Relationship Between Host Acidification and Virulence of *Penicillium* spp. on Apple and Citrus Fruit

Dov Prusky, James L. McEvoy, Robert Saftner, William S. Conway, and Richard Jones

First, second, third, and fourth authors: Produce Quality and Safety Laboratory; fifth author: Vegetable Laboratory, Henry A. Wallace Beltsville Agricultural Research Center, U.S. Department of Agriculture-Agricultural Research Service, Beltsville, MD 20705; and first author: Department of Postharvest Science of Fresh Produce, Institute for Technology and Storage of Agricultural Products, the Volcani Center, Agricultural Research Organization, Bet Dagan 50250, Israel.
Accepted for publication 7 July 2003.

ABSTRACT

Prusky, D., McEvoy, J. L., Saftner, R., Conway, W. S., and Jones, R. 2004. Relationship between host acidification and virulence of *Penicillium* spp. on apple and citrus fruit. Phytopathology 94:44-51.

Penicillium expansum, *P. digitatum*, and *P. italicum* acidify the ambient environments of apple and citrus fruit during decay development. They use two mechanisms for this: the production of organic acids, mainly citric and gluconic, and NH_4^+ utilization associated with H^+ efflux. Exposure of *P. expansum* and *P. digitatum* hyphae to pH 5.0 increased their citric acid production, compared with the production of organic acids at acidic ambient pH. In decayed fruit, both pathogens produced significant amounts of citric and gluconic acids in the decayed tissue and reduced the host pH by 0.5 to 1.0 units. Ammonium depletion from the growth medium or from the fruit tissue was directly related to

ambient pH reduction. Analysis of transcripts encoding the endopolygalacturonase gene, *pepg*1, from *P. expansum* accumulated under acidic culture conditions from pH 3.5 to 5.0, suggesting that the acidification process is a pathogenicity enhancing factor of *Penicillium* spp. This hypothesis was supported by the finding that cultivars with lower pH and citric acid treatments to reduce tissue pH increased *P. expansum* development, presumably by increasing local pH. However, organic acid treatment could not enhance decay development in naturally acidic apples. Conversely, local alkalinization with $NaHCO_3$ reduced decay development. The present results further suggest 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 modified host pH created by the pathogens.

Penicillium expansum, *P. digitatum*, and *P. italicum* are filamentous ascomycete phytopathogens that attack a wide range of deciduous and tropical fruits. The pathogens macerate the host tissue and produce significant amounts of hydrolytic enzymes, with polygalacturonase (PG) being especially abundant (44). PGs have been implicated as colonization and virulence factors in several plant-infecting fungi, particularly in diseases characterized by tissue maceration or soft rot. Abundant correlative evidence supports the role of PGs in pathogenesis (2,3,13,14,20,21,27). The disruption of PG genes reduces virulence, which suggests that this enzyme is a significant virulence factor in several plant-infecting fungi (33,34). However, in several other cases, disruption of cell wall-degrading enzymes caused only partial or no reduction in pathogenicity, suggesting that not all enzymes produced by the pathogen are required for pathogenicity (33).

Prusky et al. (29) 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 extracellular enzymes are expressed and that their products secreted under the optimal pH conditions for their functioning (9,11,29). This is consistent with the finding that, although several genes encode cell wall-degrading enzymes, only specific ones are activated during pathogenicity in vivo (29).

In the case of *Sclerotinia sclerotiorum*, the secretion of oxalic acid during infection creates the acidic environment necessary for activity of the PG produced by this fungus (19). In *Colletotrichum*

spp.–avocado fruit interactions, the alkalinization of host tissue during fruit ripening (42), and the localized alkalinization induced by the pathogen's secretion of ammonia, result in an appropriate pH for expression of *pel*B and the secretion of pectate lyase (29). Similar results were obtained for *Alternaria alternata*; it, too, induced secretion of ammonia and elicited the pH increase needed for the expression of *AaK*1, an endo-1,4-β-glucanase (11). Preliminary experiments in our laboratory also indicated that *Penicillium* spp. colonization is enhanced by low pH in the host tissue (28). 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 to (i) determine if virulence is enhanced under acidic conditions in the host, and evaluate the importance of the pathogen in the regulation of ambient pH during pathogenicity by *Penicillium* spp.; (ii) identify the mechanism or mechanisms of ambient pH modulation by fungi; and (iii) determine if ambient pH affects the transcriptional regulation of *pepg*1 in *P. expansum*. We hypothesized that ambient host pH and *Penicillium* spp.-induced host acidification control the *pepg*1 expression necessary for host tissue maceration by this pathogen.

MATERIALS AND METHODS

***P. expansum* growth conditions.** We utilized a wild-type *P. expansum* isolate obtained from a decayed 'Golden Delicious' apple at Beltsville Agricultural Research Center, U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS), Beltsville, MD. This was the isolate from which PG was purified (41) and it is routinely cultured on potato dextrose agar (PDA; Difco Laboratories, Detroit). The *P. italicum* and *P. digitatum* isolates were obtained from J. L. Smilanick, USDA-ARS, Parlier,

CA. Apple and citrus cultivars were obtained from local suppliers on the day of the experiment. To analyze the effects of ambient pH and carbon source on organic acid production, we inoculated 50 ml of M_3S medium containing, per liter, 1 g of yeast extract (Difco Laboratories), 1 g of Bacto peptone (Difco Laboratories), 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 1×10^7 spores/ml obtained from a 7- to 10-day-old sporulating culture. This liquid culture was incubated for 2 days at 25°C with shaking at 150 rpm, and then was blended with an emulsifying blender (Bonjour, Pacheco, CA) and incubated for an additional 24 h. The entire culture was harvested by vacuum filtration onto sterile filter paper (Whatman no. 1) and washed twice under vacuum with 50 ml of sterile distilled water. The washed mycelia (average wet weight, 1.2 g) were resuspended in 50 ml of fresh inducing medium containing, per liter, 4 g of yeast extract, 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, and 15 g of sucrose (yeast sucrose medium [YSM]). Four flasks were inoculated for each initial pH value of 3.0, 5.0, or 7.0 that was adjusted with 0.5 M HCl and 0.5 N NaOH. These cultures were incubated for an additional 24 h. These secondary cultures in inducing medium were harvested by vacuum filtration; the supernatants were saved for pH determination and organic acid quantification and the hyphae for dry weight determination.

In experiments in which the effect of *P. expansum* on NH_4^+ influx (depletion from the medium) was determined, the washed mycelia resulting from growth on M_3S were resuspended in 50 ml of fresh inducing medium containing, per liter, 0.5 g of NH_4Cl, 4 g of K_2HPO_4, 2 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of $CaCl_2 \cdot 2H_2O$, 0.03 g of $FeCl_3$, and 5 g of glucose, and the pH was adjusted to 5.0 with 1 N NaOH. These secondary cultures were harvested by vacuum filtration, the supernatants were saved for pH determination and NH_4^+ quantification, and the hyphae were saved for dry weight determination.

