea* reported to be involved in pectin degradation were used as query sequences (Table 1) to search for homologies within the recently published ML8L genome (Naranjo-Ortiz *et al.*, 2018), using the NCBI Genome Workbench software version 2.11.10 (https://www.ncbi.nlm.nih.gov/tools/gbench/) and the BLAST tool implemented therein, setting an expect (E) value of 10^{-3}. The identity (> 60%) and the fraction of the query sequences covered by the match region (> 50%) were used as filter criteria to select only reliable hits. Results obtained were checked by carrying out BLASTx, BLASTn and tBLASTn searches. All retrieved ML8L protein sequences were verified by a search of the NCBI Conserved Domain Database (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the Pfam 32.0 Database (https://pfam.xfam.org/). Prediction of subcellular localization was performed using the TargetP 1.1 server (http://www.cbs.dtu.dk/services/TargetP/). Finally, the theoretical isoelectric point (pI) and the molecular weight (MW) were predicted by ExPASy-Compute pI/MW tool (https://web.expasy.org/compute_pi/).

***In vitro* assays**

Mycelial growth rate in solid culture containing different carbon sources

To evaluate the growth rate of *M. laxa* as affected by glucose and pectin carbon sources, a drop of 10 µL of a conidial suspension of *M. laxa* (10^5 conidia mL^{-1}) obtained as explained above, was placed individually onto the centre of PDA (control) or PDA plates supplemented with the desired carbon source (1% (*w/v*) glucose or 1% (*w/v*) pectin). The initial pHs of the

PDA and PDA plates supplemented with glucose or pectin were 5.3, 5.4 and 3.7, respectively (see Supporting Table S3), so the effect of this parameter was also assayed by adjusting or not the pH of the plates to 4.5 with HCl or NaOH, respectively. The pH of 4.5 was chosen because it is in the optimal range for mycelial growth (pH 3.5-6.5) and sporulation (pH 4.5-5.5) of *M. laxa* (Obi *et al.*, 2018). In addition, this is a value closer to the pH of fruit. Plates were then incubated in the dark at 22 ºC for 7 days. Colony diameters were measured daily in two directions at right angles to each other for a maximum of 7 days post-inoculation (dpi). Concomitantly, cultures grown under different conditions were also phenotypically characterized (shape of margins, colour of the colony and amount of conidia and mycelia produced). Experiments were carried out twice with three replicates per condition.

Mycelial growth in liquid culture containing different carbon sources

To evaluate the effect of glucose and pectin carbon sources on mycelial growth of the fungus, conidial suspensions of *M. laxa* (10^5 conidia mL^{-1}) obtained as explained above, were inoculated into 1 L flasks containing 200 mL of a minimal medium (MM) at pH 6.5. The composition of the MM for 1 L consisted of 19 mL of a 50X salt solution (26 g/L KCl, 26 g/L $MgSO_4 \times 7H_2O$, 76 g/L g of KH_2PO_4), 1 mL of 50X trace elements (20 mg/L $Na_2B_4O_7$, 25.5 mg $CuSO_4$, 800 mg/L $FePO_4 \times 2H_2O$, 800 mg/L $Na_2MoO_4 \times 2H_2O$, 8 g $ZnSO_4 \times 7H_2O$, 800 mg $MnSO_4 \times 4H_2O$) and 0.092% (*w/v*) of $(NH_4)_2C_4H_4O_6$ as nitrogen source. The fungus was grown on MM containing 1% glucose and incubated at 22 ºC on an orbital shaker under a constant agitation of 150 rpm. After 24 h, *M. laxa* mycelium was filtered through Miracloth (Merck KGaA, Darmstadt, Germany), rinsed with the MM and transferred to a new 1 L flask containing 200 mL of MM in the absence of any supplementary carbon source to shift the fungus to a basal metabolism. The liquid cultures were incubated for 4 h under the above conditions to reprogram gene expression before the addition of the carbon source. After that,

the mycelia were filtered and rinsed again with the MM. Finally, they were transferred to a new 1 L flask containing 200 mL of MM supplemented with the desired carbon source (1% (*w/v*) glucose or 1% (*w/v*) pectin). The pH was adjusted to 4.5 with the addition of HCl or NaOH, respectively. Cultures exposed to both carbon sources were incubated at 22 ºC for 48 h under the above conditions. After 30 min, 2 h, 6 h, 24 h and 48 h post-inoculation, mycelia samples were collected, filtered, rinsed with fresh MM and immediately ground and frozen with liquid nitrogen. All samples were kept at -80 ºC until further molecular analysis. Concomitantly, the pH of the filtrate at each sampling point was measured using a pH meter (Model GLP22, Crison Instruments S.A., Barcelona, Spain) with a conventional electrode (5202 Crison). Experiments were carried out twice with three replicates per condition and carbon source.

