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Acidification by gluconic acid of mango fruit tissue during colonization via stem end infection by *Phomopsis mangiferae*

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ABSTRACT

Colonization of mango and other deciduous and tropical fruit by *Phomopsis mangiferae* was accompanied by local acidification of the host tissue. The fungus acidified the host tissue in mango and grape from pH 5.1 and 4.1, respectively, to 3.8 and 2.5. Analysis of the acidification process in colonized fruit showed that gluconic acid was the main organic acid accumulated at the infection site and in the lesion tissue. In liquid culture conditions the relative induction of transcripts of *pmgox1*, encoding for glucose oxidase (GOX) was 8–12 times greater at pH 7.0 and 8.0 than at pH 4.0. In infected fruit the detection of high levels of transcripts of *pmgox1* and the accumulation of gluconic acid and H_2O_2 in the decayed tissue, suggested that glucose oxidase contributed to the acidification of the tissue. At the same time, transcripts encoding the endopolygalacturonase gene, *pmpg1* accumulated greatly under acidic culture conditions, signifying the importance of the acidification in enhancing the pathogenicity of *P. mangiferae*. Our results indicate that ambient pH is a regulatory cue for processes linked to pathogenicity of postharvest pathogens, and that specific genes are expressed as a result of the environmental pH created by the pathogen.

1. Introduction

Phomopsis mangiferae Ahmad. is one of the main pathogens that cause stem end rots during postharvest storage of fruit. Infections occur in the orchard and are enhanced as the trees age. Hyphae of the fungus colonize the floral parts of mango trees, develop endophytically in healthy tissue of all parts of the plants, but especially in the fruit pedicel, and remain quiescent until the fruit matures. At this stage the parasite renews its development and starts to grow through the fruit stem end via the xylem vessels of the maturing fruit (Johnson et al., 1992; Prusky et al., 2009). During stem end development the pathogen produces a dark, circumpeduncular lesion with defined edges, which spreads relatively slowly but penetrates deeply into the flesh.

Levels of infected fruit after harvest are affected by periods of rain and high RH at the beginning and end of the dry season (Johnson and Sangchote, 1994). The initial systemic infection by *P. mangiferae* plays a crucial role in establishing blossom-blight infection, but secondary infection is apparently an even more important factor in development and incidence of stem end soft rots. Secondary infections occur when rain washes spores away from various inoculum sources such as leaves and stems (Saaiman, 1996; Prusky et al., 2009).

A similar pathogen, *Phomopsis cucurbitae*, which attacks cucurbits, produces significant amounts of hydrolytic enzymes, among which a polygalacturonase (PG) is especially abundant (Zhang et al., 1997). Polygalacturonase has been implicated as a virulence factor in several plant-infecting fungi, particularly in diseases characterized by tissue maceration or soft rot (Marciano et al., 1983; Prusky et al., 1989; Hadas et al., 2007).

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 genes 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* are described as pathogens that alkalinize the tissue during decay development in several subtropical fruit (Prusky and Yakoby, 2003; Alkan et al., 2008), and *Penicillium* spp., *Sclerotinia* and *Botrytis* have been described as acidifiers of the decayed tissue. *Sclerotinia sclerotiorum* (Magro et al., 1984) and *Botrytis cinerea* (Verhoeff et al., 1988) acidify their host tissues by producing oxalic acid; in the case of *Penicillium expansum*, gluconic acid is produced as the main organic acid. Organic acids are secreted during infection, and create the acidic environment necessary for the activity of the PG produced by these fungi (Magro et al., 1984; Prusky and

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Yakoby, 2003; Hadas et al., 2007). Taken together, these results suggest that environmental pH is important as a global regulator for enhancing the virulence of several postharvest pathogens.

Our objectives in the present study were (i) to determine whether *P. mangiferae* modulates the fruit environment pH during colonization; (ii) to evaluate the pathogen contribution to ambient pH modulation during pathogenic attack, and to identify the mechanism(s); (iii) to determine whether ambient pH affects the transcriptional regulation of *P. mangiferae* genes that contribute to fungal colonization. We hypothesize that during fruit ripening *P. mangiferae* induces fruit acidification by means of gluconic acid accumulation, and that this acidification contributes to PG secretion and thereby enhances tissue maceration and colonization.