Inoculation of fruit was carried out by wounding the fruit on four sides to a depth of 2 to 3 mm and placing 25 μl of spore suspension (0.5×10^6 spores/ml) into each wound. The fruit then were incubated for 4 days at 20°C and 90% relative humidity. In some experiments, fruit wound sites were treated with 25 μl of citric (3 g/liter) or gluconic (15 g/liter) acid or $NaHCO_3$ (140 mM) 1 h before pathogen inoculation. In vivo and in vitro experiments were repeated at least three times. The results of one representative experiment are presented. Standard errors (SEs) of the means were calculated.

Partial purification of PG produced by *P. expansum*. Apple fruit were washed in soapy water and surface disinfested with 80% ethanol. They then were wound inoculated with a spore suspension of *P. expansum* (0.5×10^6 spores/ml). After 13 days of incubation at 20°C, the peel over the lesion was removed and the decayed cortical tissue was collected for PG extraction. The extraction procedure, which was a modification of one previously used (44), was carried out at 5°C. The decayed cortical tissue (500 g) was homogenized in an equal volume of a solution containing 20 mM 2-[*N*-morpholino]-ethanesulfonic acid (MES), 1 M NaCl, and 1.5% water-insoluble polyvinylpyrrolidone. The homogenate was adjusted to pH 6.0, stirred for 30 min, and filtered through cheesecloth followed by Miracloth (Calbiochem-Boehring, La Jolla, CA). The filtrate was centrifuged at $20,000 \times g$ for 30 min. The supernatant was concentrated to 100 ml by a Minitan ultrafiltration system equipped with 10-kDa cut-off low protein-binding membranes (Millipore Corp., Bedford, MA). The filtrate was centrifuged at $20,000 \times g$ for 30 min and the supernatant was concentrated further to 15 ml in a stirred-cell ultrafiltration system fitted with a 10-kDa cut-off membrane (Amicon, Beverly, MA). The concentrate was dialyzed against deionized water overnight.

The activity of PG in the concentrate was determined by measuring reducing groups released from sodium polypectate, with D-galacturonic acid as the standard, according to Yao et al.

(44). One unit of PG activity was defined as the amount of enzyme required to release 1 μmol of reducing groups per minute under the assay conditions employed. The PG extract was filter sterilized and used to treat apple tissue. The sterilized crude PG extract exhibited a total activity of 320 units and a specific activity of 230 units/mg.

Organic acid quantification. Organic acid levels were measured in supernatants from cultures of *P. expansum* and *P. digitatum*, healthy and *P. expansum*-decayed apples, and healthy and *P. digitatum*-decayed grapefruit. For organic acid measurements, supernatants from cultures grown on different carbon sources and at various pH levels and filtered extracts of healthy and decayed tissues were analyzed. Samples (20 μl each) were injected onto a PLRP-S column (250 by 4.6 mm; Polymer Laboratories, Amherst, MA). The mobile phase was 0.5% perchloric acid and the flow rate was maintained at 1.0 ml/min. Oxalic acid was detected with a Model 400 electrochemical detector (EG&G, Princeton, NJ) with a glassy carbon electrode set at 1,150 mV. The peak height was quantified and compared with an external standard curve, with oxalic acid dihydrate (Sigma-Aldrich, St Louis) as the standard. For acids other than oxalic, 5 ml of 0.1 N H_2SO_4 was added to 1-g tissue samples, the mixture was homogenized for 30 s with a Polytron (Brinkmann Instruments, Westbury, NY), and the extract was passed through a 0.45-μm nylon filter. The final extract volume was 5.9 ml. Aliquots (20 μl each) of supernatants, tissue extracts, and organic acid standards were injected onto a Lonpak KC-810P precolumn (6 by 50 mm; Shodex, Tokyo) and a Lonpak KC-811 column (8 by 300 mm) maintained at 50°C. The mobile phase was 0.002 N H_2SO_4, and the flow rate was maintained at 1.5 ml/min with a Model 110B pump (Brinkmann Instruments, Columbia, MD). A Model 400 electrochemical detector (EG&G) with a glassy carbon electrode set at 750 mV was used for ascorbic acid quantitation and a Model LC-95 UV detector (Perkin-Elmer, Norwalk, CT) set at 200 nm was used for other organic acids. The peak height for each acid detected in the samples was quantified and compared with an external standard curve based on the appropriate acid standard obtained from Sigma-Aldrich. All data for organic acid analyses were collected with a Millennium 32 Chromatography Manager (Waters, Milford, CT).

pH measurements. pH was measured directly with a micro-combination pH electrode Model 9810BN (Orion, Beverly, MA) in 1- to 3-ml aliquots sampled at different times after fungal inoculation, and four replicates were tested for each treatment. Mesocarp pH was determined along a transverse cut through the infection site, made with a scalpel blade. pH measurements were taken by placing the micro pH electrode directly against the exposed tissue, as described previously (29). All measurements were repeated on 10 to 12 fruit (at least 30 measurements) on the transverse axis of the lesion of each fruit. The SEs of the means of pH measurements were never higher than 2.5%.

Detection of ammonium in the liquid medium and in the tissue. Ammonium concentration was determined in filtered culture medium or tissue extract. Tissue extract was obtained by crushing 2 to 4 g of cortical tissue with a glass rod and centrifuging the liquid through a 4-mm-diameter 0.45-μm filter (Eppendorf Scientific, Inc., Westbury, NY) at $4,000 \times g$. Ammonium was measured at different times after inoculation with an ammonia electrode Model 95-12 (Orion) in 0.1- to 1-ml aliquots (depending upon the concentration) after the pH of the samples had been adjusted to 10.0 with 10 N NaOH. Different concentrations of NH_4Cl in the range of 7 to 700 μM were used as standards. Experiments with three replications were repeated at least three times. The results of one representative experiment are presented. SEs of the means were calculated. The ATPase inhibitors, Na orthovanadate at 100 to 200 μM, and diethylstilbestrol at 20 μM (Sigma-Aldrich) were added to the ammonium medium and residual ammonium concentrations were determined 20 h later as previously described (26).

