Local Modulation of Host pH by *Colletotrichum* Species as a Mechanism to Increase Virulence

Dov Prusky,[1,2] James L. McEvoy,[1] Britta Leverentz,[1] and William S. Conway[1]

[1]Produce Quality and Safety Laboratory, Henry A. Wallace Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705, U.S.A.; [2]Department of Postharvest Science of Fresh Produce, Institute for Technology and Storage of Agricultural Products, The Volcani Center, Agricultural Research Organization, P. O. Box 6, Bet Dagan 50250, Israel

The phytopathogenic fungus *Colletotrichum gloeosporioides* produces one pectate lyase (PL) that is a key virulence factor in disease development. During growth of *C. gloeosporioides*, *Colletotrichum acutatum*, and *Colletotrichum coccodes* in acidified yeast extract medium, the fungus secreted ammonia and increased the medium pH. Ammonia accumulation and the consequent pH change increased as a function of initial pH and buffer capacity of the medium. PL secretion by *C. gloeosporioides* correspondingly increased as the pH of the medium increased. The *C. gloeosporioides* *pelB* gene-disrupted mutant was able to increase ammonia accumulation and pH of the media similarly to the wild-type isolate. *C. gloeosporioides* in avocado, *C. coccodes* in tomato, and *C. acutatum* in apple showed ammonia accumulation in the infected area where pH increased to 7.5 to 8 and PL activity is optima. In nonhost interactions where *C. gloeosporioides* was inoculated in apples, the addition of ammonia-releasing compounds significantly enhanced pathogenicity to levels similar to those caused by the compatible *C. acutatum*–apple interaction. The results therefore suggest the importance of ammonia secretion as a virulence factor, enhancing environmental pH and pathogenicity of the *Colletotrichum* species.

Resistance of unripe fruits to *Colletotrichum* spp. quiescent infections depends on i) the presence of preformed antifungal compounds that decline in ripening fruits, ii) inducible antifungal compounds, and iii) the lack of activation of fungal pathogenicity factors during fruit ripening (Prusky 1996). Significant data suggests the importance of preformed and inducible antifungal compounds in fruits (Prusky 1996; Prusky and Keen 1993). Few reports have described the possibility that fruit resistance to fungal attack is dependent upon the lack of activation of fungal pathogenicity factors (Prusky 1996), including pectolytic enzymes responsible for maceration of the plant cell wall leading to decay. The inhibition of pathogenicity factor production might be the result of direct inhibition by specific inhibitors such as polygalacturonase-inhibiting protein (Benhamou 1996) and phenols (Wattad et al. 1994), which inhibit fungal attacks during specific periods of fruit development. Yakoby et al. (2000) suggested that the absence of fungal colonization also might depend on inhibiting the secretion of a specific enzyme involved in virulence.

Pectate lyase (PL) from *Colletotrichum gloeosporioides* is secreted at pH values higher than 5.8, but *pel*B was already expressed at pH values of 5.1 (Yakoby et al. 2000). The differential timing of *pel*B expression and PL secretion suggests that PL is translated, but the protein remains in the mycelium until a secretion-permissive pH level is reached. Additionally, PL was not secreted if the medium was adjusted to pH levels lower than 5.7. When mycelium grown in pectolytic-inducing media (PEIM) at pH 6.5 was transferred to fresh PEIM at a pH lower than 5.7, no PL secretion was detected. The lack of PL secretion in PEIM at low pH, following the transfer of mycelium from conditions where PL was being secreted, further supports the regulation of PL secretion by a pH-dependent mechanism.

pH affects a series of regulatory processes in fungi and yeast (De Bernardis et al. 1998; Denison 2000; Espeso et al. 1993; Keller et al. 1997; Macheroni et al. 1997; Muhlschlegel and Fonzi 1997; Orejas et al. 1995; Otero and Gaillardin 1996; St. Leger et al. 1999). Regulation of genes encoding extracellular enzymes by ambient pH is useful for fungi such as *Aspergillus nidulans*, which can grow over a wide pH level of 2.5 to 9.0. Such a pH regulation system ensures that extracellular enzymes, permeases, and other exported metabolites are produced under pH conditions where they can function (Denison 2000). The natural increase of host pH during fruit ripening regulates *pel*B expression and PL secretion by *C. gloeosporioides* in avocado fruits. Our findings indicate, however, that host environmental pH also is influenced directly by the pathogen. In the present report, we describe the capability of the *Colletotrichum* species to secrete ammonia locally into the host tissue, resulting in a pH increase that enables enzymatic secretion and enhanced virulence.