2. Materials and methods

2.1. P. mangiferae growth conditions

We utilized a wild-type *P. mangiferae* isolate (PM-10) taken from decayed 'Keitt' mango fruit, obtained in Israel. This isolate was routinely cultured on potato dextrose agar (PDA; Difco, Detroit, MI). To analyze the effects of ambient pH and carbon source on organic acid production, we inoculated 50 mL of M_3S medium (Mathur's medium containing, per liter, 1 g of yeast extract (Difco), 1 g of Bactopeptone (Difco), 10 g of sucrose, 2.7 g of KH_2PO_4, and 2.5 g of $MgSO_4{\cdot}7H_2O$ at pH 5.5, in a 125-mL flask with 10^6 spores/mL obtained from a 7-d-old sporulating culture. This liquid culture was incubated for 2 d at 25 °C, with shaking at 150 rpm. The entire culture was harvested by vacuum filtration onto sterile filter paper and washed twice under vacuum with 50 mL of sterile distilled water. The washed mycelia (average wet weight, 1.2 g) were resuspended and incubated for an additional 24 h, in 50 mL of fresh inducing medium containing, per liter: 1 g of K_2HPO_4, 0.5 g of $MgSO_4{\cdot}7H_2O$, 7 g of $NaNO_3$, 3 g of peptone, 0.5 g of KCl and 10 g of sucrose (secondary medium, SM). These secondary cultures were harvested by vacuum filtration; the supernatants were saved for pH determination and the hyphae for dry weight determination.

2.2. Fruit inoculation and treatments

Inoculation of all the fruit described in the present study was done by wounding the fruit on four sides to a depth of 2–3 mm, diameter 2 mm, and placing 10 μL of spore suspension containing 10^6 spores/mL into each wound. The inoculated fruit were then incubated for 7 d at 20 °C and 90% relative humidity. These fruit included mango cv. Keitt, persimmon cv. Triumph, grapefruit cv. Prime, orange cv. Valencia, clementine cv. Or, nectarine cv. Flavor Top, lemon cv. Villa Franca, apple cv. Top Red and Golden Delicious, pear cv. Spadona, tomato cv.1402, avocado cv. Fuerte and pepper cv. Maor.

To determine the effect of an inorganic acid, hydrochloric acid, on colonization, fruit wound sites were dip-treated with several concentrations of HCl 1 h prior to pathogen inoculation. The experiments were repeated at least three times with similar results. The results of one representative experiment are presented. Standard deviations (SDs) of the means were calculated.

2.3. Determination of organic acid concentrations in P. mangiferae colonized mango fruit and liquid media

2.3.1. Sample preparation

Fresh infected flesh tissue of mango fruit and the supernatant of *P. mangiferae*-inoculated secondary medium were used for extraction. The infected flesh tissue was first squeezed though miracloth to obtain the fruit juice, and the secondary medium was used "as is" for the analysis. All samples were then filtered through a 0.22 μm nylon filter and cleaned with solid-phase extraction (SPE) anion-exchange sorbent (Phenomenex Strata X-AW, 33 μm, 200 mg/mL). The SPE sorbents were conditioned with 2 mL of methanol and equilibrated with 2 mL of water. Aliquots of 100–500 μL of filtered sample were loaded onto the cartridge, which was then washed with 2 mL of water followed by 2 mL of methanol. Anions were eluted with 2 mL of acidic ethanol/acetonitrile (1% HCl). Purified samples were diluted 1:10 with acetonitrile/water (50/50), and the proper peaks were identified by comparison with commercial organic acids. The samples were quantified by comparison with weighed samples of the commercial organic acids.

2.4. Mass spectrometry analysis

Samples were analyzed with an Orbitrap Discovery mass spectrometer (Thermo Scientific, Bremen, Germany), equipped with an electrospray ion source. The mass spectrometer was operated in negative-ionization mode with the following settings: spray voltage, 3 kV; sheath gas flow, 35 (arbitrary units); capillary temperature, 250 °C; capillary voltage, 6 V. Data were acquired in the 50- to 1000-Da mass range. Samples were injected directly into the ion source with an infusion pump at a flow rate 5–15 μL/min.

2.5. pH measurement

The pH was measured in vitro with a Thermo-Orion Model 9810BN microcombination pH electrode (Thermo Fisher Scientific, Waltham, MA), in 0.5 mL aliquots that were taken at various times after fungal inoculation. For in vivo measurements, 1 mL aliquots were sampled 4 d after fungal inoculation, and four replicates were tested. All measurements were repeated on 10–12 fruit (at least 20 measurements), on the transverse axis of the lesion on each fruit. The standard deviation (SD) of the means of pH measurements was never higher than 2.5%.

For qualitative assessment of pH changes caused by the fungus on solid mango fruit extract media, the fungus was grown on solid media containing 100 g ripe flesh ground in 100 mL of DDW and 2% agar, brought to pH 7.0 with NaOH. The changes of pH induced by the fungus were detected by the addition of 50 μL of 0.1% Alizarin Red S (Prusky et al., 2004).

