

## Effect of Ammonia Production by *Colletotrichum gloeosporioides* on *pelB* Activation, Pectate Lyase Secretion, and Fruit Pathogenicity

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Received 27 April 2005/Accepted 10 November 2005

**The accumulation of ammonia and associated tissue alkalization predispose avocado fruit to attack by *Colletotrichum gloeosporioides*. Secretion of ammonia by *C. gloeosporioides* in the presence of KNO<sub>3</sub> was induced by decreasing the pH from 7.0 to 4.0. When the fungus was grown at pH 4.0 or 6.0 in the absence of a nitrogen source, ammonia did not accumulate, and neither *pelB* (encoding pectate lyase) transcription nor pectate lyase secretion was detected. Under these nitrogen starvation conditions, only transcriptional activation of *areA*, which encodes the global nitrogen regulator, was detected. *pelB* transcription and pectate lyase secretion were both detected when *C. gloeosporioides* was grown at pH 6.0 in the presence of ammonia accumulated from different nitrogen sources. The early accumulation of ammonia induced early *pelB* expression and pectate lyase secretion. As the external pH increased from 4.0 to 6.0, transcripts of *pac1*, the *C. gloeosporioides* *pacC* homolog, also could be detected. Nit mutants of *C. gloeosporioides*, which cannot utilize KNO<sub>3</sub> as a nitrogen source, did not secrete ammonia, alkalize the medium, or secrete pectate lyase. If Nit mutants were grown at pH 6.0 in the presence of glutamate, then pectate lyase secretion was induced. Infiltration of 0.1 M ammonium hydroxide at pH 10 into ripening avocado fruits enhanced the activation of quiescent infection and symptom development by *C. gloeosporioides*. These results suggest that ambient pH alkalization resulting from ammonia accumulation and the availability of ammonia as a nitrogen source independently regulate *pelB* expression, pectate lyase secretion, and virulence of *C. gloeosporioides*. These data suggest that alkalization during *C. gloeosporioides* infection is important for its transformation from the quiescent biotrophic stage to the necrotrophic stage of fungal colonization in the fruit host.**

*Colletotrichum gloeosporioides* is a filamentous ascomycete plant pathogen that attacks the fruits of a variety of pre- and postharvest hosts. *C. gloeosporioides* germinates on avocado (*Persea americana* Miller var. *drymifolia* [Schlechtendal and Chamisso] S. F. Blake) fruit peel and forms appressoria. Infection hyphae from the appressoria penetrate the epidermal cells of the avocado exocarp but remain quiescent until the fruit ripens. The resistance of unripe fruit to fungal attack during quiescence is related to the presence of preformed or inducible antifungal compounds and the lack of secretion of fungal pathogenicity factors. The activation of quiescent infection results from a decline in the concentration of preformed antifungal compounds followed by the activation of fungal pathogenicity factors (27, 28). Pectate lyase (PL) has been implicated as a virulence factor of *C. gloeosporioides* in avocado fruit (26), and its expression is strongly affected by alkalization (8). Alkalization of the tissue occurs naturally during fruit ripening, where the pH of the pericarp increases from 5.2 to 6.1. However, the pathogen also helps increase the amount of ammonia accumulated by the host (16). This increase in accumulated ammonia helps create the alkaline environment

necessary for the activation of some extracellular lyase enzymes, including PL (3, 4, 5, 6, 25, 26). The increase in external pH may change the expression of regulatory genes, including *pacC*, the terminal component of the pH signaling pathway and a transcriptional regulator of pH-dependent genes which is known to regulate the expression of *pelB*, the gene encoding PL (8). Thus, activation of quiescent infection, which requires alkalization of the tissue and colonization by *C. gloeosporioides*, may depend on the availability of exogenous nitrogen sources that can be converted to NH<sub>3</sub>.

It is not clear, however, whether the environmental conditions that affect PL secretion are determined only by the effect of ammonia on pH or if ammonia also has a direct effect on the regulation of *pelB* expression by *C. gloeosporioides*. Our objective in this study was to determine the importance of ammonia accumulation in the secretion of PL. We hypothesized that ammonia and ambient pH are independent signals for the transcriptional regulation of genes required for the disease process of *C. gloeosporioides* during decay development in avocado fruits. The accumulation of large amounts of ammonia in the decaying tissue ensures transformation from the quiescent biotrophic to the necrotrophic stage of *Colletotrichum* colonization.