Polymerase chain reaction subcloning to generate hybridization probes for *pepg*1 from *P. expansum*. Two DNA fragments corresponding to nucleotides 1007 to 1386 and 1705 to 2018 from the *P. expansum pepg*1 gene (accession no. AF047713) were amplified by polymerase chain reaction (PCR) of genomic DNA with the following primers: PE1006 (TGAGGG-AACCACTACTTTCG) plus PE1385 (CGTTCCAGAATTG-ATAGCAAG) and PE1704 (TGTCAAGGCTGTCTCTGG) plus PE2017 (AAGTGCCGGAAGGTGC), respectively. The fragments were cloned into pCRII with a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) to generate pCRPG2 and pCRPG, respectively. The 423-bp fragment in pCRPG2 corresponded to exon 2 and the 313-bp fragment in pCRPG1 corresponded to exon 4 of the 3′ untranslated region (UTR) of *pepg*1. Both probes were used separately to repeat and confirm the Northern analyses. Genomic DNA was isolated from the mycelium of *P. expansum* with a DNeasy QIAGEN kit (Qiagen, Santa Clarita, CA).

Sequence analysis of the PCR-generated fragments of *pepg1*. Plasmid pCRPG containing the genomic sequences of *pepg*1 were used as DNA sequencing templates. The sequence of both strands of each subcloned fragment were confirmed (DNA Sequencing Facility, University of Maryland, College Park). Homology to the *pepg*1 was determined with the BLAST algorithm (accession no. AF047713) and was conducted with the Wisconsin Sequence Analysis Program 9.1 (Genetics Computer Group, Madison, WI).

Northern analysis. Primary cultures were grown and harvested as described above. For experiments to determine the effect of ambient pH on *pepg*1, harvested cultures were transferred to fresh YSM buffered with 0.5 M citric acid–sodium phosphate buffer to achieve initial pH values between 3.0 and 7.0; the actual initial pH value for each culture was determined after autoclaving. These secondary cultures were incubated for 2, 5, or 7 h, harvested by vacuum filtration, and quickly frozen in liquid nitrogen. Total RNAs were prepared with the FastRNA Kit-RED (BIO 101, Carlsbad, CA). Total RNAs (20 μg/lane) were separated in a 1.2% agarose, 0.36 M formaldehyde/MOPS gel, transferred to a Hybond-N$^+$ nylon membrane (Amersham, Arlington Heights, IL), fixed by incubating for 2 h at 80°C, hybridized overnight in a hybridization incubator (Robbins Scientific, Sunnyvale, CA) with 1% bovine serum albumin, 0.25 M Na_2HPO_4, and 7% sodium dodecyl sulfate (SDS) solution at 65°C, followed by three washes of 45 min each at 65°C (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] + 0.5% SDS; 1× SSC + 0.5% SDS; and 0.1× SSC + 0.5% SDS) as described by Church and Gilbert (7), and autoradiographed essentially as described by Ausubel et al. (1). An RNA ladder standard (GibcoBRL) was used to estimate the lengths of the RNAs. Probes were synthesized with a random priming kit with ^{32}P-dATP as the label (Boehringer Mannheim, Indianapolis, IN). For all hybridizations, a ^{32}P(dATP)-labeled probe was diluted to 1×10^6 dpm/ml. The sequences used for hybridization were two probes of the endopolygalacturonase-encoding *pepg*1 coding sequence (41) (accession no. AF047713) and the rDNA repeat sequence from *Neurospora crassa* from pMF2 (12). The washed blot was autoradiographed and exposed to a Fuji BAS (Bio Analyzing System) sample screen. Images were captured with a Fuji BAS reader (Fujifilm, Tokyo). Hybridization signals were quantified with MacBAS software version 2.3 (Fujifilm). The reported relative level of signals in each lane was corrected based on the background intensity and hybridization signal from the rDNA probe.

RESULTS

The relationship between pH decrease and decay development by *Penicillium* spp. in apple and citrus fruit. *P. expansum* decayed 'Granny Smith', 'Gala', 'Red Delicious', 'Fuji', and 'Golden Delicious' apple fruit within 4 to 6 days after inoculation. The pH in nondecayed tissue ranged between 4.0 and 4.5 (Table 1); whereas, in the center of the decayed tissue (≈15-mm in diameter), the pH decreased to 3.6 to 4.1. The pH values at the edge of the decayed tissue and the adjacent edge of the healthy tissue were very similar to those observed in the decayed and healthy tissue, respectively (data not shown). *P. digitatum* and *P. italicum* isolates caused decay symptoms 5 to 6 days after inoculation of citrus fruit and decreased the pH of the tissue from 4.6 to 4.8 in the healthy tissue to 3.0 to 3.2 in the decayed lesion (Table 1).

The relationship between apple flesh pH and colonization by *P. expansum* was examined by using cultivars with differing initial pH and by local modulation of the tissue pH. The sizes of the decay lesions caused by *P. expansum* infection in the three apple cvs. Fuji, Rome, and Granny Smith increased as the pH of the untreated tissue decreased from 4.5 to 3.8 (Table 2). Similarly, treatment of Fuji fruit, which has an untreated tissue pH of 5.3, with citric or gluconic acid enhanced *P. expansum* decay development, but the same treatment did not affect decay development in Golden Delicious apples, which had a pH of 4.4 (Fig. 1). However, treatment of Golden Delicious apples with a 140-mM $NaHCO_3$ solution resulted in an increase of pH to 7.1 and to reduced *P. expansum*-induced decay (Fig. 2). Similar results were obtained in $NaHCO_3$-treated Fuji fruit (data not shown).

TABLE 2. The relationship between apple pH and *Penicillium expansum* decay development[z]

Cultivar	pH at inoculation	Decay diameter (mm)
Fuji	4.46 ± 0.01	12 ± 0.2
Rome	3.77 ± 0.03	17 ± 0.1
Granny Smith	3.45 ± 0.02	21 ± 0.5

[z] Average pH values ± standard error. pH was measured directly with a microcombination pH electrode Model 9810BN (Orion, Beverly, MA). All measurements were repeated on 10 to 12 fruit (at least 30 measurements) on the transverse axis of the lesion of each fruit.

TABLE 1. pH levels in healthy and *Penicillium*-decayed fruit

Penicillium sp.	Host	Cultivar	pH value ±SE[z]		
			Healthy	Decayed	Δ_{ph}
P. expansum	Apple	Granny Smith	3.95 ± 0.06	3.64 ± 0.01	0.31
		Gala	4.31 ± 0.06	3.88 ± 0.03	0.43
		Red Delicious	4.44 ± 0.03	4.07 ± 0.02	0.37
		Fuji	4.44 ± 0.06	3.96 ± 0.02	0.48
		Golden Delicious	4.54 ± 0.06	3.88 ± 0.03	0.88
P. digitatum	Orange	Naval	4.77 ± 0.45	3.12 ± 0.07	1.65
	Grapefruit	Oro Blanco	4.74 ± 0.05	3.10 ± 0.14	1.64
P. italicum	Orange	Naval	4.77 ± 0.07	3.02 ± 0.13	1.75
	Grapefruit	Oro Blanco	4.55 ± 0.13	3.23 ± 0.17	1.32

[z] pH changes induced by *P. expansum*, *P. digitatum*, *and P. italicum* on different cultivars of apple and citrus fruit; SE = standard error. pH was measured directly with a microcombination pH electrode Model 9810BN (Orion, Beverly, MA). All measurements were repeated on 10 to 12 fruit (at least 30 measurements) on the transverse axis of the lesion of each fruit. Measurements were taken 7 days after inoculation.