2.6. Analysis of expression of polygalacturonase (pmpg1) and glucose oxidase (pmgox1) by P. mangiferae

2.6.1. Cloning of pmpg1 and pmgox1

DNA fragments of 830 base pairs of partial coding sequence (NCBI Acc. No. GQ18049) from the *P. mangiferae pmpg1* gene were amplified by PCR on genomic DNA. The following primers were designed based on (NCBI Acc. No. GQ18049) genomic DNA fragments, using primer express software: F-(TCTTGTGGAATCGCTCTGGTG); R-(GACGTGCTCCACAATGTCAAAC).

DNA fragments of 449 base pairs of partial coding sequence (NCBI Acc. No. GQ18048) from the *P. mangiferae pmgox1* gene were amplified by PCR on genomic DNA. The following primers were designed based on (NCBI Acc. No. GQ18048) genomic DNA fragments, using Primer Express software: F-(TCTTGTGGAATCGCTCTGGTG); R-(GACGTGCTCCACAATGTCAAAC).

Primers of 18S were designed based on (NCBI Acc. No. FN386273) sequence, using Primer Express software: F-(ATCTCTTGGTTCTGGCATCG); R-(GCTTGAGGGTTGAAATGACG).

For sequencing of *pmpg1* and *pmgox1*, degenerative custom-made oligonucleotide primers were designed based on alignment between different PG and GOX genes of different fungi, and were sequenced by the DNA Sequencing Facility of the Weizmann Institute of Science, Rehovot, Israel. Homology to the *pepg1* and *pmgox1* was determined with the BLAST algorithm. Multiple amino acid

sequence alignment with *P. mangiferae pmpg1* was conducted by multiple sequence alignment (Corpet, 1988).

For the qRT-PCR, mycelia of *P. mangiferae* were grown on M_3S liquid medium for 3 d and transferred to a sucrose secondary medium. The mycelia were harvested by vacuum filtration, washed as described in Section 2.1, frozen at −80 °C and dried by lyophilization. To test the expression of *pmpgox1* during fungal colonization, 'Prime' grapefruit were inoculated as described before, and 7 d later, tissue was sampled and frozen at −80 °C pending RNA analysis. Mycelia samples were extracted for RNA with the SV Total RNA Isolation System (Promega, Madison, WI). Reverse-transcription was performed on 10 μg of total RNA with the Reverse-It First-Strand Synthesis Kit (ABgene, Surrey, UK). Up to 1 g of frozen tissue was ground to a fine powder in liquid N_2 with a pre-cooled pestle and mortar, and total RNA was extracted with cetyltrimethylammonium bromide (CTAB) according to Liao et al. (2004).

Samples of cDNA were diluted 1:10 (v/v) to the final template concentration for qRT-PCR, with the RotorGene 3000 system (Corbett Research, Sydney, Australia). PCR amplification was applied to 3.5 μL of cDNA template in 10 μL of a reaction mixture containing 5 μL of Syber-Green Amplification Kit (ABgene, Surrey, UK) and 300 nM of primers. The PCR conditions were initial denaturation for 15 min at 94 °C, 40 denaturation cycles of 10 s at 94 °C, annealing at 60 °C for 15 s, extension at 72 °C for 20 s (cycling A). The samples were subjected to melting-curve analysis with the RotorGene program. All samples were normalized to *18S* rRNA gene levels in the same qRT-PCR, and the values were expressed as the increase or decrease of the levels relative to a calibration sample. Each experiment was repeated at least three times with similar results; the results of one experiment are presented.

2.7. Detection of polygalacturonase activity

2.7.1. Polygalacturonase activity assay

All operations were carried out at 2–5 °C. The culture filtrate was concentrated by ultrafiltration, and the ultrafiltrate was dialyzed overnight against 10 mM sodium acetate buffer, pH 5.0, and loaded onto a CM-cellulose column (21 cm × 2.7 cm) equilibrated with the same buffer. A 1 mL aliquot of dialysis solution was mixed with 1 mL of substrate comprising 0.5% sodium polypectate in 0.01 M sodium acetate, pH 5.5. The mixture was incubated at 47 °C for 3 h and the control samples were incubated at −20 °C. After the incubation period the reaction was stopped by boiling for 10 min and the amount of product was measured by spectrophotometry at 660 nm (Nelson, 1944). An additional standard graph was used to determine the PG concentration necessary for catalyzing 0.5% of substrate to make reduced ends (parallel to 1 mg D-glucose), as described by Nelson (1944).