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### MATERIALS AND METHODS

**Strains, media, and growth conditions.** Single-spore cultures of a Cg-14 isolate of *C. gloeosporioides*, obtained from a decayed *Persea americana* cv. Fuerte avocado fruit in Israel, were routinely cultured on MS agar containing 250

mg/liter chloramphenicol. The fungus was grown in 40 ml MS medium (23) at pH 5.0 (primary culture) at an inoculation density of  $1 \times 10^6$  spores/flask. MS medium contains the following reagents (per liter): 2.5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.7 g  $\text{KH}_2\text{PO}_4$ , 1.5 g peptone Bios D (Biolife, Milan, Italy), 1.5 g Bacto yeast extract (Difco, Detroit, MI), 15 g sucrose, and 250 mg chloramphenicol. Cultures were incubated at 22 to 24°C in a shaking incubator at 150 rpm for 4 days. Cultures were harvested by filtration through a sterile Büchner funnel fitted with filter paper. The hyphal mat was washed twice by filtering with 40 ml of sterile distilled water each time. The washed mycelia were resuspended in 40 ml of fresh medium (secondary medium) containing the following reagents (per liter): 4 g  $\text{K}_2\text{HPO}_4$ , 2 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 g  $\text{KNO}_3$ , 0.3 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 10 mg  $\text{FeCl}_3$ , and 10 g glucose. The medium was buffered with 50 mM phthalate-hydroxide buffer (Sigma, St. Louis, MO) to obtain an initial pH between 4.0 and 7.0. The initial pH for each flask was determined after the medium was autoclaved but prior to inoculation. Experiments were repeated at least three times, with the results of a single representative experiment shown here. Differences between average values for each treatment in each experiment did not differ by more than 2 to 3% between experiments. To create nitrogen starvation conditions, a non-nitrogen-containing medium was used as an intermediate medium between the primary and secondary media. The washed mycelia were resuspended in 40 ml of fresh intermediate medium containing the following reagents (per liter): 4 g  $\text{K}_2\text{HPO}_4$ , 2 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.3 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 10 mg  $\text{FeCl}_3$ , and 10 g glucose.

Four *nit* mutants were obtained from the wild-type isolate Cg-14 as spontaneous mutants growing on potato dextrose agar plates amended with  $\text{KClO}_3$  (10). When transferred to secondary medium, the *nit* mutants were grown in the presence of 3 mM glutamate amended with amounts of  $\text{KNO}_3$  similar to those described above.

Ammonia was detected with an ammonium test kit (Merck KGaA, Darmstadt, Germany) based on a colorimetric reaction. Samples (0.5 ml) from the culture media were diluted with 4.5 ml of double-distilled water, and the concentration of ammonia was determined according to the manufacturer's instructions. In brief, the sample containing ammonium was adjusted to pH 13 so that ammonium was transformed to ammonia and could be detected as ammonia in a colorimetric reaction. Concentrations are reported as mmol ammonia.

The pH was measured with a microcombination pH electrode (model 9810BN; Orion, Beverly, MA) in 0.5-ml aliquots sampled at various times after fungal inoculation.

**Fruits, ripening parameters, and fruit treatments.** Freshly harvested avocado Fuerte fruits from an orchard at Kibbutz Givat Brenner, Israel, were used for fruit treatment and inoculations. The effect of  $\text{NH}_4\text{OH}$  on decay development in vivo was determined by infiltration experiments.  $\text{NH}_4\text{OH}$  at a concentration of 0.1 M in 0.05 M phthalate buffer (pH 10.0) was infiltrated into freshly harvested avocado fruits ca. 12 h prior to inoculation with *C. gloeosporioides* conidial suspensions. Infiltration was performed by dipping fruits in the solution, reducing the atmospheric pressure to 20 mm Hg within 60 s, and then releasing the vacuum. Other fruits, harvested at the same time, were similarly infiltrated with  $\text{H}_2\text{O}$  as a control (8). Fruits were inoculated by placing 10  $\mu\text{l}$  of a conidial suspension ( $10^6$  conidia/ml) on six longitudinally spaced inoculation spots, with three on each side, of the 10 different fruits per treatment (60 inoculation replicates per treatment). Following inoculation, fruits were incubated at 22°C in 95% rH for 24 h in covered plastic containers containing wet paper towels. They were then transferred to ambient (70 to 80%) rH at 22°C until fruit ripening and symptom development were observed (16). The average decay diameter for 10 fruits is reported. Inoculation experiments were repeated three times.