Corresponding author: D. Prusky;. Telephone: +972 3 968 3880; Fax: +972 3 968 3622; E-mail: prusky33@netvision.net.il

RESULTS

pH changes induced by *Colletotrichum* species during growth in the presence of yeast extract.

Growth of *C. gloeosporioides* on 1% yeast extract solid medium increased the pH from 4.5 to 5.8, as detected by the change of Alizarin red S dye from yellow to red and direct pH measurement of the medium (Fig. 1). *C. acutatum* and *C. coccodes* increased pH of the medium several millimeters out from the leading edge of the colony, similar to *C. gloeosporioides* (results not shown). A *pel*B gene-disrupted mutant
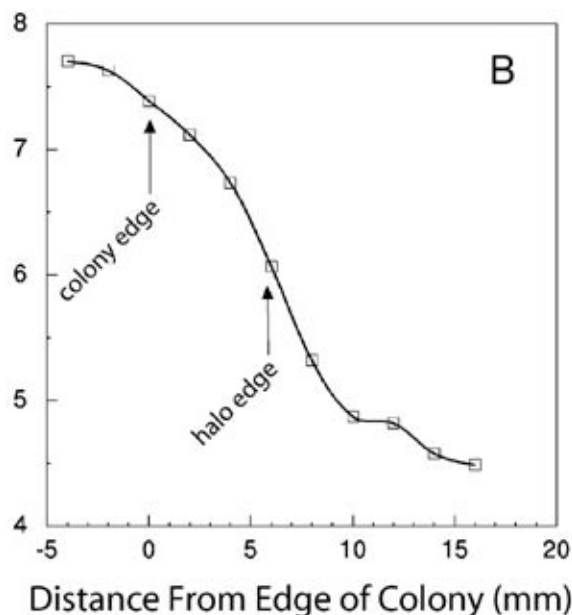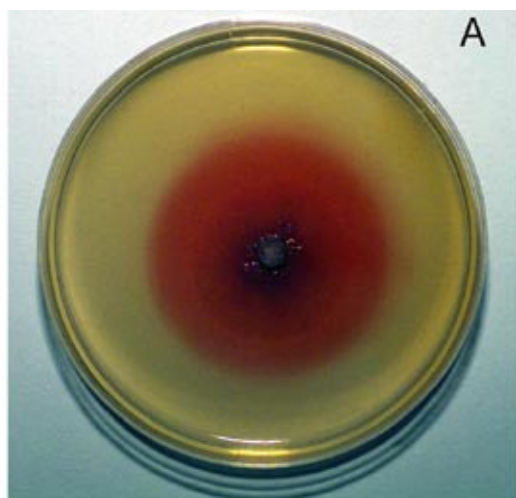
Fig. 1. pH increase induced by *Colletotrichum gloeosporioides* on acidified yeast extract medium. **A,** Yeast extract plates at pH 4.0 were inoculated with *C. gloeosporioides,* incubated at 25°C for 5 days, then the plates were stained with 0.2% Alizarin red S to determine pH change. pH higher than 6.0 was indicated by red, whereas a pH of 4.0 or less was indicated by yellow. **B,** Direct pH measurements on the growth plate.

induced the same pH changes as the wild-type isolate of *C. gloeosporioides* (result not shown).

Induced increase of ammonia and pH in yeast extract medium by *C. acutatum*, *C. gloeosporioides*, and the *pel*B gene-disrupted mutant.