2.8. Fluorescence and staining methods

Changes in tissue ROS were detected by using the DCF D-399 fluorescence probes (Molecular Probes, Invitrogen, Eugene, Oregon, USA). Infected (7 d after inoculation) and uninfected fruit tissue samples comprising 5 mm × 5 mm pieces of decayed and undecayed pericarp with 4 mm deep mesocarp tissue were stained for ROS evaluation by exposing the tissue sample to 10 μM DCF for 15 min in the dark at 24 °C and then rinsing it twice in phosphate buffer saline (PBS). The tissue was sampled for ROS evaluation by slicing strips of parenchyma, 0.5 mm thick, 5 mm long and parallel to the surface, from the fruit pericarp and mesocarp tissue. The strips were viewed immediately after staining, with a Olympus model IX81 laser-scanning confocal microscope.

2.9. Statistical analysis

Data were analyzed with the JMP software package, version 3.2.6 (SAS Institute, Inc, Cary, NC). Mean values of *pmgox1*, *pmpg1* expression levels and PG activity were compared by using least significant difference, according to the Tukey–Kramer Multiple Comparison Test at $P \leq 0.05$. Mean values with different letters present in figures are significant.

3. Results

3.1. pH changes induced by P. mangiferae during colonization of fruit and cultures

Inoculation of various deciduous and subtropical fruit with spores of *P. mangiferae* elicited decreases in the pH of the colonized tissue, ranging from 0.2 pH units in lemon fruit up to 1.3 and 1.6 pH units in mango and grape, respectively (Fig. 1). Growth of *P. mangiferae* on mango extract solid medium also reduced the pH from 7.0 to 4.5, as indicated by the color change in Alizarin Red dye (Fig. 1B). *P. mangiferae* also reduced the pH of the secondary medium to an extent related to the initial pH: the pH fell by 3.3 pH units from an initial pH 8.0, but by only 1.5 pH units from pH 6.0 (Fig. 2). All of this suggests that *P. mangiferae* changes the pH in a pH-dependent manner.

3.2. Mechanism of acidification during colonization

3.2.1. Organic acid production by P. mangiferae in vitro

Analysis of organic acids secreted by *P. mangiferae* during 24 h of growth in secondary media whose initial pH ranged from 5.0 to 8.0 suggested that the main product was gluconic acid, together with minor amounts of citric and fumaric acids (Table 1). The highest accumulations of gluconic acid were observed at initial pH 7.0–8.0, and ranged from 465 μg/mL at pH 8.0 to 580 mg/mL at pH 7.0. Minor concentrations of citric acid were detected, with a maximum accumulation of 50 μg/mL at pH 7.0 (Table 1).

3.2.2. Organic acid accumulation induced by P. mangiferae in mango fruit

Analysis of organic acids found in mango fruit infected by *P. mangiferae* showed concentration increases in decayed tissue, from 45 to 150 μg/mL for citric acid and from 60 to 350 μg/mL for gluconic acid (Table 2). No significant production of fumaric and malic acids was detected in decayed mango tissue.

3.3. The relation between ambient pH, and pmgox1 and pmpg1 expression of P. mangiferae

The influence of culture ambient pH on the accumulation from *P. mangiferae* of transcripts of *pmgox1* encoding for glucose oxidase and of *pmpg1* encoding for endopolygalacturonase enzyme was examined by qRT-PCR analysis. Analysis of *pmgox1* expression of *P. mangiferae* was tested in infected grapes, which are one of the main hosts of the pathogen, similarly to mango fruit. It was found that inoculated grape fruit showed a 4.5-fold increase in relative expression in the decayed tissue (Fig. 3A). In vitro analyses of mycelial cultures on secondary media at a series of unbuffered pH values indicated that *pmgox1* transcript levels showed that *pmgox1* relative expression was highest at pH 8.0, declined at pH 6.0, and was lowest at pH 4.0 (Fig. 3B). It is also interesting that ROS accumulated at the hyphae in the infection site of fruit inoculated with *P. mangiferae* (Fig. 3C).