The preformed antifungal compound 1-acetoxy-2-hydroxy-4-oxo-heneicos-12,15 diene (diene) was extracted from the avocado pericarp (2 g of tissue, 1- to 2-mm-thick slices). Pericarp tissue was homogenized in 95% ethanol in a Sorval Omni-Mixer (Kendro, Asheville, NC) at full speed for 3 min as previously described by Yakoby et al. (28). The collected samples were dried, dissolved in 1 ml of running solvent, and analyzed by high-performance liquid chromatography. Unripe fruits were harvested at a firmness of 120 N, and the fruits were considered ripe when the firmness decreased to below 10 N. Firmness, expressed in newtons (N), was measured with a Chatillon digital force gauge (model DFGS50; Ametek Chatillon, Brooklyn, NY).

**Detection of PL in liquid medium.** Hyphae grown on secondary medium were separated by filtration (see above), washed twice with sterile water, frozen with liquid  $\text{N}_2$ , lyophilized, and stored at  $-80^\circ\text{C}$  until used for RNA or protein extraction. The culture medium filtrate was concentrated on a Rotavapor machine (Buchi, Flawil, Switzerland) at 30°C to 5 ml, dialyzed with a SnakeSkin pleated dialysis tube (10,000-molecular-weight cutoff; Pierce, Rockford, IL) for 24 h against 5 liters of 50 mM Tris-HCl, pH 8.5, reconcentrated to 1 ml, lyophilized, and resuspended in 150  $\mu\text{l}$  sterile water. Protein samples were

quantified by the Bradford reagent (Bio-Rad Laboratories, Hercules, CA) protein assay, with bovine serum albumin (Sigma) as the standard.

Samples (2.5  $\mu\text{g}$  of secreted proteins) were loaded onto a 12.5% sodium dodecyl sulfate-polyacrylamide gel (Mini-Protein II; Bio-Rad) and run for 1.5 h at a constant 100 V. Western blot analysis was performed with PL antiserum as previously described (2, 27, 28). Blot analyses were repeated three times, and the results from one representative experiment are presented here.

**RNA extraction and Northern blot analysis.** For Northern analysis, samples of hyphae were first lyophilized and then homogenized with a Mini Beadbeater (Biospec Products, Bartlesville, OK) in the presence of 1 g zirconia beads for three periods of 20 s each, and total RNA was extracted with 1 ml of Tri-Reagent (Sigma) for every 25 mg of lyophilized hyphae. RNA samples were prepared according to the manufacturer's instructions (Tri-Reagent technical bulletin MB-205, Sigma). RNAs were quantified by GeneQuant (Pharmacia Biotech, Cambridge, United Kingdom).

Northern blot analysis was conducted by running 10  $\mu\text{g}$  of total RNA in a 1.1% formaldehyde denaturing agarose gel (19). The RNA was blotted onto a Hybond+ nylon membrane (Amersham, Buckinghamshire, United Kingdom) by the capillary method (19) with  $20\times$  SSC ( $1\times$  SSC is 17 mM NaCl and 170 mM sodium citrate). RNAs were fixed by baking for 2 h at 80°C and then subjected to hybridization. All hybridizations were carried out at 65°C, and reaction mixtures were washed with  $0.1\times$  SSC. Probes were synthesized by using the Prime a Gene labeling system (Promega, Madison, WI) with [ $^{32}\text{P}$ ]dCTP. The hybridization probes were the 1.1-kb full-length *pelB* clone (GenBank accession no. U32942), the 1.2-kb *pacI* coding sequence from *C. gloeosporioides* (GenBank accession no. AF539700) (8), and the rRNA gene repeat sequence from *Neurospora crassa* pMF2 (9). 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, Japan).