C. acutatum and *C. gloeosporioides* increased ammonia concentration in yeast extract medium, at 40 h postinoculation, to 6,000 µmol per g of dry weight. It increased, 11 h later, to 12,000 µmol per g of dry weight (Fig. 2). Ammonia production by both Colletotrichum strains was closely related to fungal growth (Fig 3A). A significant increase, from 0 to 6,000 µmol per g of dry weight, in ammonia corresponded to an increase in pH from 4.0 to 5.0. Following that breakpoint, the pH increased by 1 U for approximately 1,500 µmol per g of dry weight of ammonia accumulated (Fig. 3B). The *pel*B gene-disrupted mutant induced an increase in ammonia, and the corresponding increase in pH of the media was similar to the wild type *C. gloeosporioides* (results not shown).

Initial pH and buffer concentration affects ammonia accumulation and induction of pH changes by *C. gloeosporioides*.

To determine the effect of the initial pH on the induction of pH changes and ammonia production, *C. gloeosporioides* was grown in ammonia-noninducing medium M_3S for 3 days, then exposed to the inducing acidified yeast extract medium. In the presence of 50 µM phthalate buffer and an initial pH of 6.2, the growth of *C. gloeosporioides* for 40 h resulted in an ammonia accumulation of 1,050 µmol per g of fungal dry weight and a pH increase to 7.2 (Fig. 4). In the uninoculated acidified yeast extract medium, the ammonia concentration was 1,000 times lower. The pH level induced by *C. gloeosporioides* in a similar buffer concentration (50 µM phthalate buffer)
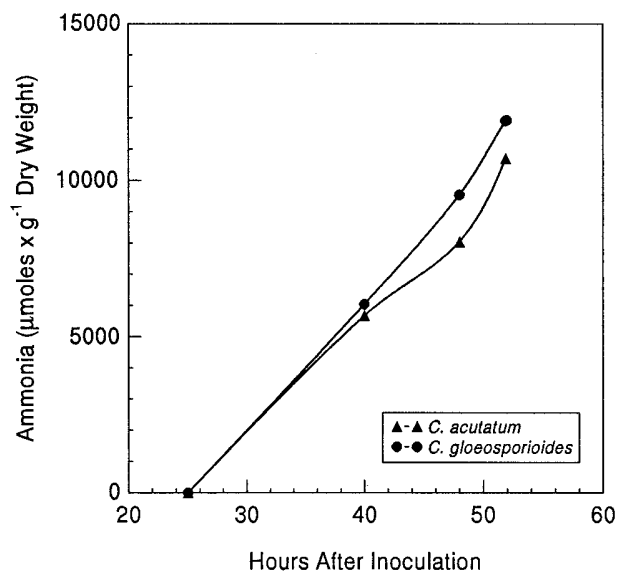
Fig. 2. Accumulation of ammonia in acidified yeast extract liquid medium used to grow *Colletotrichum gloeosporioides* and *Colletotrichum acutatum.*