In contrast, analysis of the *pmpg1* transcript levels 20 h after induction in buffered secondary media showed that *pmpg1* accumulation was highest at pH 4.0 and significantly lower at higher

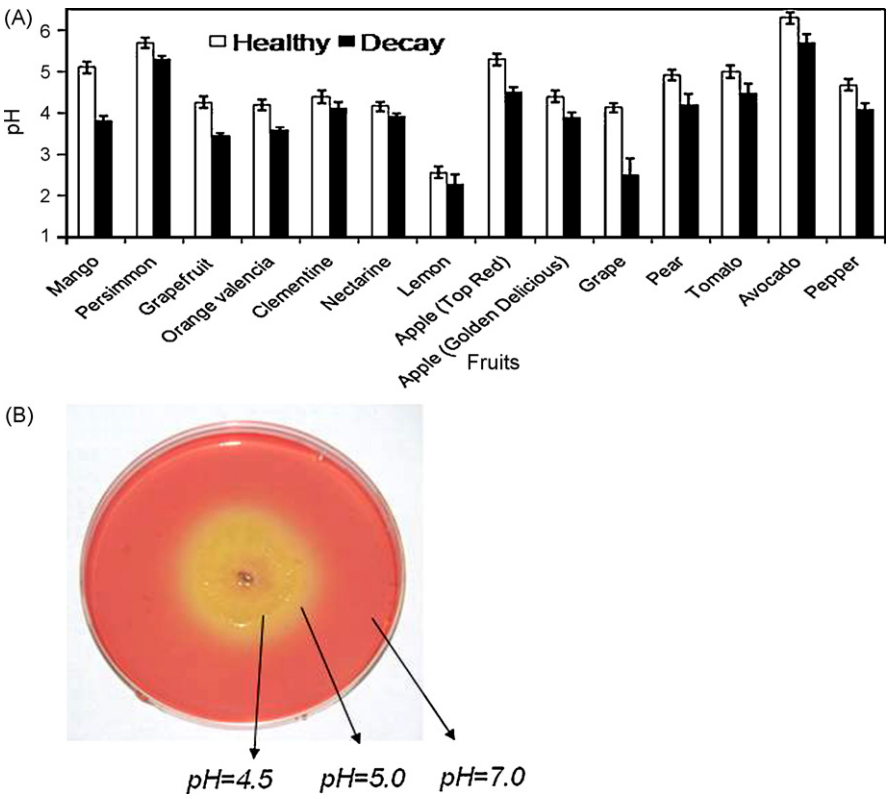

Fig. 1. pH changes induced by *Phomopsis mangiferaee* during colonization of (A) various subtropical and decidous fruit and (B) solid media containing mango fruit extract buffered to pH 7.0. pH monitored with Alizarin Red. Measurements were taken 7 d after inoculation of fruit and 5 d after inoculation of solid media. Results represent one out of four different experiments.

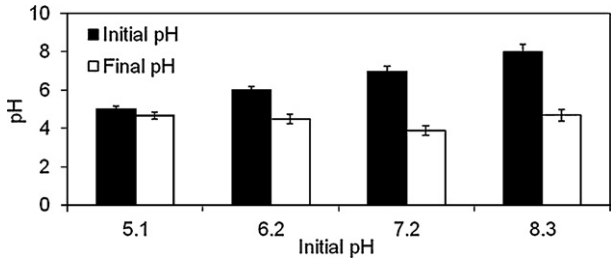

Fig. 2. Effect of initial pH on the acidification of secondary media by *P. mangiferae*. Spores of *P. mangiferae* were inoculated into primary media, and transfered to secondary media at various pH level after 4 d of growth. pH was evaluated 24 h after transfer to secondary media.

pH values (Fig. 4B). Similar results were obtained 24 h after induction (data not shown). The PG activity assay of the extracts obtained 20 h after transfer of hyphae to buffered secondary media showed a slightly different pattern from that of gene expression, with a maximal activity at pH 4.0–5.0 and lower activities at pH 6.0–7.0 (Fig. 4A). To demonstrate the importance of acidification for enhancement of *P. mangiferae* pathogenicity we treated inoculated fruit with an inorganic acid, such as hydrochloric acid (Fig. 5). Development of *P. mangiferae* decay was significantly enhanced when the inoculated mango fruit were treated with 10–25 mM HCl, which indicates the importance of acidification in fruit colonization.

4. Discussion

Postharvest pathogens of fruit and vegetables colonize infected tissue, causing significant maceration and decay. A key factor for pathogenicity of postharvest pathogens is the secretion of pectolytic enzymes, which is initiated during the transition from quiescent to active infection, and that results in tissue maceration (Miyara et al., 2008). Prusky et al. (2001) analyzed the mechanism of activation of pathogenicity factors of postharvest pathogens, and suggested that postharvest pathogens are involved in the shift in host environment pH that leads to conditions better suited for pathogen gene expression and enzymatic degradation of plant cell walls (Prusky and Lichter, 2008). Whereas *Colletotrichum* spp. locally increased ambient pH values in many hosts by secretion of ammonia (Prusky and Yakoby, 2003; Alkan et al., 2008), *Penicillium* spp., *Botrytis* spp., and *S. sclerotiorum* showed increased virulence in the acidic environments in which they cause disease (Rollins

Table 1
Effects of initial pH on the secretion of organic acids in culture media inoculated with *P. mangiferae*. Measurements were taken 24 h after transfer to secondary inducing media. Results represent the averages of four replications per treatment.