For real-time PCR, RNAs were extracted from 80 mg (dry weight) of mycelium with an RNeasy Plant mini kit (QIAGEN Sciences, Hilden, Germany) and further purified by treatment with RNase-free DNase (QIAGEN). The reverse transcription reaction was performed with a Reverse-it first-strand synthesis kit (ABgene, Surrey, United Kingdom). cDNA samples were diluted 1:10 to the final template concentration for real-time PCR. Real-time detection was performed with Absolute SYBR green ROX mix in an ABI Prism 7000 machine (Applied Biosystems [ABI], Foster City, CA). The endogenous control was a  $\beta$ -tubulin gene, and the calibrator was a no-nitrogen sample. The primers for *pelB* were 5'-CACAAGCCCCGACTACAGCT-3' (forward) and 5'-AGCCTTACCTTGGAGGAGCC-3' (reverse); the primers for *areA* (accession no. AY699608) were 5'-ACAGACCACAGGCATTGCAA-3' (forward) and 5'-TGTGGAGACGAAACCCTGAAG-3' (reverse); and the primers for  $\beta$ -tubulin were 5'-GCAACAACTGGGCCAAGG-3' (forward) and 5'-GCGGACAACATCGAGAACCT-3' (reverse). The primer concentration was 200 nM. Primer efficacy was examined by running different solutions of template and verifying a slope of  $-3.2 \pm 0.2$  for a curve of cycle threshold ( $C_T$ ) values versus the log values of template concentrations. Mixtures of all cDNAs used for all the treatments were used as templates for calibration curves. Relative quantification was calculated by the method using  $\Delta\Delta C_T$  (ABI). The  $\Delta C_T$  value was determined by subtracting the  $C_T$  results for the target gene from the  $C_T$  results for the endogenous control gene and then normalized as suggested by ABI ( $\Delta\Delta C_T$ ). The final relative quantification value is  $2^{-\Delta\Delta C_T}$ , which represents the level of expression of the gene in relation to the control (no-nitrogen) treatment (relative quantification). Each experiment was repeated three times with similar results, and results for one experiment are presented. The averages presented are the results of two independent cDNA measurements for different RNA samples from the same treatments. The variation between the quantification levels for the same treatment ranged between 10 and 15%.

## RESULTS

**Effect of pH on induction of ammonia accumulation.** Cultures in primary medium were transferred to secondary medium at a range of pH levels, from 4.0 to 7.0 (Fig. 1). Two hours after transfer of the hyphae to pH 7.0, the amount of accumulated ammonia reached 0.05 mM; at pH 4.0, however, the accumulated amount was 22-fold higher (1.1 mM). Two hours later, hyphae grown at pH 4.0 had secreted more ammonia, reaching up to 1.2 mM, while exposure of the hyphae to higher pH values (5 to 7) resulted in the accumulation of <0.1

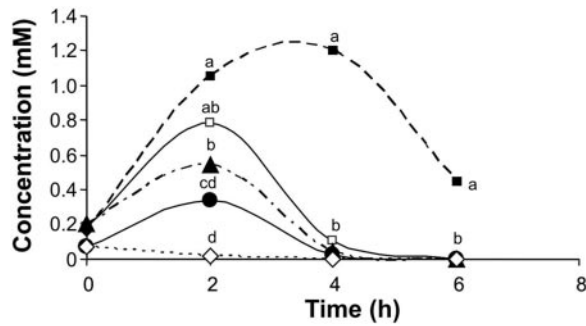


FIG. 1. Effect of pH on ammonia secretion by *C. gloeosporioides*. Samples of medium were quantified for ammonia accumulation at different times after transfer to secondary medium containing  $\text{KNO}_3$ , buffered with phthalate buffer to a pH range of 4.0 to 7.0. Symbols: ■, pH 4; □, pH 5; ▲, pH 6; ●, pH 6.5; ◇, pH 7. Significance was calculated by comparing the concentrations of ammonia at the different initial pHs on each sampling day and was analyzed statistically by analysis of variance of three replications. Values for points on the same day labeled with the same letter were not statistically different ( $P < 0.05$ ).

mM ammonia. This differential accumulation of ammonia raised the question of whether the amount of ammonia present could differentially activate PL secretion. Ammonia also activated *pelB* expression and PL secretion (Fig. 2A and B). Growth of the fungus in secondary medium with increasing concentrations of  $\text{NH}_4\text{Cl}$  (6 to 25 mM) at pH 6.0 enhanced the increased secretion of PL by *C. gloeosporioides* (Fig. 2C).

**Effect of nitrogen sources on ammonia accumulation, *pelB* expression, and PL secretion.** Nitrogen starvation for 15 h before the transfer to secondary medium induced strong expression of *areA* (3.3-fold higher than that in controls), but this expression level dropped to approximately that of the control 9 h after transfer to secondary medium containing  $\text{KNO}_3$  or glutamate (results not shown). Under these conditions (secondary medium buffered at pH 6.0 in the presence of  $\text{KNO}_3$ ), ammonia accumulation and *pelB* expression began earlier than if the fungus was grown in culture medium with glutamate as the nitrogen source (Fig. 3).