but at an initial pH of 4.2 was only up to 4.8. The ammonia accumulation increased only to 400 μmol per g of dry weight. If *C. gloeosporioides* was transferred to medium containing phthalate buffer at a similar low pH of 4.2 but at lower concentrations of 10 and 5 μM, ammonia accumulated and the pH increased as the buffer concentration declined. PL secretion into the medium containing 50 μM phthalate buffer was highest when the initial pH of the inducing medium was 6.2, whereas no secretion was observed at pH 4.2. As the concentration of the phthalate buffer decreased to 10 and 5 μM and the final pH of the medium increased, PL secretion increased as well.
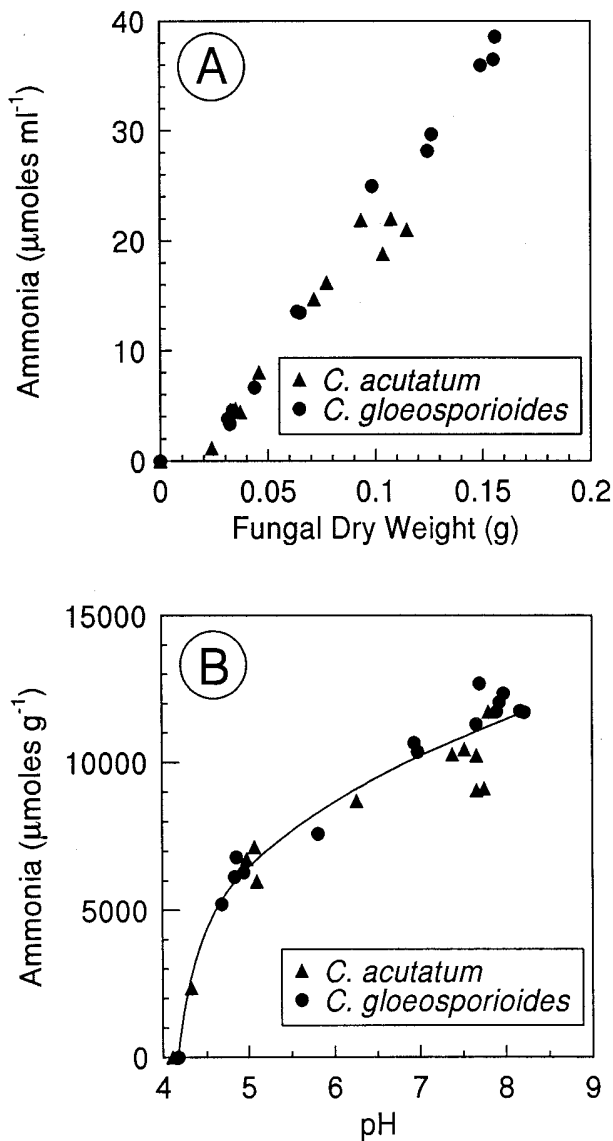

Fig. 3. Relationship between growth, ammonia accumulation, and pH changes in cultures of *Colletotrichum gloeosporioides* and *Colletotrichum acutatum*. Acidified yeast extract was inoculated with *C. gloeosporioides* and *C. acutatum*. Ammonia concentration, pH, and fungal dry weight were determined at various intervals. **A,** Relationship between the dry weight of *Colletotrichum* species and ammonia production. **B,** Relationship between ammonia production and pH changes.

Ammonia concentrations and pH changes induced by the avocado pathogen *C. gloeosporioides* and the tomato pathogen *C. coccodes*.

C. gloeosporioides isolate Cg-14 decayed ripe avocado fruits within 4 days of fruit ripening. Direct pH measurements in the healthy part outside of the decayed mesocarp tissue showed pH values of 6.5 to 6.7 (Fig. 5A). In the center or at the edge of the decayed tissue, however, the pH values were about 7.0 to 7.2. The concentration of ammonia in the extract from healthy avocado mesocarp was 550 μM, with a pH value of 6.9, whereas in the decayed part, ammonia concentration reached 3,350 μM, with a pH of 8.5 (Fig. 5B). Avocado fruits infected with *C. acutatum* also showed characteristic symptoms of decay with a pH increase to 8.2 and an ammonia concentration of 1,025 μM (results not shown). *C. coccodes* caused significant decay development on ripe tomato fruits within 5 days. Direct pH measurements of healthy tomato pericarp tissue (OE and OH in Fig. 6A) showed pH values of 5.1 to 5.5. The pH in the center or the edge of the decayed area (DC and DE in Fig. 6A), however, was approximately 8.0. The concentration of ammonia in the healthy tomato pericarp was 300 μM, with a pH value of 4.5, whereas in the decayed portion, the concentration of ammonia reached 3,600 μM, with a pH of 8.6 (Fig. 6B).

Ammonia concentration and pH changes induced in apples by apple pathogen *C. acutatum* and non-apple pathogens *C. gloeosporioides* and *C. coccodes*.