RNA extraction

Total RNA extraction corresponding to the mycelium at each sampling point was performed using the reagent TRI Reagent® (Sigma, St. Louis, MO, USA) following the manufacturer's recommendation. Briefly, 100 mg of each frozen sample were grinded using a mortar and pestle and transferred to a 2 mL Eppendorf tube. One mL of TRI Reagent was added to each sample and after homogenization, samples were allowed to stand for 5 min at room temperature (RT) to ensure complete dissociation of nucleoprotein complexes. After the addition of 0.2 mL of chloroform, samples were incubated for 5 min at RT and centrifuged at 12,000 rpm for 15 min at 4 ºC. The aqueous phase containing the RNA was transferred to a fresh tube and 0.5 mL of isopropanol were added and mixed until completely homogenisation. Then, samples were incubated 10 min at RT and centrifuged at 12,000 rpm for 15 min at 4 ºC. The pellet obtained was washed by adding 1 mL of 75% ethanol and subsequently mixed and centrifuged at 7,500 rpm for 5 min at 4 ºC. The supernatant was

removed and the RNA pellet was left to dry for 10 min and resuspended with RNase-free water. To facilitate its dissolution, samples were incubated for 10 min at 60 ºC. Experiments were carried out twice and total RNA was extracted from 2 biological replicates of each carbon sources and time condition.

RNA quantity was determined spectrophotometrically using a NanoDrop 2000 spectrophotometer (Thermo Scientific DE, USA). Contaminant DNA was removed by treating RNA extracts with Turbo DNA-free DNase (Ambion, TX, USA), following the manufacturer's recommendations. Both RNA integrity and the absence of DNA were assessed after electrophoresis on an agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA).

In vivo assay

Plant material

Experiments were conducted with 'Merryl O'Henry' peaches (*Prunus persica* (L.) Batch) harvested at optimal commercial maturity and obtained from an organic orchard located in Vilanova de Segrià (Lleida, Catalonia, NE Spain). Fruit free of physical injuries and rot were picked at optimal commercial maturity, based on grower's recommendations, and immediately transported to IRTA facilities.

Fruit quality

For the evaluation of quality parameters, a sample of 20 fruit were randomly selected and assessed for index of DA-Meter (I_{AD}), weight, cheek diameter (CD), flesh firmness (FF), soluble solids content (SSC) and titratable acidity (TA) (see Supporting Table S1), according to the methodology described by Baró-Montel *et al.* (in press).

Fruit inoculation and experimental design

'Merryl O'Henry' peaches were placed on plastic holders in simple, lidded, storage boxes containing water at the bottom (not in contact with the sample) and inoculated by applying 6 drops of 50 µL each of a conidial suspension of the strain ML8L at 10^6 conidia mL^{-1} onto the surface of non-wounded fruit. After that, fruit were kept in a chamber at 22 ºC and 100% relative humidity (RH). Experiments were carried out with three replicates of five fruit for each sampling point (24, 38, 48 and 72 hours post-inoculation (hpi)). After each incubation time, 90 cylinders of peel and pulp tissue (10 mm diameter and 5 mm deep) encompassing the inoculation sites were obtained from 15 individual fruit (3 biological replicates of 5 fruit each), using a cork borer. All samples were immediately frozen with liquid nitrogen prior to being kept at -80 ºC until further molecular analysis. Concomitantly, the pH of the both healthy and infected mesocarp were measured using a pH meter (Model GLP22, Crison Instruments S.A., Barcelona, Spain) with a penetration electrode (5231 Crison).

RNA extraction

Total RNA corresponding to the infected fruit at each sampling point was extracted following the protocol described by Ballester *et al.* (2006) with some modifications. RNA quantity was determined spectrophotometrically using a NanoDrop 2000 spectrophotometer (Thermo Scientific, DE, USA). Contaminant DNA was removed by treating RNA extracts with Turbo DNA-free DNase (Ambion, TX, USA), following the manufacturer's recommendations. Both RNA integrity and the absence of DNA were assessed after electrophoresis on an agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA).

Primers design and validation

The oligonucleotide primers used for both *in vitro* and *in vivo* quantitative real-time polymerase chain reaction (qRT-PCR) analysis, were designed using Primer3Plus version 2.4.2, with a length of 18-23 bp and GC content of 50-60%. When possible, at least one intron was included, which allowed design of the primers at the exon-exon junction and thus, minimised the amplification of contaminant genomic DNA. Amplicon sizes ranged between 160-250 bp.

Annealing temperature conditions for each pair of primers of both target and reference genes were optimised in the melting temperature range of 58-62 ºC using the Verity Thermal Cycler 96-wells Fast (Applied Biosystems, Foster City, CA). Additionally, for *in vivo* assay, non-amplification of the cDNA derived from the fruit was also verified. Primer efficiency was determined by serial dilution method, using a mix of all cDNA samples as a template (see Supporting Table S2).