Organic acid	pH 5.0	pH 6.0	pH 7.0	pH 8.0
Acid content (μg/mL)				
Gluconic	190 ± 45	375 ± 40	580 ± 75	465 ± 60
Citric	16 ± 1.8	35 ± 3.2	50 ± 5	40 ± 1.6
Malic	Nd	Nd	Nd	Nd
Fumaric	14 ± 1.8	22 ± 4.5	26 ± 4.5	20 ± 5

Table 2
Detection of organic acid in decayed mango fruit cv. Keitt inoculated with *P. mangiferae*. Measurements were taken 7 d after inoculation of mango fruit. Results represent the averages of four replications per treatment.

Organic acid	Healthy (fruit)	Decayed (fruit)
Acid content (μg/mL)		
Gluconic	60 ± 30	350 ± 35
Citric	45 ± 22	150 ± 30
Malic	1820 ± 370	2010 ± 680
Fumaric	2 ± 0.3	13 ± 2

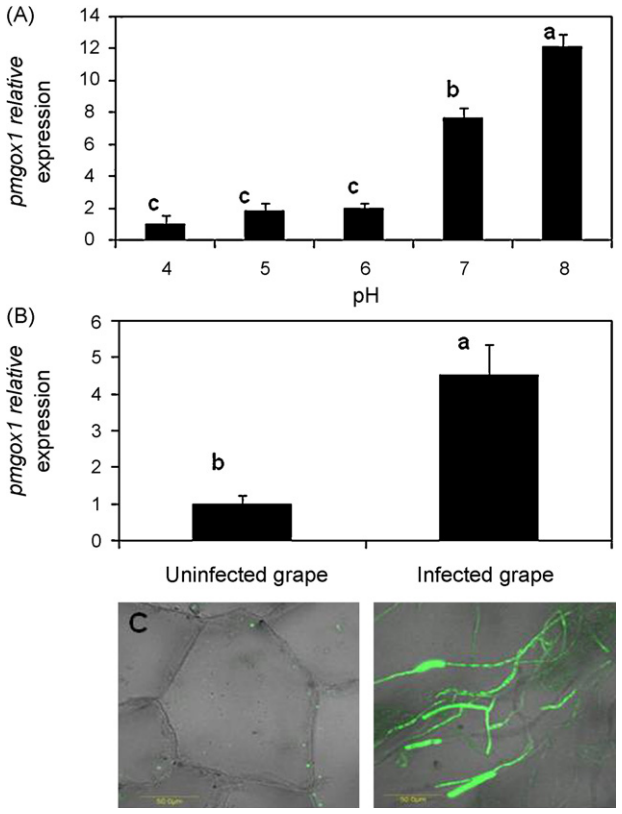

Fig. 3. Effect of initial pH on *pmgox1* relative expression by *P. mangiferae*. (A) *pmgox1* relative expression in vitro. (B) *pmgox1* relative expression in vivo. (C) ROS accumulation in *P. mangiferae* hyphae at the infection site in grapes cv. Prime. For in vitro experiment spores of *P. mangiferae* were inoculated into primary media, and transfered to secondary inducing media at various pH levels after 3 d of growth. For in vivo experiment, 7 d after inoculation of *P. mangiferae* into grapes, RNA was extracted from fruit and relative gene expression measured by qRT-PCR.