The mechanism of acidification during colonization. *In vitro organic acid production by* P. expansum *and* P. digitatum. Analysis of organic acids secreted by *P. expansum* and *P. digitatum* during 24 h of growth in YSM indicated that citric acid was the main product, while minor amounts of fumaric and oxalic acid also were detected. For both *Penicillium* spp., when accumulation of citric acids was compared among cultures having the initial pH values of 3, 5, and 7, the greatest amount of citric acid was produced at pH 5 after 24 h (Table 3).

In vivo organic acid accumulation induced by P. expansum *and* P. digitatum. In grapefruit decayed by *P. digitatum*, citric acid content increased by 52% and gluconic acid content by 150% (Table 4). In *P. expansum*-decayed apple fruit, the accumulation of gluconic and fumaric acids increased from 0 to 1,586 and 6.0 μg g^{-1} fresh weight, respectively, and citric acid content increased by 64%. In contrast, ascorbic acid declined significantly in these same experiments (Table 4). No significant amount of oxalic acid production was detected in decaying citrus or apple tissue. The addition of purified PG from *P. expansum* to wounded apple tissue did not induce any organic acid accumulation up to 48 h after the treatment in the macerated tissue (data not shown), suggesting that the host–pathogen interaction is needed for organic acid accumulation.
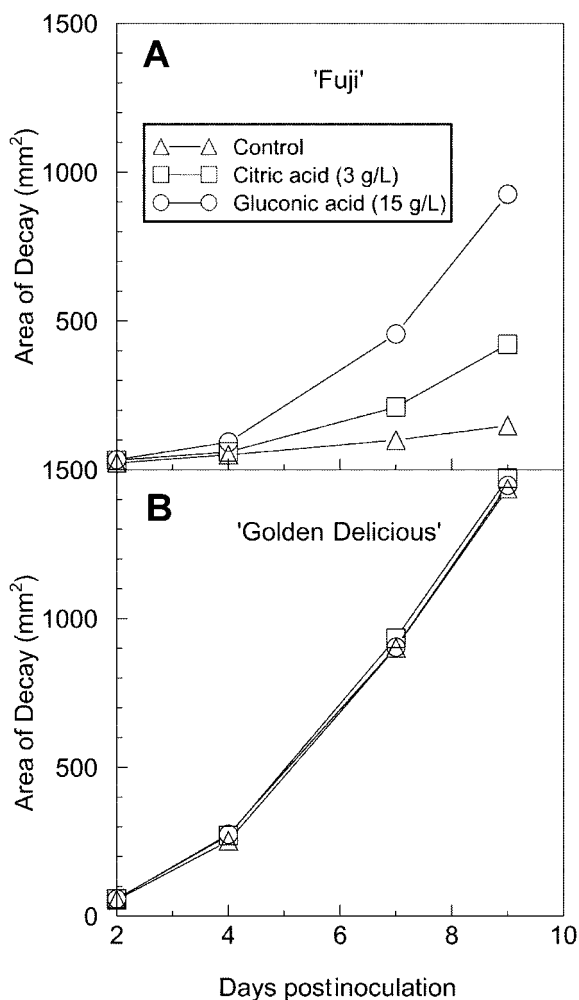
Ambient pH and NH$_4^+$ influx induced by *P. expansum*, *P. digitatum*, and *P. italicum*. The possibility that another mechanism of acidification is induced by *Penicillium* spp. was tested. When *P. digitatum* and *P. italicum* were transferred, during the inducing period, to a secondary medium containing 11 mM NH$_4$Cl at pH 4.8, the NH$_4^+$ concentration in the medium decreased by >75% in both *P. digitatum* (Fig. 3) and *P. italicum* (data not shown) cultures. During the same period, the pH of the culture media of *P. digitatum* and of *P. italicum* decreased to 3.8 and 3.1, respectively. In similar experiments in which *P. expansum* was transferred, during the inducing period, to a secondary medium containing NH$_4$Cl at pH 5.0, the NH$_4^+$ concentration in the medium decreased by >99% over a period of 17 h. At the same time, the pH decreased from 5.0 to 2.4 (data not shown). Exposure of *P. expansum* hyphae to 100 to 200 μM Na-orthovanadate or 20 μM diethylstilbestrol partially inhibited NH$_4^+$ depletion from the medium, with levels decreasing by 47 and 29%, respectively.

Analysis of the ammonium content in the decayed tissue of three apple cultivars showed >60% reduction in ammonium concentration and pH decreases of up to 0.6 units compared with healthy tissue in the same fruit (Table 5). Similar analysis of decayed grapefruit tissue showed an ≈50% reduction in ammonium concentration.

Ambient pH and *pepg*1 expression of *P. expansum*. The influence of ambient culture pH on the accumulation of transcripts encoding the endopolygalacturonase enzyme from *P. expansum* was examined by Northern blot analysis. Mycelia were transferred from primary cultures with an average pH of 5.5 to fresh secondary cultures with a series of pH values from 3.0 to 7.0. Analysis of the *pepg*1 transcript levels with the 3′ UTR probe, 5 h after induction, showed that *pepg*1 accumulation ranged between 3.5 and 5.0, but was highest at pH 4.0 (Fig. 4). Minor expression was observed at pH values higher than 5.0 (data not shown).

DISCUSSION

Host pH modulation by pathogens recently was suggested as an important factor in enhancing the virulence of postharvest patho-

Fig. 1. Effect of citric and gluconic acid treatment on *Penicillium expansum* virulence in apples fruit of various initial pH values. **A,** Decay development of *P. expansum* in cv. Fuji with a pH at inoculation of 5.3. **B,** Effect of acid treatments on decay development of cv. Golden Delicious with a pH at inoculation of 4.4. Ten fruit were wounded on four sides (40 inoculations) and 50 μl of citric acid or gluconic acid was applied to the wound. One hour later, 25 μl of *P. expansum* (0.5 × 10^6 spores/ml) in water was applied to the wound. Lesion size was measured every second day during the first 9 days after inoculation.
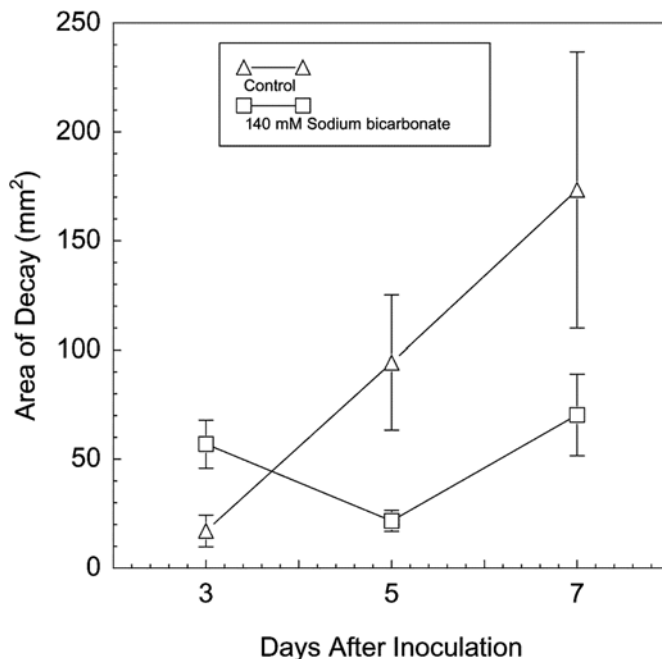