Gene expression analysis

First-strand cDNA synthesis was performed on 1 µg of DNase-treated RNA using the SuperScript IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). Oligonucleotides used for qRT-PCR analysis were designed as described above. qRT-PCR was performed using a 7500 Real Time PCR System (Applied Biosystems). The reaction mix consisted of 10 µL of GoTaq® qPCR Master Mix for Dye-Based Detection (Promega, Madison, USA), 300 nM of each primer and 2 µL of the diluted cDNA according to standard curves. Thermal conditions applied were as follows: i) hot start activation at 95 ºC for 2 min, ii) 40 cycles of denaturation at 95 ºC for 15 s, and iii) annealing/extension at 60 ºC for 1 min. To determine the melting curve, a final amplification cycle at 95 ºC for 15 s, 60 ºC for 1 min, 95 ºC for 30 s and 60 ºC for 15 s was applied. In all cases, a non-template control (NTC) was

included using DNAse free water instead of DNA. *M. laxa ACTIN* (*MlACT*) and *HISTONE H3* (*MlHISH3*) were used as independent reference genes in all the experiments. Relative expression levels were determined using the standard Cq method (Pfaffl, 2001). Expression values are presented relative to the glucose condition at 0.5 hpi time point or 24 hpi for the *in vitro* and *in vivo* analysis, respectively, and normalised to the geometrical mean of reference genes. Three technical replicates were analysed for each biological replicate for both the target and the reference genes.

Statistical analysis

Data were collated and statistically analysed with JMP® software version 13.1.0 (SAS Institute Inc., Cary, NC, USA). Means were compared by analysis of variance (ANOVA). When the analyses were statistically significant, Tukey's test at the level $p < 0.05$ was performed for separation of means, while comparisons between pH (adjusted or non-adjusted) for each medium at specific days, and comparisons between liquid media (glucose or pectin) at each time post-inoculation were done using the least significance difference value test (LSD; $p < 0.05$) using critical values of *t* for two-tailed tests. For phenotypic tests, growth rate was determined as the slope of a linear polynomial regression obtained by plotting growth diameter (mm) *vs*. time (days).

Results

Identification of two major family members involved in *M. laxa* pectin degradation

Following the methodology described, and considering the potentially secreted proteins in *B. cinerea* (Blanco-Ulate *et al.*, 2014), a BLAST search against the *M. laxa* genome was conducted. Among all the hits obtained, a total of three PMEs and six RG-HYDs were retrieved from the *M. laxa* genome and functionally annotated (Table 1). A coverage greater

than 88% was obtained for all the retrieved sequences with identities over 69% (Table 1). Theoretical isoelectric points were relatively low in all cases, ranging from 4.6 to 6.9, except for Histone H3, a well-known basic protein (Table 1). The molecular function, based on identification of conserved domains, demonstrated the presence of pectinesterase domains for the three PMEs identified and, as expected, confirmed the classification of the RG-HYDs into the GH28 family (Table 1). Notably, *MlRG-HYD4* and *MlRG-HYD5* also contain a PL-6 domain as observed for their homolog in *B. cinerea*.

Effect of different carbon sources on *in vitro* mycelial growth rate and phenotype

There were large differences among mycelial growth rates on the different media at 22 ºC. When the pH was not adjusted (Fig. 1a), the growth rate was greatest on the glucose-containing medium, with an average of 10.2 mm day^{-1} (see Supporting Table S3), and the largest colony diameter at 7 dpi was achieved. In contrast, adding pectin to the medium diminished the growth of *M. laxa* throughout the incubation period, compared to the control (PDA) or glucose-containing media. Concerning the morphological features (Fig. 1a, insert), a markedly lobed colony margin was observed under all growing conditions. Stromata were almost cream due to the sparsely produced conidia.

When the pH was adjusted to 4.5, however, the effect of the media on mycelial growth was completely different (Fig. 1b), and significant differences among the three media were found at all sampling points, except 1 dpi. At this pH, colony diameters were significantly greater ($p < 0.0001$) at 7 dpi on the pectin-containing media, than on PDA or the glucose-containing media. Similarly, the average growth rate on the pectin-containing media was greater than on the glucose-containing medium or the control (see Supporting Table S3). In the presence of pectin as the carbon source, sporulation was favoured and hence, the *M. laxa* colonies were greyish in colour, although no concentric ring of sporulation was observed (Fig. 1b, insert).

Moreover, the enhanced growth in the presence of pectin resulted in a diminished lobed morphology due to the increased formation of flabelliform outgrowths of mycelium which eventually coalesced to form a definite zone.