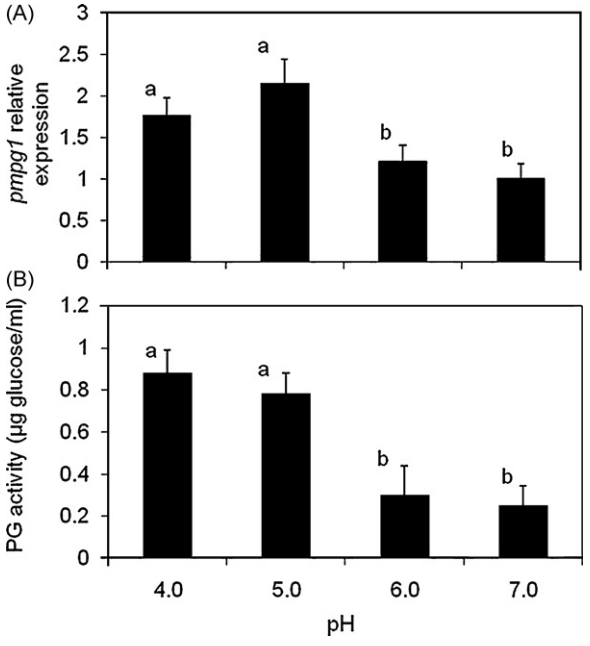

Fig. 4. Effects of pH conditions on the relative expression of *pmpg1* and enzyme activity of polygalacturonase in *P. mangiferae*. (A) Relative expression of *pmpg1* and (B) polygalacturonase activity. PG activity was detected in the filtered supernatant 24 h after transfer to a fresh secondary medium buffered with citrate–phosphate to the indicated pH values. Total RNA isolated from *P. mangiferae* mycelia was used for RT-PCR analysis, using the 475-bp segment of *pmpg1*, with a ribosomal DNA (rDNA) fragment as a control.

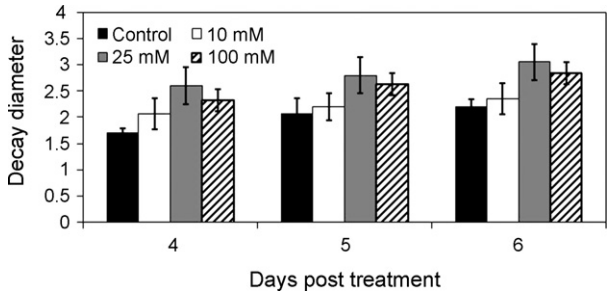

Fig. 5. Effect of hydrochloric acid treatment on *P. mangiferae* colonization in mango fruits cv. Keitt. Mango fruit were inoculated with 2.5×10^3 *P. mangiferae* spores, and 48 h later the fruit was dip-treated in various concentrations of hydrochloric acid. Lesion size was measured 4–6 d post-treatment.

and Dickman, 2001; Hadas et al., 2007). *Phomopsis mangiferae*, a key postharvest pathogen that causes stem end rots of mango fruit, also actively reduced the pH of the tissue by 1.2–1.6 pH units during decay development. Examination of the various inoculated fruit showed that in fruit such as mango and grape, in which the reduction in pH was sharpest, the extent of *P. mangiferae* attack was generally greatest.

How does *P. mangiferae* acidify the host tissue? Growth of *P. mangiferae* in liquid media in the presence of 1.5% sucrose at pH 5.0 was accompanied by accumulation of gluconic acid at about 200 µg/mL, which increased to 580 µg/mL as the initial pH of the medium was higher up to pH 7.0. Under the same conditions the concentrations of other organic acids, such as citric and fumaric, did not increase. However, analysis of organic acids in the decayed tissue showed not only an increase of gluconic acid but also small increases in citric acid, ranging from 16 to 50 µg/mL, suggesting that the fungal metabolism differs between in vivo and in vitro conditions. Also in *Penicillium* a similar behavior was observed, i.e., slight accumulation of citric acid in vivo, but strong accumulation of gluconic acid both in vivo and in vitro (Hadas et al., 2007). This accumulation of gluconic acid in decayed mango fruit infected by *P. mangiferae* suggests, as found for *Penicillium* infection of apple and grapefruit (Prusky et al., 2004), that the acidification of the tissue resulted mainly from the accumulation of gluconic acid, with a possible contribution of citric acid. Gluconic acid is produced by the enzyme glucose oxidase (GOX), which catalyzes the oxidation of β-D-glucose to H_2O_2 and D-glucono-1,5-lactone, which hydrolyze spontaneously to gluconic acid (Anastassiadis et al., 2003). Hadas et al. (2007) suggested that gluconic acid accumulation might facilitate fungal pathogenicity by chelating Ca^{2+} ions and thereby weakening the host cell wall (Martell and Calvin, 1952; Magro et al., 1984) or directly causing cell death by H_2O_2 accumulation (Dutton and Evans, 1996; Sillanpaa et al., 2003).