**Effect of nitrogen and pH on *pac1* and *pelB* expression and PL secretion.** The growth of *C. gloeosporioides* at pH 6.0 in the presence of  $\text{KNO}_3$  as the nitrogen source enhanced the transcript levels of *pac1* and *pelB*, as well as PL secretion, 16 h after exposure to the secondary medium. If the nitrogen source was not included in the secondary medium but the pH was kept at 6.0, the *pac1* transcript was present at levels similar to those seen when the fungus was grown in medium without nitrate, but no *pelB* signal or PL secretion was observed. At pH 4.0, the *pac1* signal was considerably reduced, and neither the *pelB* signal nor PL was secreted (Fig. 4).

**Effect of deficiency of ammonia secretion by *C. gloeosporioides* on alkalization.** We made four Nit mutants, which are unable to utilize  $\text{NO}_3^-$  as a nitrogen source (10). These mutants grew well in the primary medium, which contains peptone and yeast extract as potential nitrogen sources. PL secretion by the wild type and the Nit mutants was similar if they were grown in 50 mM glutamate-amended secondary medium at pH 6.0. Under these conditions, both the Nit mutants and the wild-type strain secreted similar amounts of ammonia into the medium

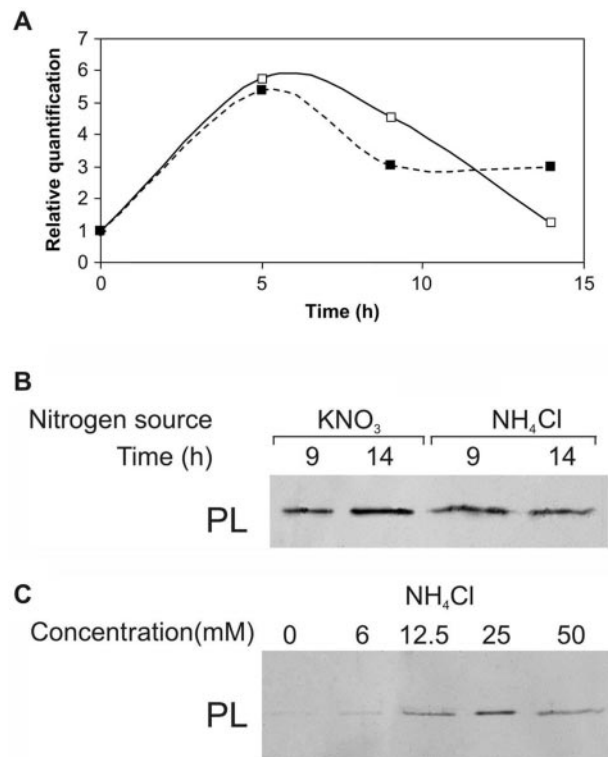


FIG. 2. Effect of  $\text{NH}_4\text{Cl}$  as a nitrogen source on *pelB* expression and PL secretion during growth of *C. gloeosporioides* in culture medium. At different times after transfer to secondary medium buffered with phthalate buffer (pH 6.0) in the presence of 50 mM  $\text{NH}_4\text{Cl}$  (■), the level of secreted PL was analyzed by Western blot analysis of concentrated, dialyzed culture medium, and *pelB* expression was analyzed in the mycelia. Growth of the fungus in the presence of 50 mM  $\text{KNO}_3$  (□) was used as a control. (A) *pelB* expression by real-time PCR. (B) PL secretion in the presence of  $\text{NH}_4\text{Cl}$ . (C) PL secretion in the presence of increasing concentrations of  $\text{NH}_4\text{Cl}$  16 h after transfer to secondary medium. Blot analyses were repeated three times, and results from a representative experiment are presented.

(0.12  $\mu\text{g/ml}$  and 0.11  $\mu\text{g/ml}$ , respectively). Growth of the Nit mutants and the wild type in 3 mM glutamate-amended secondary medium did not affect fungal growth compared to that with higher glutamate concentrations, but alkalization did not occur. The addition of  $\text{KNO}_3$  to the 3 mM glutamate-containing, nonbuffered secondary medium at pH 4.0 or 6.0 did not cause the Nit mutants to accumulate ammonia, increase the pH of the medium, or secrete PL. The wild-type progenitor strain, Cg-14, increased the amount of ammonia secreted, the pH of the medium (from 4 to 6.25), and the amount of PL secreted.