Effect of different carbon sources on in vitro pectin-modifying gene expression

To understand the effect that glucose and pectin had at a molecular level, transcriptional responses of *M. laxa* pectinases grown in liquid media enriched with these carbon sources were analysed by qRT-PCR. In parallel, the pH of the filtrate in which the fungus was grown was monitored at each sampling point (Fig. 2). Data obtained revealed acidification for both glucose- (from 4.79 to 2.74) and pectin-containing media (from 4.58 to 3.54). Regarding gene expression analysis, both *PMEs* and *RG-HYDs* gene families were detected *in vitro* expressed at different levels (Fig. 3 and 4). In relation to the *MlPME* family, no significant differences either over time or between carbon sources were observed in the case of *MlPME1* (Fig. 3a), although there was a tendency towards up-regulation in the presence of glucose and pectin at 6 and 48 hpi. Gene expression levels of *MlPME2* (Fig. 3b) did not change over time in the presence of glucose as a carbon source, but in the presence of pectin, there was a tendency to up-regulation from 0.5 to 6 hpi and at 48 hpi the increase in *MlPME2* transcript levels was significant compared with glucose (Fig. 3b). On the other hand, *MlPME3* showed the highest relative expression levels compared to the other *PME* family members (Fig. 3c), and these were similar with both glucose and pectin. This gene showed a tendency to up-regulation during the time-course of the experiment up to 10.1-fold and 10.9-fold after 48 hpi for glucose and pectin, respectively. These last results indicate that, in general, expression levels were not dependent on the presence of pectin, since they were quite similar regardless of the carbon source.

In the case of the *MlRG-HYD* family, the pectin-containing medium triggered a long-term up-regulation of *MlRG-HYD1* levels (30.4-fold) at 48 hpi when compared to glucose at 0.5 hpi, pointing to a carbon source-dependent expression profile (Fig. 4a). In fact, at all sampling points significant differences were found between glucose and pectin. In contrast, the primers designed for *MlRG-HYD2* gave non-specific results (data not shown). Regarding the expression levels of *MlRG-HYD3* (Fig. 4b), a slight glucose-induced repression was observed until 6 hpi, compared to pectin, but expression levels started to increase after 24 hpi and were sharply induced (14.8-fold) at 48 hpi. With regards to pectin, a significant induction (approx. 7.5-fold compared with the same treatment at 0.5 hpi) was observed at 48 hpi. Contrary to the trend observed with *MlRG-HYD3*, the expression levels of *MlRG-HYD4* were clearly dependent on the carbon source (Fig. 4c). Thus, while glucose-containing media barely affected the expression levels of *MlRG-HYD4* over time, the presence of pectin enhanced the induction of the transcript levels of this gene. Significant up-regulation was observed from 0.5 to 6 hpi (from 1.5-fold to 6.5-fold induction, respectively) followed by a decrease in expression levels. For *MlRG-HYD5*, while the presence of glucose barely affected the expression levels, the pectin-containing media apparently repressed the expression of this gene over time, from 0.8–fold at 6 hpi to 0.3-fold at 48 hpi, but this trend was not statistically significant (Fig 4d). Finally, the expression profile of the last member of the family, *MlRG-HYD6*, was similar to that observed for *MlRG-HYD3* (Fig. 4e), but on a much smaller scale and without differences between glucose and pectin, except for 2 hpi. In general, up-regulation was observed for both carbon sources, reaching 3-fold and 4.2-fold for glucose and pectin, respectively, at 48 hpi.

Taken together, specific and different expression levels, even within the members of the same pectinase family, were observed with both carbon source and time-dependent expression profiles.

Pectin-modifying gene expression during *M. laxa* infection on peaches

Based on the *in vitro* gene expression results, a more in-depth *in vivo* study was conducted in order to gain insight into the pathogenesis of *M. laxa* when infecting fruit, covering the different infection stages, from cell wall maceration to penetration and fruit colonization (Fig. 5). After 24 hpi, epidermal discoloration in the form of brown spots was visible (Fig. 5a); although the fruit cell wall had softened and started to macerate, the fungus was not yet able to penetrate. A few hours later, penetration was detected (Fig. 5b), but at a very superficial level, and brown spots at the inoculation sites had started to darken, becoming smooth grey. At 48 hpi, the infected area had expanded over the flesh tissue (Fig. 5c) encompassing the inoculated sites. Thereafter, at 72 hpi, the entire area was necrotic and completely colonized by the pathogen (Fig. 5d). Furthermore, the spread of brown rot was accompanied by local acidification of the host tissue. Overall, the fungus decreased the pH of the peach from 4.0 to 3.4 (data not shown).

Analogous to what occurred in the *in vitro* study, all the genes from the *MlPMEs* and *MlRG-HYDs* families were expressed at different levels during the infection process on peaches (Fig. 6). Results regarding *MlPME1* (Fig. 6a) showed that, although no significant differences were observed among different time points, there was a clear tendency towards up-regulation at 72 hpi (72.9-fold compared to 24 hpi). A significant up-regulation over time was observed for *MlPME2* (Fig. 6b) and *MlPME3* (Fig. 6c), indicating a similar role for the three PMEs, but primarily at the more advanced stages of the disease.