Fig. 2. Modulation of *Penicillium expansum* virulence by Na-bicarbonate in apple fruit, cv. Golden Delicious. Ten fruit were wounded on four sides (40 inoculations) and 50 μl of Na-bicarbonate (140 mM) was applied to the wound. One hour later, 25 μl of *P. expansum* (0.5 × 10^6 spores/ml) in water was applied to the wound. Lesion size was measured daily during the first 8 days after inoculation.

gens. Alkalinization of the ambient pH was shown to modulate pectolytic gene expression of hydrolases in *Colletotrichum gloeosporioides* (29) and of glucanases in *A. alternata* (11). *C. gloeosporioides*, *C. acutatum*, and *C. coccodes* locally increased ambient pH values by the secretion of ammonia in avocado, apple, and tomato fruit, respectively (29). In the present study, *P. expansum*, a broad-host-range phytopathogenic fungus, showed increased virulence as the pH of the apple fruit decreased. *P. expansum* also actively reduced the pH of the tissue by 0.3 to 0.6 during decay development in five apple cultivars. This is not the first observation that a pathogen decreased host pH during disease development. *S. sclerotiorum* reduced host pH during pathogenicity by secreting large amounts of oxalic acid, thus creating an acidic environment in which to grow and cause disease (15, 23,31). However, the present findings suggest that fungal virulence was affected by a combined effect of host and pathogen on ambient pH as found in avocado fruit (43). Examination of apple cultivars after storage indicated that, as the pH of the apple fruit became more acidic, *P. expansum* colonization increased. Furthermore, *P. expansum* colonization could be enhanced by treatments with exogenous citric and gluconic acids, or suppressed by NaHCO₃ treatment, findings which, together, suggest that tissue acidification is a significant factor in determining virulence.

How do *Penicillium* spp. acidify the host tissue? The growth of *P. expansum* in the presence of 1.5% sucrose resulted in the accumulation of high levels of citric acid and a pH decrease from 7.0 and 5.0 to 4.5 and 4.2, respectively. The secretion of citric acid in culture by other *Penicillium* spp. has been reported previously (8). However, in the present study, the accumulation of citric and gluconic acids in decayed apple and grapefruit tissue suggest that the acidification of the tissue results, at least in part, from the accumulation of these acids. The concentration of citric acid in decayed apple was twice that found in healthy fruit; and, in citrus fruit, where the basal level of citric acid is much higher than in apple, the citric acid concentration increased by 50% during decay. Although gluconic acid production by *P. expansum* or *P. digitatum* was not detected under in vitro conditions, its accumulation during *P. expansum*- and *P. digitatum*-induced decay in apple and citrus fruit was higher than that of citric acid. In contrast to previous studies with *Sclerotinia* spp., oxalic acid accumulated only in minor amounts in *Penicillium* cultures and was not detected in decayed tissue. When *P. expansum* and *P. digitatum* were grown in the presence of sucrose, the highest citric acid production was observed at pH 5.0, and lower amounts were found at neutral pH, suggesting a reduced efficiency to acidify fruit tissue having a pH higher than 7.0. Whether the pathogen itself or the host tissue, under induction by the pathogen, is the source of the organic acids being produced during decay is not clear. The occurrence of gluconic acids in decayed tissue might suggest the host contribution when affected by the pathogen. Our results suggest, however, that PGs produced by *P. expansum* are not the inducers of organic acid accumulation by the fruit.

P. expansum has developed a parallel mechanism for tissue acidification that involves the NH_4^+ uptake from the apple tissue. Ammonium concentrations decreased significantly in the acidified, decayed tissue. Ammonium is one of the preferred nitrogen sources for fungi, and *Penicillium* spp. take up NH_4^+ efficiently (32) accompanied by stoichiometric H^+ excretion. Ross and Luckner (32) suggested the presence of an H^+-translocating ATPase in *P. cyclopium* that functions as a proton pump under specific physiological conditions. It might be assumed that the plasma membrane possesses pathways for the permeation of the cationic species (22). The extent of the NH_4^+ depletion from the medium and from decayed tissue suggests that *P. expansum* and *P. digitatum* have a significant ammonium-transport system or systems that may affect ambient pH. The finding that ATPase inhibitors, such as Na orthovanadate and diethylstilbestrol, partially inhibited NH_4^+ influx in *P. expansum* further supports this suggestion.

What is the importance of tissue acidification for *Penicillium* spp. virulence? McCallum et al. (25) indicated that aggressive *P. expansum* isolates reduced pH faster than the weaker isolates. The capability of pathogens to acidify the environment has resulted in the expression of genes and the secretion of many hydrolytic enzymes encoding genes (4), including PGs (17,31,38). Transcript analysis of the endoPG-encoding gene *pepg*1 from *P. expansum* shows that it occurred between 3.5 and 5.0, with the highest transcript level observed at pH 4.0. *pepg*1 was shown to be expressed in vivo by Yao et al. (44) in decayed tissue that generally had a pH ranging between 3.6 and 4.0. Furthermore, our present results indicate that colonization of the tissue was faster in cultivars having a lower untreated tissue pH or if they were treated with organic acid. Organic acid treatment, however, could not enhance colonization in those cultivars where their untreated tissue pH was already low. Both results support the hypothesis that acidification of the tissue provides a better pH for *pepg*1 expression.

TABLE 4. Organic acid presence in healthy and decayed 'Oro Blanco' grapefruit and 'Golden Delicious' apple inoculated with *Penicillium digitatum* and *P. expansum*, respectively

Tissue	Organic acids concentration (µg g⁻¹ fresh weight)ᶻ			
	Citric	Fumaric	Ascorbic	Gluconic
Grapefruit				
Healthy	5,979 ± 234	0	42 ± 41	6,320 ± 2,470
Decayed	9,097 ± 362	0	473 ± 520	15,832 ± 2,980
Apple				
Healthy	591 ± 280	0	4 ± 3 0	0
Decayed	970 ± 256	6 ± 1	0	1,586 ± 165

ᶻ Average values ± standard error. pH was determined with a microcombination pH electrode Model 9810BN (Orion, Beverly, MA). Organic acid concentrations were determined by high-performance liquid chromatography using a pure sample as a standard. All measurements were repeated four times from the decayed lesion of different fruit.