C. acutatum decayed Golden Delicious apples. Within 7 days postinoculation, the average lesion diameter was 4.2 ± 0.3 mm. *C. gloeosporioides* and *C. coccodes* caused only me-
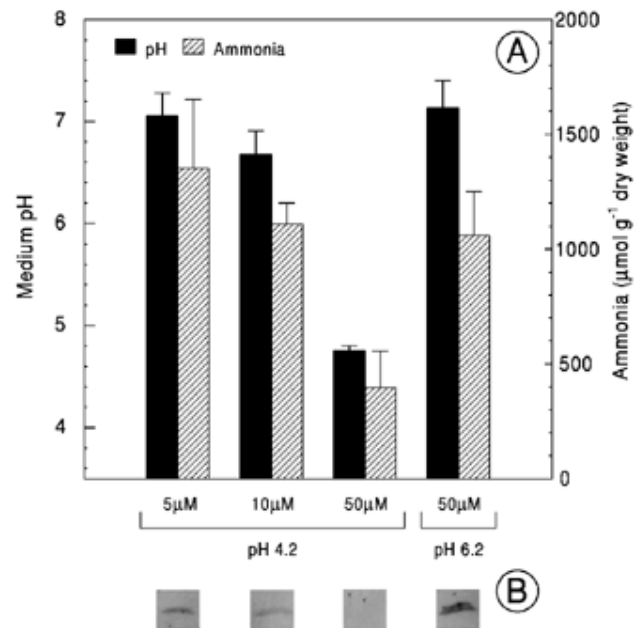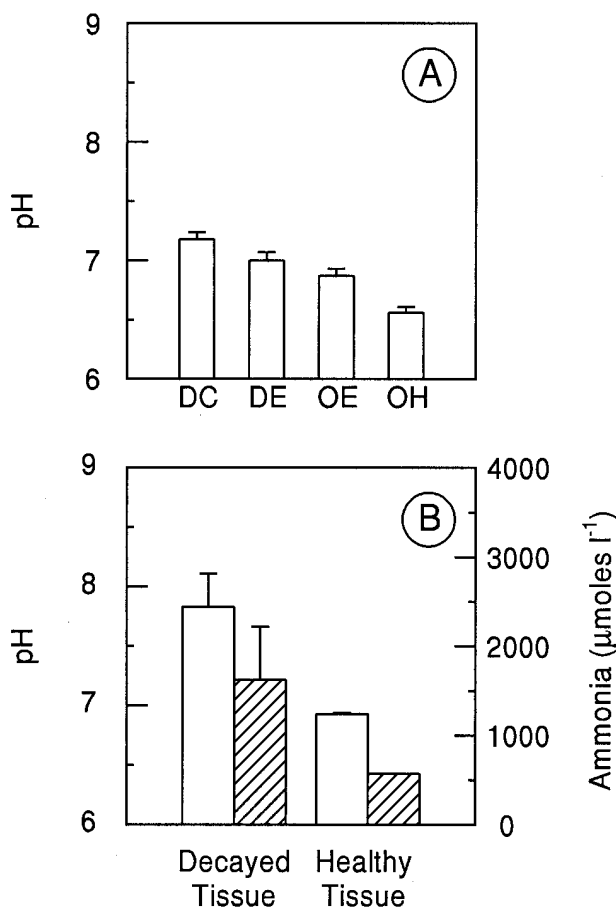

Fig. 4. Effect of pH and buffer molarity on the induction of ammonia accumulation, pH increase, and pectate lyase (PL) secretion by *Colletotrichum gloeosporioides* in yeast extract medium. **A,** Effect of initial medium pH and buffer molarity on final pH and ammonia accumulation. **B,** PL detection by Western blot analysis, following the concentration of growth medium.

dium and minor lesions, however, with average decay diameters of 1.5 ± 0.3 and 0.7 ± 0.2 mm, respectively.

Direct pH measurement of decayed apple tissue inoculated with *C. acutatum* indicate a very localized pH change only in the infected area (Fig. 7). pH measurements of healthy apple tissue (OE and OH in Fig. 8) had pH values from 3.9 to 4.1, but in the decayed tissue, the pH increased to 6.5. Similar pH values were detected in the center (DC in Fig. 8) or inner edge (DE in Fig. 8) of the decayed tissue. The concentration of ammonia in the healthy part of the apple was 90 μM, with a pH value of 3.8, whereas in the decayed tissue, ammonia concentration increased almost 2.8-fold (250 μM), with a pH of 6.9.

pH values in the decayed tissue of apples inoculated with *C. gloeosporioides* and *C. coccodes* were lower than for *C. acutatum*, reaching only 4.0 to 5.1 (Fig. 8). In the healthy part of the apple, the values were similar to those in apples inoculated with *C. acutatum*. The concentration of ammonia and the pH in the juice of apples decayed by *C. gloeosporioides* and *C. coccodes* was 80 and 60 μM, respectively, with a pH of 4.0 to 5.5. In the healthy tissue, the concentration of ammonia was 50 to 60 μM, with pH values of approximately 4.0 to 4.3.