Results obtained from the *RG-HYD* family showed different expression profiles for each member (Fig. 7). Regarding *MlRG-HYD1* (Fig. 7a), low levels of expression were detected in the early stages of infection, but a 151.3-fold up-regulation occurred at 72 hpi. For *MlRG-HYD2* (Fig. 7b), a transient increase up to 10.5-fold was detected at 48 hpi compared to 24 and 38 hpi, but by 72 hpi, expression levels had declined to earlier levels. A similar

expression pattern to that observed for *MlRG-HYD1* was noted for *MlRG-HYD3* (Fig. 7c), in which expression was negligible from 24 hpi to 48 hpi, but was dramatically up-regulated at 72 hpi (164.9-fold). With respect to *MlRG-HYD4* (Fig. 7d), a slightly repression was observed at both 38 and 48 hpi, but a tendency towards up-regulation occurred at 72 hpi, this was not significant. Expression of *MlRG-HYD5* was significantly reduced over time (Fig. 7e), especially at 48 and 72 hpi, when expression levels were significantly lower compared to 24 hpi (1.7-fold and 2.3-fold, respectively). Finally, results obtained for *MlRG-HYD6* (Fig. 7f) showed an expression profile quite similar to that obtained for *MlRG-HYD4*, resulting in an early repression (38 and 48 hpi), followed by an increase in transcript levels at 72 hpi, although not significantly different.

Discussion

The ability of fungi to infect a specific host is partly determined by the secreted enzymatic machinery, responsible for the degradation of the complex web of carbohydrates, glycoproteins and phenolic compounds of the plant cell wall (Mendgen *et al.*, 1996). Hence, the first challenge to understanding the pathogenic mechanisms occurring during plant-pathogen interactions is to assess which enzymes might be important and thus worth investigating. In this context, enzymatic degradation accomplished by pectinases is of particular interest due to their ability to weaken the cell wall, causing tissue maceration, the characteristic symptom of soft-rot diseases (Walton, 1994). Indeed, further evidence of the importance of pectin-degrading enzymes was provided by Blanco-Ulate *et al.* (2014), who showed that endo- and exo-PGs and RGases were the most abundant CAZymes among those expressed during infection of lettuce leaves, tomato and grape berries by *B. cinerea*.

Among the diverse arsenal of CWDEs produced by fungi, GHs are the largest and most diverse group of enzymes involved in degradation of the components of the plant cell wall

(Murphy *et al.*, 2011). The GH28 subfamily includes the well-studied PGs (Kubicek *et al.*, 2014). GH28 also contains less thoroughly studied enzymes such as RG-HYDs, that cleave the α-1,2-glycosidic bonds formed between D-galacturonic acid and L-rhamnose residues in the hairy regions by both exo- and endo-mechanisms (van den Vrink & de Vries, 2011). PMEs, belonging to the CE8 subfamily, also have a major role since they are assumed to start pectin de-esterification into methanol and polygalacturonic acid, allowing the subsequent action of the other depolymerising enzymes (Reignault *et al.*, 2008). However, hardly anything is known about their contribution in *Monilinia* spp. pathogenesis, although their activity may be of major importance for complete degradation of pectin by PGs and PLs (Valette-Collet *et al.*, 2003). The present study therefore focused on understanding the role that both PMEs and RG-HYDs might play in *M. laxa*-stone fruit interaction.

Gene identification in the recently available *M. laxa* genome sequence (Naranjo-Ortiz *et al.*, 2018) allowed identification of all the genes already described as being secreted by the closest homolog *B. cinerea* during the interaction with its host (Blanco-Ulate *et al.*, 2014). In the present study, results obtained *in vitro* demonstrated that the three *MlPMEs* were differentially expressed in liquid culture containing different carbon sources. Besides this, all the genes showed different and distinct regulation patterns. Thus, while the expression of *MlPME1* was unaffected by the carbon source, *MlPME2* and *MlPME3* seemed to be dependent on the presence of pectin, especially at 48 and 6 hpi, respectively. These results agree with those of Shah *et al.* (2009), who reported that the secretion of most of the pectinases in *B. cinerea* depended on the carbon substrate used by the fungus, with the exception of two PMEs that were independent of the carbon substrate. Consistent with those observations, Valette-Collet *et al.* (2003) reported that *Bcpme1* was expressed in a glucose-containing medium to the same extent as in a pectin-containing medium, as has been demonstrated for *MlPME1* in the study presented here. Notwithstanding, the results presented

here have shown that the presence of pectin is important, at least, for *MlPME2* and *MlPME3 in vitro* gene expression. Indeed, this is confirmed by the results of the phenotypic test on solid media since pectin increased *M. laxa* growth, probably because conditions were closer to the host cell environment. Taken together, these results indicated *MlPME2* and *MlPME3* as key to degradation of the host cell wall *in vivo*. In agreement with this, a global transcriptomic study of the three most common *Monilinia* spp. revealed that a PME from *M. laxa* was included among the differentially expressed transcripts (De Miccolis Angelini *et al.*, 2018). Furthermore, this PME (*B. cinerea* protein ID: BCIN_03g03830) is coincident with the putative functionally annotated *MlPME2* studied in this work.