**Effect of ammonia on virulence.** The direct effect of ammonia on *C. gloeosporioides* virulence was analyzed by measuring the diameter of decay on avocado Fuerte fruit caused by the addition of ammonia to fruits inoculated with the wild-type Cg-14 strain. Infiltration of fruits with a 0.1 M  $\text{NH}_4\text{OH}$  solution in 50 mM phthalate buffer, pH 10, following inoculation with Cg-14 significantly enhanced decay development (Fig. 5). The decay development of fruits infiltrated with 50 mM phthalate buffer at pH 10.0 was similar to that of water-treated fruits.  $\text{NH}_4\text{OH}$  did not affect fruit ripening, as measured by fruit

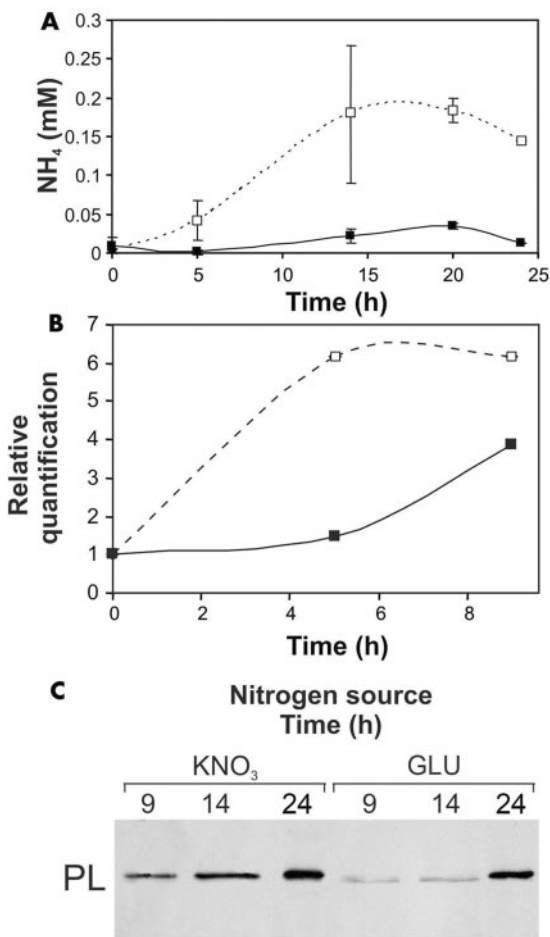


FIG. 3. Ammonia secretion, transcript levels of *pelB*, and PL secretion by *C. gloeosporioides* at pH 6.0 in the presence of glutamate (GLU) (■) or KNO<sub>3</sub> (□) as the nitrogen source in the culture medium. (A) Ammonia accumulation. (B) *pelB* transcript levels. (C) PL secretion. Following growth on primary medium, mycelia were transferred for 15 h to a medium lacking nitrogen and then transferred to the secondary medium. At different times after transfer to the secondary medium, the following were analyzed: ammonia accumulation, *pelB* expression (by real-time PCR), and PL secretion (by Western blot analysis). Western blot analysis of secreted PL was performed on concentrated, dialyzed culture medium. Blot analysis was repeated three times, and the results of one representative experiment are presented.

firmness or ethylene or CO<sub>2</sub> evolution on different days after harvesting (results not shown). The levels of the antifungal compound (diene) in NH<sub>4</sub>OH- and control-treated fruits declined similarly, from 1,320 to 330 μg<sup>-1</sup> g fresh weight of fruit peel, suggesting that differential decay development is not affected by a differential decline in the level of the preformed antifungal diene.

**DISCUSSION**

*C. gloeosporioides* is a broad-host-range plant-pathogenic fungus that secretes ammonia as a mechanism of tissue alkalinization (8). An environmental pH of 6.0 significantly increases both *pelB* expression and the quantity/activity of the specific protein species secreted compared to those secreted at