TABLE 3. Influence of pH on organic acid accumulation by *Penicillium expansum* and *P. digitatum*ᶻ

pH(i)	pH(f)	Organic acid (µg/ml)		
		Citric acid Δ_{24-0h}	Fumaric acid Δ_{24-0h}	Oxalic acid Δ_{24-0h}
Secreted by *P. expansum*				
3.0	3.1	181 a	5.7 a	1.1 a
5.0	4.2	737 b	11.0 b	1.2 a
7.0	4.5	593 b	8.0 b	52.6 b
Secreted by *P. digitatum*				
3.0	2.9	174 b	6.8 a	…
5.0	4.2	602 c	4.3 a	…
7.0	6.3	31 a	6.0 a	…

ᶻ pH was determined with a microcombination pH electrode Model 9810BN (Orion, Beverly, MA). pH(i) = pH of the culture before mycelia inoculation and pH(f) = pH of the culture 24 h after mycelia inoculation. Within treatments, the final pH values varied by 0.1 or less. Different letters indicate significant difference at $P \leq 0.05$, between the average of four replications of organic acid accumulation after transfer to inducing media with three different pH values. Experiments were repeated at least three times. The results of one representative experiment are presented.

It should be pointed out that freshly harvested apple fruit and those stored commercially in optimal conditions (controlled atmosphere) usually show decay development only after a long storage period (4 to 6 months). This may suggest that the pH regulation of the environment would become critical following a decline of possible resistance mechanisms (e.g., preformed compounds, phenolic content, cell wall resistance, and so on). Under these conditions, the acidification of the environment by citric and gluconic acids, along with strong chelating activity of calcium and other ions (23), might have a synergistic effect and enhance the expression of genes and secretion of specific enzymes needed to facilitate fungal attack (29). Citric and gluconic acids accumulation were shown to decrease calcium concentration in the intercellular spaces of plant tissues and alter mineral balances and would, thereby, affect the stability of cell membranes and cell wall pectate polymers (19,21). Destabilization of cell membranes and cell walls would enhance the sensitivity to pathogen-produced pectolytic enzymes similarly to what has been reported for oxalic acid (24). The secretion of organic acids also might have an indirect effect on pathogenesis by the suppression of fruit resistance. It was reported that the secretion of oxalate by *S. sclerotiorum* suppresses the plant oxidative burst (6). If this is combined with the reduction in host pH which inhibits the activities of plant-produced polyphenol oxidase (21,25), this could result in a relatively broad and significant role in pathogenesis.

The extent of the effect of ambient pH on different pathogenic systems might suggest that pH is a regulator of gene expression
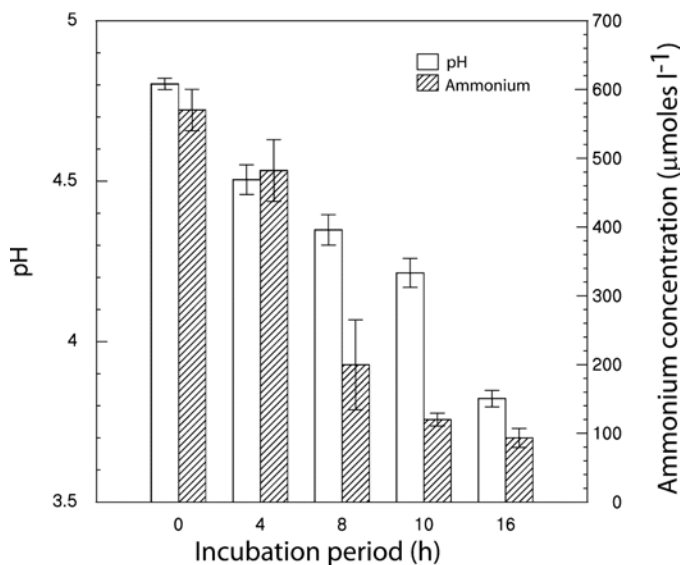
(5,11,29,31). In specific cases, the pathogen activates the mechanism of alkalinization and, in others, the mechanism of acidification. The principle behind the idea of ambient pH regulation is that this mechanism ensures that genes encoding extracellular enzymes are expressed and that their metabolites are secreted under the pH conditions at which they function best (9,29). This would indicate that only specific enzymes out of the battery of enzymes produced by the pathogen are specifically involved. The pathogen would then actively change the environment to enhance the specific involvement of the preferred enzymes. However, the observation that optimal activity of the pure enzyme are not close enough to the optimum pH for gene expression suggests that disease incidence probably is dependent on gene expression and protein secretion rather than optimal enzyme activity. In those cases, the effect on the stability of cell membranes and cell wall pectate polymers created by the accumulation of oxalic (25), gluconic, or citric acid would enhance the sensitivity to pathogen-produced pectolytic enzymes in addition to the environmental conditions created by the ambient pH to secrete the protein. These findings suggest that *P. expansum*, *P. digitatum*, *P. italicum*, *C.*
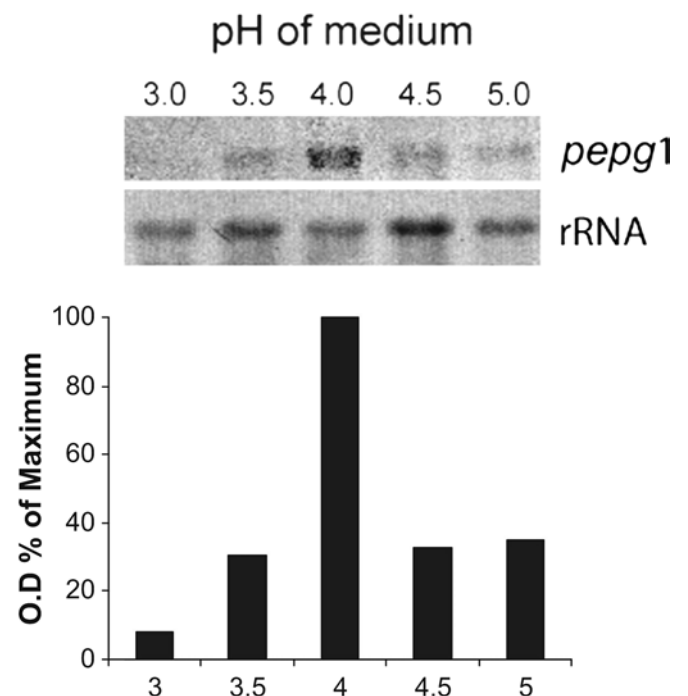

Fig. 3. Ammonium concentration and pH levels induced by *Penicillium digitatum* on M$_3$Cl-containing media. Following growth in M$_3$S primary cultures, the hyphae were transferred to yeast sucrose medium, pH 4.8. Direct pH and ammonium measurements were taken. Average of three replications and standard errors are presented. Experiments were repeated at least three times. The results of one representative experiment are presented.