Interestingly, the results from the present study also confirmed the role of pH in PMEs' performance, since the enhanced growth of *M. laxa* on pectin media was only evident at pH 4.5, which led us to hypothesize that this pH was optimal for pectinase production. In fact, Obi *et al.* (2018) previously showed that *M. laxa* grew better in moderately acidic conditions (pH 6.40 to 4.21). It is known that pH values at which PMEs are active range from 4 to 8, but fungal PMEs have a narrower optimum pH range (between pH 4 and 6) than those of bacterial origin (Jayani *et al.*, 2005). Accordingly, not only the carbon source, but the pH may have an effect on PMEs' activity. In this sense, the results of gene expression under *in vitro* conditions presented here supported this because acidification of the culture medium occurred when *M. laxa* was grown either on glucose or pectin, and may suggest that the tendency of *MlPME3* to up-regulation could be favoured by low pH rather than by the carbon source itself. Regarding the diverse RG-HYDs analysed in the present study, an equally complex expression pattern was obtained. Remarkably, expression levels of the *MlRG-HYD* family were higher in most cases than for the *MlPME* family and occurred later, with the exception of *MlRG-HYD4*. This shared later induction may be explained by the strong decrease in pH (from 6 to values below 3.5) during *M. laxa* growth in liquid media. The pH is

one of the most important parameters of aqueous environments since it affects the activity of individual enzymes and thus, the activity of virulence factors secreted by the pathogen (Prusky & Yakoby, 2003). The involvement of pH-controlled gene expression in fungal virulence has been documented in several pathosystems. For instance, in the case of the PGs, Wubben *et al.* (2000) reported the effect of low pH on the positive regulation of the *B. cinerea BcPG3* gene.

The increasing amounts of the enzymes that break down pectic substances of the middle lamella bring about maceration, the characteristic symptom of brown rot, as well as other soft rot diseases (Bateman & Millar, 1966). In the present study, maceration of 'Merryl O'Henry' peaches infected with *M. laxa* was observed around 38 hpi, coinciding with a significant increase in expression levels of *MlPME2* and *MlPME3*, especially the former. In *Botrytis*, some controversy exists in the case of PMEs since while Valette-Collet *et al.* (2003) proposed that *PME1* is the determinant for *B. cinerea* virulence on different hosts, Kars *et al.* (2005b) reported that *BcPME1* and *BcPME2* were not required for virulence. The results from our study indicated the importance of these enzymes during invasion of fruit. In addition, since enhanced *MlPME2* and *MlPME3* expression was observed when the pH decreased, we hypothesise that these genes may be up-regulated by the pH. In this sense, it has been reported that fruit colonization is enhanced by differential pH modulation by the pathogen as a host-dependent mechanism (Bi *et al.*, 2016). In this way, fungi adjust their ambient pH in order to optimise the activity of their enzymatic arsenal (Alkan & Fortes, 2015). In fact, during the *M. fructicola* infection, gluconic acid has been reported as the main organic acid associated with peach acidification (De Cal *et al.*, 2013), and hence, could act synergistically with PMEs, which are known to be pH-sensitive (Pelloux *et al.*, 2007).

In agreement with the above studies, the *in vivo* results presented here also showed that brown rot spread was accompanied by a decrease in the pH. In this context, fungal PMEs may be responsible for acidification due to their action on some components of HG such as D-galacturonic acid. As a result of hydrolysis by these enzymes, de-esterified galacturonic acid residues are released, exposing negative charges at the polymer surface (Höfte & Voxeur, 2017). Indeed, D-galacturonic acid could cause the drop in pH levels, as reported by Vilanova *et al.* (2014) in the *Penicillium digitatum*-apples interaction when gluconic and galacturonic acids were detected, which may have contributed to the tissue pH decrease. This decrease in pH, initially mediated by the action of PMEs, may lead to the activation of other enzymes such as RG-HYDs known to have a lower optimum pH range and a later induction, as mentioned above.