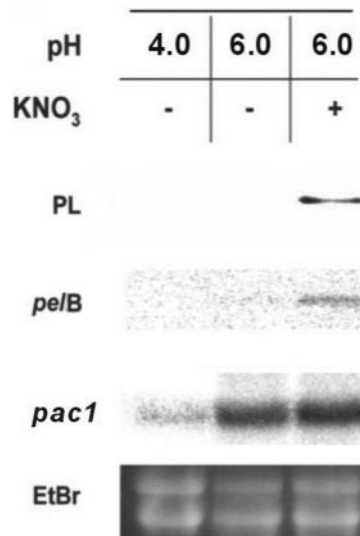


FIG. 4. Transcriptional activation of *pelB* and *pac1* and PL secretion by *C. gloeosporioides* as a function of pH levels and nitrogen amendment. The different factors were analyzed 16 h after transfer to the secondary medium buffered with phthalate buffer at pH 4.0 or 6.0 in the presence or absence of KNO<sub>3</sub>. Northern blots of total RNA were probed with *pelB* and then sequentially stripped and reprobated with *pac1* and rRNA gene (bottom panel) probes. Secreted PL was analyzed by Western analysis. Ethidium bromide staining was used as a loading control.

pH 4.0 (8, 28). The finding that, during alkalinization, *pelB* and *pac1* transcript levels increase in parallel suggests that an ambient pH signal transduction pathway (regulated by the *pacC* homolog) exists in *C. gloeosporioides* and modulates *pelB* (6, 8, 17, 20).

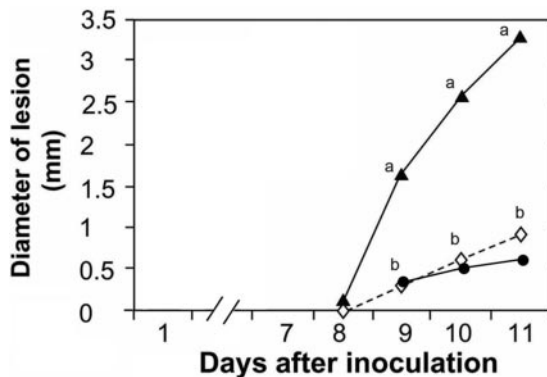


FIG. 5. Effect of NH<sub>4</sub>OH treatment on decay development by *C. gloeosporioides* on avocado fruits. Symptoms of decay by *C. gloeosporioides* wild-type Cg-14 on avocado Fuerte fruits were examined after infiltration with 0.1 M NH<sub>4</sub>OH in 0.05 M phthalate, pH 10. Fruits were inoculated 12 h after infiltration. Lesion development in NH<sub>4</sub>OH-treated fruits (▲) was compared to changes occurring on H<sub>2</sub>O (◇)- or 0.05 M phthalate (pH 10) (●)-infiltrated fruits. Each experiment contained 10 repetitions (fruits), with a total of 30 replications (10 fruits × 3 replications per fruit). Significance was calculated from comparisons of the diameters of lesion decay by *C. gloeosporioides* on NH<sub>4</sub>OH-treated and nontreated fruits on each sampling day and was analyzed statistically with a Student *t* test. Averages for points on the same day labeled with different letters were statistically different (*P* < 0.05).

Drori et al. (8) suggested that the secretion of PL is also subject to nutritional signals, such as the presence of nitrogen. The regulation of nitrogen assimilation is both complex and important for disease development (13). In *Colletotrichum acutatum*, nitrogen starvation stimulates synchronous preinfection development (11). In *Magnaporthe grisea*, nitrogen starvation enhances the expression of *PTH11* and *MPG1*, genes affecting appressorium differentiation and formation, respectively (7, 21). In *Colletotrichum lindemuthianum*, however, mutants of the AREA fungal nitrogen regulators are not affected during the biotrophic stage but must function during the necrotrophic stage (15).

For *C. gloeosporioides*, the presence of a nitrogen source seems to be a key element in the expression of virulence factors. Nutritional deprivation of primary nitrogen sources has shown that they are critical for PL secretion. PL secretion was observed only with the combination of a suitable environment (pH 6.0) and ammonia production. Increasing the amount of ammonia added to the secondary growth medium also increased the amount of PL secreted. Differential rates of ammonia accumulation at pH 6.0 by *C. gloeosporioides* in the presence of  $\text{KNO}_3$  and glutamate resulted in differential activation of *pelB* expression and PL secretion. In vivo, it is possible that the nitrogen source nutrients for the pathogen's initial growth are provided by protease activity breaking down structural glycoproteins in the plant cell wall (18). *C. gloeosporioides*, *C. acutatum*, and *Colletotrichum coccodes* probably utilize these degraded proteins to secrete up to 3,350  $\mu\text{M}$  ammonia into decayed tissue (16). Ammonia may therefore have the following two key functions: (i) alkalization of tissue and (ii) direct activation of *pelB* expression and PL secretion (16, 28). The effect of ammonia on alkalization of the medium in the presence of  $\text{NO}_3$  resulted in an increase in the pH level from 4 to 6.5 (16). Alkalinization of the host's decayed tissue also has been observed for apples, tomatoes, and other infected hosts (16). This alkalization represents the basic signal for activation of the pH-dependent transcriptional regulator *pac1*, which modulates the activation of *pelB* and the secretion of PL.