Fig. 4. *pepg1* transcript accumulation in response to different ambient pH conditions. Shown is a Northern analysis of total RNA isolated from *Penicillium expansum* mycelia 5 h after transfer to a fresh secondary yeast sucrose medium buffered with citrate-phosphate at the indicated pH values. The blot was probed with the 379-bp segment of *pepg1* and a ribosomal DNA (rDNA) fragment as indicated. The column at the bottom of each ribosomal DNA blot indicates the corrected percent intensity value of *pepg*1 based on the background intensity and hybridization signal from the rDNA probe as determined with MacBAS software version 2.3 (Fujifilm).

TABLE 5. Ammonium concentrations and pH levels in healthy and *Penicillium* spp.-decayed fruit[z]

Penicillium sp. and fruit cultivar	Ammonium (µM)		pH value	
	Healthy	Decayed	Healthy	Decayed
P. expansum in apple				
Golden Delicious	201 ± 34	37 ± 5	4.06 ± 0.14	3.48 ± 0.02
Granny Smith	141 ± 32	52 ± 30	3.43 ± 0.07	3.17 ± 0.02
Red Delicious	165 ± 21	54 ± 24	4.04 ± 0.07	3.45 ± 0.04
P. digitatum in grapefruit				
Oro Blanco	103 ± 5.5	52 ± 15	4.74 ± 0.05	3.10 ± 0.14

[z] Averages ± standard error of the means of three replications are presented. pH was determined with a microcombination pH electrode Model 9810BN (Orion, Beverly, MA).

gloeosporioides, and *A. alternata* could use the ambient pH environment where organic acids and ammonium is secreted as a regulatory cue for disease development. We recently found that *Botrytis cinerea* also decreased the pH of the decayed tissue (D. Prusky, *unpublished data*). Transcript levels of *Bcpg3* encoding for PG in *B. cinerea* (41) also were activated under acidic conditions. In this case, the presence of a large endoPG gene family may enable the differential expression of the family members under different growth or host conditions. Together, these results suggest that local pH modulation by pathogens may be a more common mechanism than previously thought for increasing virulence during postharvest attack.