In vivo results from *MlRG-HYDs* were similar to those observed *in vitro* with pectin-containing medium and noticeably, *MlRG-HYD2* was detected. As reported by Prusky *et al.* (2001), although several genes encode CWDEs, only specific ones are activated during pathogenicity *in vivo*. This was also true for *B. cinerea*, as among all PGs only *BcPG2* was shown to be a virulence factor (Kars *et al.*, 2005a). Furthermore, the induction of *MlRG-HYD2* was maximum after 48 hpi, whilst, in general, other RG-HYDs reached their maxima later. The comparison of the expression patterns also showed that *MlRG-HYD1* was significantly over-expressed, unlike *MlRG-HYD5* that was downregulated over time, coinciding with the decrease in pH. It is apparent, therefore, that among several other factors, pH decrease could act as a significant determinant of the *in vivo* activity of *MlRG-HYD5*. This raises the possibility that regulation occurred through the zinc finger protein denoted PacC, involved in the regulation of pH-controlled genes in filamentous fungi (Peñalva *et al.*, 2008). PacC activates transcription of those pathogenicity factors depending on the environmental pH in several host-pathogen interactions (Alkan *et al.*, 2013; Barad *et al.*,

2016). All these differences among the members of RG-HYDs family would provide the fungus with versatile and complementary tools for degrading substrates under different conditions during degradation of the cell wall. Moreover, both the host itself and the phenological stage of the fruit could affect the activation of the different members of the pectinase families.

On the basis of this study it can be concluded that PMEs and RG-HYDs have a large impact on the *M. laxa*-stone fruit interaction and further studies are needed. In this context, much effort has been directed to obtaining pectinase-deficient mutants. Obtaining gene knockout mutants is considered the most direct way to confirm the involvement of a gene (Tian *et al.*, 2016). However, there are many cases in which the absence of one gene is counteracted by another member, especially in multigene families, such as those of CWDEs. Thus, studies based on proteomic analysis could help to unravel the role of each enzyme at the different infection stages and might explain if certain pectinases are important determinants of *M. laxa* virulence. Moreover, considering that penetration success relies largely on the result of the combined action of a diverse arsenal of CWDEs produced by fungi (Glass *et al.*, 2013), research focused on other relevant enzymes, apart from PMEs and HYDs, should be considered to provide a complete overview of the decay development.

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Supporting Information legends

Table S1 Maturity date, minimum and maximum values of single index of absorbance difference (I_{AD}), cheek diameter (CD), flesh firmness (FF), soluble solids content (SSC) and titratable acidity (TA) of 'Merryl O'Henry' peach cultivar. Data represent the mean ± the standard deviation of the mean (n = 20)

Table S2 Quantitative real-time polymerase chain reaction (qRT-PCR) primer set

Table S3 Comparison of *Monilinia laxa* growth rate at different media (potato dextrose agar (PDA) or PDA supplemented with 1% (*w/v*) glucose or pectin), initial pH value of the non-adjusted media (pH_0) and goodness-of-fit (R^2) statistic of the regression

Figure legends

Figure 1 Effect of different carbon sources on *Monilinia laxa* growth rate (mm) at different pHs (non-adjusted (a) and adjusted to 4.5 (b)). Plates of potato dextrose agar (PDA) ( ) or PDA supplemented with 1% (*w/v*) glucose ( ) or pectin ( ) were inoculated by applying a drop of 10 µL at 10^5 conidia mL^{-1} and incubated in the dark at 22 ºC for 7 days. Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 6). For each day post-inoculation (dpi), asterisks denote significant differences among media according to analysis of variance (ANOVA) and Tukey's HSD test ($p < 0.05$). The inserted images in Fig. (a) and (b) reported phenotypic differences among the three media (from left to right: PDA, PDA + Glucose and PDA + Pectin) at 5 dpi.

Figure 2 Changes in pH of *Monilinia laxa* liquid cultures supplemented with 1% (*w/v*) glucose ( ) or pectin ( ), inoculated at 10^5 conidia mL^{-1} and incubated for 0.5, 2, 6, 24 and 48 hours post-inoculation (hpi) at 22 ºC on an orbital shaker under a constant agitation of 150 rpm. Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 2).

Figure 3 Changes in *in vitro* gene expression levels of *Monilinia laxa* pectin methyl esterases (*MlPME1* (a), *MlPME2* (b) and *MlPME3* (c)) after exposure for 0.5, 2, 6, 24 and 48 hours post-inoculation (hpi) at 22 ºC under glucose- ( ) or pectin-containing liquid media ( ). Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 2). Lowercase and uppercase letters indicate significant differences for glucose- or pectin-containing liquid media, respectively, over the post-inoculation period according to analysis of variance (ANOVA) and Tukey's test ($p < 0.05$). For each time post-inoculation,