In the present study, activity of GOX could not be detected in healthy mango tissues but was measured in the decayed area of *P. mangiferae*-infected fruit. Genes encoding *gox* have been cloned from several strains of *Aspergillus* and *Penicillium* spp. as well as from *Talaromyces flavus* and *B. cinerea* (Frederick et al., 1990; Hatzinikolaou et al., 1996): one putative gene, *gox1*, encoding GOX was detected in *P. mangifera* and showed high homology to GOX from *A. niger* (Frederick et al., 1990; Hatzinikolaou et al., 1996). In the present study the expression of *pmgox1* increased with increasing pH, up to pH 8.0. It is possible that *pmgox1* is induced in the fruit under natural conditions, as ripening results in alkalinization and increased sugar availability (Prusky and Lichter, 2008), conditions that trigger the activation of the pathogenic process of the pathogen. Under these conditions, acidification of the environment by citric and gluconic acids could increase (Hadas et al., 2007). These phenomena might have synergistic effects that could enhance the expression of genes and secretion of specific enzymes needed to

facilitate fungal attack (Prusky et al., 2001). Accumulation of citric and gluconic acids was shown to decrease calcium activity in the intercellular spaces of plant tissues, and to alter mineral balances; it would thereby affect the stability of cell membranes and cell wall pectate polymers (Martell and Calvin, 1952; Marciano et al., 1983; Magro et al., 1984). Destabilization of cell membranes and cell walls would enhance sensitivity to pathogen-produced pectolytic enzymes, similarly to what has been reported for oxalic acid (Maxwell and Lumsden, 1970). Secretion of organic acids might also have an indirect effect, through suppression of fruit resistance. It was reported that the secretion of oxalate by *S. sclerotiorum* suppressed the plant oxidative burst (Cessna et al., 2000). If this were combined with reduction in host pH, which would inhibit the activities of plant-produced polyphenol oxidase (Marciano et al., 1983; McCallum et al., 2002), it could make a relatively broad and significant contribution to pathogenesis.

What is the importance of tissue acidification for *P. mangiferae* virulence? Hadas et al. (2007) and McCallum et al. (2002) indicated that aggressive *P. expansum* isolates reduced the pH faster than weaker ones. The capability of pathogens to acidify the environment has led to the expression of genes encoding secretion of many hydrolytic enzymes (Bateman and Beer, 1965), including PGs, in *Penicillium* and *Botrytis* infections (Ten Have et al., 1998; Rollins and Dickman, 2001). Transcript analysis of the endoPG-encoding gene *pmpg1* from *P. mangiferae* shows that it occurred at pH levels between 3.0 and 4.0, with the highest transcript level observed at pH 4.0. Furthermore, the fact that the most common hosts of *P. mangiferae* are mango and grape, which showed the steepest pH falls, supports the hypothesis that acidification of the tissue enhances *pmpg1* expression and host colonization.

The production rate of GOX and the synthesis of gluconic acid have been shown to be affected by various conditions such as glucose concentration, pH value (Anastassiadis et al., 2003) and oxygen level (Sankpal and Kulkarni, 2002). Sucrose accounts for almost 50% of the total soluble solid (TSS) concentration, a factor that determines fruit sweetness. The TSS concentration in mango and grape usually increases from 8.0–9.0% to 13–14% during fruit growth and postharvest ripening (Lurie and Klein, 1990), conditions under which *P. mangiferae* commonly attacks (Prusky et al., 2009). Hadas et al. (2007) showed that GOX activity and gluconic acid production by *P. expansum* during growth at pH 8.0 were 33 and 220 times, respectively, higher than those measured at pH 4.0. Also in *P. mangiferae*, expression of *pmgox1* was 12 times greater at pH 8.0 than at pH 4.0. This is a peculiar observation, in light of the present findings that the pH of most of the infected fruit was below 6.0, and that in some fruit the pH decreased by only by 0.2 U; it suggests that the pathogen somehow induces GOX activity, gluconic acid accumulation and pH decrease when the environmental pH value is suboptimal. This relationship between the induced decrease in pH and the GOX activity suggests that the extent of pH decline is an important factor but not the only one in *P. mangiferae* pathogenicity.

To summarize, gluconic acid production is a key factor contributing to the pH decrease in infected fruit tissue, that enables the induction of pathogenic factors such as degrading enzymes which, in turn, results in damage to the host cell walls and complete tissue necrotization (Prusky et al., 2001). Since *P. mangiferae* develops in wounded stem tissue, it is possible that the host's wound responses are critical to activation of fungal *gox* through the oxidative environment. In this connection, Castoria et al. (2003) reported that ROS generation was detected during the first 4 h after apple fruit wounding, which suggests that ROS production in the stem might activate fungal GOX. We have shown in the present study that GOX was active during necrotrophic colonization by *P. mangiferae*, and that there was significant ROS accumulation inside the hyphae. ROS may also contribute to the activation of the fungal necrotrophic colonization by inducing host cell death (Govrin and Levine, 2000; Alkan et al., in press) and thereby modulate the transition from quiescent to active necrotrophic infection (Prusky and Lichter, 2008).

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