The finding that ambient pH may play a major regulatory role in *pepg*1 expression suggests the importance of ambient pH regulation by *Penicillium* spp. An ambient pH signal transduction pathway has been characterized in *Aspergillus nidulans*, and several components of this pathway, including the pH-dependent transcriptional regulator *pac*C, have been cloned and characterized in *S. sclerotiorum* (31,39) and *C. gloeosporioides* (10,28). *pac*C homologs also have been identified in *P. expansum* (Hadass, Pines, and Prusky, accession no. AY225524) and in the closely related filamentous fungus *A. niger* (18), in *P. chrysogenum* (37), in yeasts (16,30,36,40), and, more recently, in *Fusarium oxysporum* f. sp. *lycopersici* (5). Ambient pH sensing and gene regulation may be one basis for viable strategies for blocking disease development by a broad range of postharvest pathogens.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1991. Current Protocols in Molecular Biology. John Wiley & Sons, Inc., New York.
2. Barmore, C. R., and Brown, G. E. 1981. Polygalacturonase from citrus fruit infected with *Penicillium italicum*. Phytopathology 71:328-331.
3. Bateman, D. F., and Basham, H. G. 1976. Degradation of plant cell walls and membranes by microbial enzymes. Encycl. Plant Physiol. New Ser. 4:316-355.
4. Bateman, D. F., and Beer, S. V. 1965. Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotium rolfsii*. Phytopathology 58:204-211.
5. Caracuel, Z., Roncero, M. G, Gonzales-Verdejo, C., and Di Pietro, A. 2002. The pH response transcription factor PacC controls virulence in *Fusarium oxysporum*. (Abstr. 368) 6th Eur. Conf. Fungal Genet. Pisa, Italy.
6. Cessna, S., Sears, V., Dickman, M., and Low, P. 2000. Oxalic acid, a pathogenicity factor of *Sclerotinia sclerotiorum*, suppresses the host oxidative burst. Plant Cell 12:2191-2199.
7. Church, G. M., and Gilbert, W. 1984. Genomic sequencing. Proc. Natl. Acad. Sci. USA 81:1991-1995.
8. Cunningham, J. E., and Kuiack, C. 1992. Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium bilaii*. Appl. Environ. Microbiol. 58:1451-1458.
9. Denison, S. H. 2000. pH regulation of gene expression in fungi. Fungal Genet. Biol. 29:61-71.
10. Drori, N., Kramer,-Haimovich, H., Rollins, J., Dinoor, A., Okon, Y., Pines, O., and Prusky, D. 2003. External pH and nitrogen source affect secretion of pectate lyase by *Colletotrichum gloeosporioides*. Appl. Environ. Microbiol. 69:3258-3262.
11. Eshel, D., Miyara, I., Ailinng, T., Dinoor, A., and Prusky, D. 2002. pH regulates endoglucanase expression and virulence of *Alternaria alternata* in persimmon fruits. Mol. Plant-Microbe Interact. 15:774-779.
12. Free, S. J., Rice, P. W., and Metzenberg, R. L. 1979. Arrangement of genes coding for ribosomal ribonucleic acids in *Neurospora crassa*. J. Bacteriol. 137:1219-1226.
13. Jones, T. M., Anderson, A. J., and Albersheim, P. 1972. Host-pathogen interactions. IV. Studies on the polysaccharide-degrading enzymes secreted by *Fusarium oxysporum* f. sp. *lysopersici*. Physiol. Plant Pathol. 2:153-166.
14. Karr, A. L., and Albersheim, P. 1970. Polysaccharide-degrading enzymes are unable to attack plant cell walls without prior action by "wall-modifying-enzyme." Plant Physiol. 46:69-80.
15. Kubicek, C. P., Schreferl-Kunar G., Wöhrer, W., and Röhr, M. 1988. Evidence for a cytoplasmic pathway of oxalate biosynthesis in *Aspergillus niger*. Appl. Environ. Microbiol. 54:633-637.
16. Lambert, M., Blanchin-Roland, S., Le Louedec, F., Lépingle, A., and Gaillardin, C. 1997. Genetic analysis of regulatory mutants affecting synthesis of extracellular proteinases in the yeast *Yarrowia lipolytica*: Identification of a *RIM101/pacC* homolog. Mol. Cell. Biol. 17:3966-3976.
17. Lumsden, R. D. 1976. Pectolytic enzymes of *Sclerotinia sclerotiorum* and their localization in infected bean. Can. J. Bot. 54:2630-2641.
18. MacCabe, A. P., van den Hombergh, J. P. T. W., Tilburn, J., Arst, H. N., Jr., and Visser, J. 1996. Identification, cloning and analysis of the *Aspergillus niger* gene *pac*C, a wide domain regulatory gene responsive to ambient pH. Mol. Gen. Genet. 250:367-374.
19. Magro, P., Marciano, P., and Di Lenna, P. 1984. Oxalic acid production and its role in pathogenesis of *Sclerotinia sclerotiorum*. FEMS Microbiol. Lett. 24:9-12.
20. Mankarios, A. T., and Friend, J. 1980. Polysaccharide-degrading enzymes of *Botrytis allii* and *Sclerotiorum cepivorum*: Enzyme production in culture and the effect of enzymes on isolated onion cell walls. Physiol. Plant Pathol. 17:93-104.
21. Marciano, P., Di Lenna, P., and Magro, P. 1983. Oxalic acid, cell wall-degrading enzymes and pH in pathogenesis and their significance in the virulence of two *Sclerotinia sclerotiorum* isolates on sunflower. Physiol. Plant Pathol. 22:339-345.
22. Marini, A. M., Boudekou, S. S., Vissers, S., and Andre, B. 1997. A family of ammonium transporters in *Saccharomyces cervisiae*. Mol. Cell. Biol. 17:4282-4293.
23. Martell, A. E., and Calvin, M. 1952. Pages 516 and 541 in: Chemistry of the Metal Chelate Compounds. Prentice-Hall, Inc., New York.
24. Maxwell, D. P., and Lumsden, R. D. 1970. Oxalic acid production by *Sclerotinia sclerotiorum* in infected bean and in culture. Phytopathology 60:1395-1398.
25. McCallum, J. L., Tsao, R., and Zhou, T. 2002. Factors affecting patulin production by *Penicillium expansum*. J. Food Prot. 65:1937-1942.
26. Oren-Samir, M., Pick, U., and Avron, M. 1989. Involvement of the plasma membrane ATPase in the osmoregulatory mechanism of the alga *Dunaliella salina*. Plant Physiol. 89:1258-1263.
27. Prusky, D., Gold, S., and Keen, N. T. 1989. Purification and characterization of an endopolygalacturonase produced by *Colletotrichum gloeosporioides*. Physiol. Mol. Plant Pathol. 35:121-133.
28. Prusky, D., McEvoy, J. L., and Conway, W. S. 2002. Local pH modulation by pathogens as a mechanism to increase virulence. (Abstr. 319) 6th Eur. Conf. Fungal Genet. Pisa, Italy.
29. Prusky, D., McEvoy, J. L., Leverentz, B., and Conway, W. S. 2001. Local modulation of host pH by *Colletotrichum* species as a mechanism to increase virulence. Mol. Plant-Microbe Interact. 14:1105-1113.
30. Ramon, A. M., Porta, A., and Fonzi, W. A. 1999. Effect of environmental pH on morphological development of *Candida albicans* is mediated via the PacC-related transcription factor encoded by *PRR*2. J. Bacteriol. 181:7524-7530.
31. Rollins, J. A., and Dickman, M. B. 2001. pH signaling in *Sclerotinia sclerotiorum*: Identification of *pac*C/RIM1 homolog. Appl. Environ. Microbiol. 67:75-81.
32. Ross, W., and Luckner, M. 1984. Relationship between proton extrusion and fluxes of ammonium ions and organic acids in *Penicillium cyclopium*. J. Gen. Microbiol. 130:1007-1014.
33. Scott-Craig, J. S., Panacccione, D. G., Cervone, F., and Walton, J. D. 1990. Endopolygalacturonase is not required for pathogenicity of *Cochliobolus carbonum* on maize. Plant Cell 2:1191-1200.
34. Shieh, M., Brown, R. L., Whitehead, M. P., Carey, J. W., Cotty, P. J., Cleveland, T. E., and Dean, R. A. 1997. Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of *Aspergillus flavus* in cotton bolls. Appl. Environ. Microbiol. 63:3548-3552.
35. St. Leger, R. A., Joshi, L., and Roberts, D. 1998. Ambient pH is a major determinant in the expression of cuticle-degrading enzymes and hydrophobin by *Metarhiziyum anisopliae*. Appl. Environ. Microbiol. 64:709-713.
36. Su, S. S. Y., and Mitchell, A. 1993. Molecular characterization of the yeast meiotic regulatory gene *RIM*1. Nucleic Acids Res. 21:3789-3797.
37. Suárez, T., and Peñalva, M. A. 1996. Characterization of a *Penicillium chrysogenum* gene encoding a PacC transcription factor and its binding sites in the divergent *pcbAB-pcbC* promoter of the penicillin biosynthetic cluster. Mol. Microbiol. 20:529-540.
38. ten Have, A., Mulder, W., Visser, J., and van Kan, J. A. L. 1998. The endopolygalacturonase gene Bcpg1 is required for full virulence of *Botrytis cinerea*. Mol. Plant-Microbe Interact. 11:1009-1016.

39. Tilburn, J., Sarkar, S., Widdick, D. A., Espeso, E. A., Orejas, M., Mungroo, J., Peñalva, M. A., and Arst, H. N., Jr. 1995. The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acidic- and alkaline-expressed genes by ambient pH. EMBO J. 14:779-790.

40. Wilson, R. B., Davis, D., and Mitchell, A. P. 1999. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. J. Bacteriol. 181:1868-1874.

41. Wubben, J. P., ten Have, A., van Kan, J. A. L., and Visser, J. 2000. Regulation of endopolygacturonase gene expression in *Botrytis cinerea* by galacturonic acid, ambient pH and carbon catabolite repression. Curr. Genet. 37:152-157.

42. Yakoby, N., Beno-Moualem, D., Keen, N. T., Dinoor, A., Pines, O., and Prusky, D. 2001. *Colletotrichum gloeosporioides pel*B, is an important factor in avocado fruit infection. Mol. Plant-Microbe Interact. 14:988-995.

43. Yakoby, N., Kobiler, I., Dinoor, A., and Prusky, D. 2000. pH regulation of pectate lyase secretion modulates the attack of *Colletotrichum gloeosporioides* on avocado fruits. Appl. Environ. Microbiol. 66:1026-1030.

44. Yao, C., Conway, W. S., and Sams, C. E. 1996. Purification and characterization of a polygalacturonase produced by *Penicillium expansum* in apple fruit. Phytopathology 86:1160-1166.