asterisks denote significant differences between glucose- and pectin-containing liquid media according to ANOVA and LSD test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Figure 4 Changes in *in vitro* gene expression levels of *Monilinia laxa* hydrolases (*MlRG-HYD1* (a), *MlRG-HYD3* (b), *MlRG-HYD4* (c), *MlRG-HYD5* (d) and *MlRG-HYD4* (e)) after exposure for 0.5, 2, 6, 24 and 48 hours post-inoculation (hpi) at 22 ºC under glucose- ( ) or pectin-containing liquid media ( ). Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 2). Lowercase and uppercase letters indicate significant differences for glucose- or pectin-containing liquid media, respectively, over the post-inoculation period according to analysis of variance (ANOVA) and Tukey's test ($p < 0.05$). For each time post-inoculation, asterisks denote significant differences between glucose- and pectin-containing liquid media according to ANOVA and LSD test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Figure 5 Visualisation of brown rot spread in 'Merryl O'Henry' peach fruit inoculated by applying 6 drops of 50 µL each of a conidial suspension of *Monilinia laxa* at 10^6 conidia mL^{-1} and incubated for 24 (a), 38 (b), 48 (c) and 72 (d) hours post-inoculation (hpi) at 22 °C and 100% relative humidity (RH).

Figure 6 Changes in *in vivo* gene expression levels of *Monilinia laxa* pectin methyl esterases (*MlPME1* (a), *MlPME2* (b) and *MlPME3* (c)) after 24, 38, 48 and 72 hours post-inoculation (hpi). Fruit were inoculated by applying 6 drops of 50 µL at 10^6 conidia mL^{-1} and incubated at 22 ºC and 100 % relative humidity (RH). Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 3). For each gene, different letters indicate significant differences according to analysis of variance (ANOVA) and Tukey's test ($p < 0.05$).

Figure 7 Changes in *in vivo* gene expression levels of *Monilinia laxa* hydrolases (*MlRG-HYD1* (a), *MlRG-HYD2* (b), *MlRG-HYD3* (c), *MlRG-HYD4* (d), *MlRG-HYD5* (e), and *MlRG-HYD6* (f)) after 24, 38, 48 and 72 hours post-inoculation (hpi). Fruit were inoculated by applying 6 drops of 50 µL at 10^6 conidia mL^{-1} and incubated at 22 ºC and 100 % relative humidity (RH). Each point represents the mean and vertical bars indicate the standard deviation of the mean (n = 3). For each gene, different letters indicate significant differences according to analysis of variance (ANOVA) and Tukey's test ($p < 0.05$).

Table 1 Putative *Monilinia laxa* genes involved in pectin degradation. Columns indicate: putative *Botrytis cinerea* ortholog; gene unique identifier (ID); gene name; BLAST coverage; BLAST identity; conserved domains; putative protein function; subcellular localization, isoelectric point (pI) and molecular weight (MW) according to an annotation based on homology searches and manual inspection of the sequences.

***B. cinerea* protein ID**	***M. laxa* ID**	**Gene name**	**Coverage**	**Identity**	**Conserved domain**	**Putative protein function**	**Subcellular localization**	**pI**	**MW (Da)**
BCIN_16g02020	Monilinia_011730	*MlACT*	100.00	100.00	Nucleotid-binding domain of the sugar kinase/HSP70/actin	Actin	Other	5.45	41639.50
BCIN_13g04410	Monilinia_062010	*MlHISH3*	100.00	100.00	H4	Histone H3	Other	11.15	15318.80
BCIN_08g02970	Monilinia_037510	*MlPME1*	99.13	81.40	Pectinesterase	Pectin methyl esterase	Secretory pathway	6.59	37528.63
BCIN_03g03830	Monilinia_038540	*MlPME2*	99.14	79.31	Pectinesterase	Pectin methyl esterase	Secretory pathway	5.08	37153.04
BCIN_14g00860	Monilinia_000370	*MlPME3*	100.00	84.40	Pectinesterase	Pectin methyl esterase	Secretory pathway	6.89	34350.99
BCIN_06g02140	Monilinia_004100	*MlRG-HYD1*	88.83	69.06	GH28	Rhamnogalacturonan hydrolase	Secretory pathway	6.98	41403.98
BCIN_05g04950	Monilinia_041700	*MlRG-HYD2*	95.82	74.46	GH28	Rhamnogalacturonan hydrolase	Secretory pathway	5.08	56394.30
BCIN_10g05010	Monilinia_017980	*MlRG-HYD3*	100.00	91.95	GH28	Rhamnogalacturonan hydrolase	Secretory pathway	5.62	47332.55
BCIN_12g01100	Monilinia_006410	*MlRG-HYD4*	99.77	90.29	GH28/PL-6	Rhamnogalacturonan hydrolase	Secretory pathway	5.89	46484.12
BCIN_13g02640	Monilinia_069910	*MlRG-HYD5*	99.64	86.69	GH28/PL-6/Atrophin 1	Rhamnogalacturonan hydrolase	Secretory pathway	6.00	60283.38
BCIN_10g06130	Monilinia_023000	*MlRG-HYD6*	98.61	80.79	GH28	Rhamnogalacturonan hydrolase	Secretory pathway	4.67	46652.10