Several experiments were performed to demonstrate the importance of ammonia accumulation and pH on the expression of pathogenicity factors. When the pathogen was grown at pH 6.0 without nitrogen sources, ammonia did not accumulate and *pelB* expression and PL secretion were not detected; only the pH-dependent transcription activator *pac1* was expressed. The addition of a nitrogen source to the same secondary medium resulted in the accumulation of ammonia, *pelB* expression, and PL secretion. However, the growth of *C. gloeosporioides* in nitrogen-containing medium at pH 4.0 prevented *pac1* and *pelB* transcript expression and, consequently, PL secretion.

In a second set of experiments in which *C. gloeosporioides* Nit mutants were grown in the presence of the easily assimilated nitrogen source glutamate, the mutants secreted ammonia, alkalized the medium, and secreted PL at pH 6.0 as well. Growth of the mutants under similar conditions in the presence of  $\text{NO}_3$  as the nitrogen source did not enhance the secretion of ammonia or PL or increase the pH.

A second postulated function for ammonia is the direct activation of *pelB* expression and PL secretion, as observed when ammonia was included as the sole nitrogen source. This

hypothesis was supported by the differential accumulation of *pelB* transcripts and PL secretion when the fungus, grown in the presence of two other nitrogen sources ( $\text{KNO}_3$  and glutamate), produced different amounts of ammonia. These results suggest that the accumulation of large amounts of ammonia at the infection site during decay development represents a specific condition that is recognized by the pathogen and that activates virulence factors such as *pelB* expression and PL secretion. Furthermore, infiltration of ammonia into ripening avocado fruits caused a clear increase in decay development by *C. gloeosporioides*, supporting the importance of ammonia during fungal pathogenicity.

The signal that activates the mechanism of ammonia accumulation and the activation of *pelB* expression and PL secretion is still not known. The mechanism responsible for reduced fungal sensitivity to high levels of ammonia during fungal attack is also not known. Our present results suggest that one of the signals affecting the process of ammonia secretion is a low environmental pH (4.0). Ammonium is an important nitrogen source for bacteria, fungi, and plants, but it is toxic to animals (22). The ammonium transport proteins (methylamine permeases/ammonium transporters) are ubiquitous in bacteria, archaea, and eukarya (12), are specific for ammonium, and function in the utilization of ammonium as a source of nitrogen (14), but their amounts and activities during fungal pathogenicity have not been described. Members of the MEP/amount family may transport  $\text{NH}_4^+$  across the cytoplasmic membrane and concentrate it in an energy-dependent manner (24). Cell membrane channels with high affinities for ammonium transport that function in both the uptake and excretion of ammonium have recently been described (22). Further studies of these transporters could help explain the mechanism underlying ammonia accumulation by *Colletotrichum* species.

Our results support the hypothesis that both ambient pH and nitrogen availability are major regulators of *C. gloeosporioides pelB* transcriptional activation and PL secretion. PL secretion is transcriptionally regulated by pH, and at least one component of a conserved regulatory pathway mediating pH-regulated gene expression, *pac1*, exists in this fungus (8). Our data suggest that alkalization by the presence of ammonia is also important for the activation of quiescent *C. gloeosporioides* infection and for its transformation from the biotrophic to the necrotrophic stage of fungal colonization in the fruit host (16). The accumulation of specific amino acids during fruit ripening (1) could contribute to substrate availability to the pathogen to ensure ammonia production. The ability of the pathogen to accumulate large amounts of ammonia in decaying tissue ensures the activation of *pelB* expression under optimal pH and nutritional conditions for PL secretion. Altering ammonia secretion and pH-regulated processes, such as *pelB* expression, could form the basis for a viable strategy to delay, reduce, or prevent disease development by this plant-pathogenic fungus.

#### ACKNOWLEDGMENTS

This research was supported by grants to D.P. from BARD. H.K.-H. and E.S. were awarded personal scholarships from the Israel Fruit Marketing Board/Ministry of Agriculture & Rural Development.

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