Enhanced virulence of *C. acutatum, C. gloeosporioides,* and *C. coccodes* by enhanced ammonia production.

Decay development of *C. acutatum, C. gloeosporioides,* and *C. coccodes* differed significantly on inoculated apples with *C. acutatum* being the most pathogenic, although buffered at pH 7.0 (Fig. 9). Decay development of all *Colletotrichum* species was enhanced when apples were inoculated with spores of the three species in the presence of 1% yeast extract or 15 μM ammonium chloride at pH 7.0. Yeast extract and ammonium chloride enhanced decay development of *C. gloeosporioides* on apples to values similar to those produced by the pathogen *C. acutatum* but could not enhance decay development of *C. coccodes* to the same levels as that of *C. acutatum.*

DISCUSSION

Resistance of unripe avocado fruits to *C. gloeosporioides* depends on the presence of significant concentrations of antifungal compounds (Prusky 1996; Prusky and Keen 1993; Prusky et al. 1988; Simmonds 1941). Yakoby et al. (2000), however, reported that ripe fruits from specific cultivars with
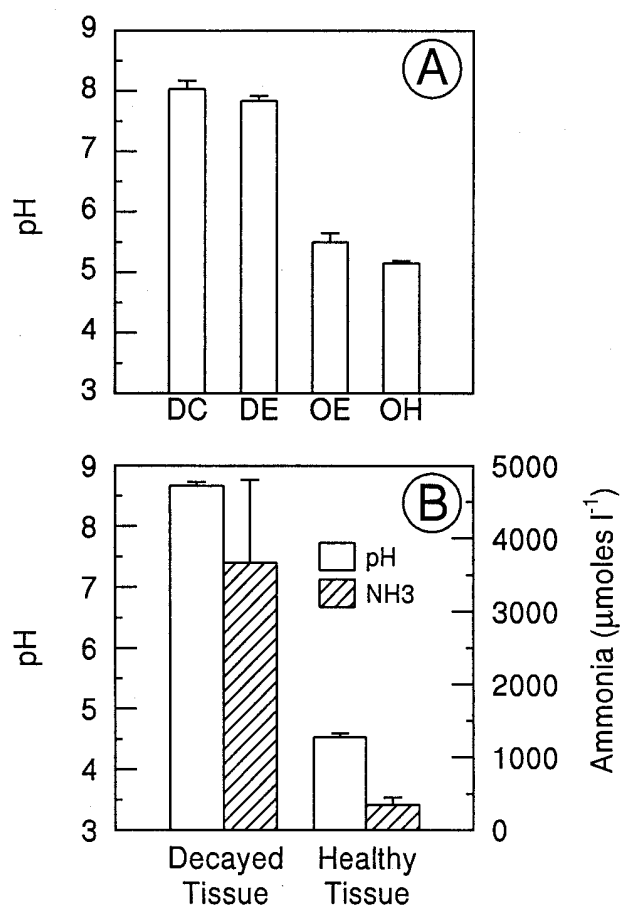

Fig 5. pH changes and ammonia accumulation induced by *Colletotrichum gloeosporioides* on avocado fruits cv. Hass. **A,** Direct pH measurements. **B,** pH and ammonia concentration in the juice of the fruit. DC, decay center; DE, decay edge; OE, outside healthy edge; OH, outside healthy tissue. Measurements were taken 7 days postinoculation. Results are from one of four different experiments.

Fig 6. pH changes and ammonia accumulation induced by *Colletotrichum coccodes* on tomato cv. Roma fruits. **A,** Direct pH measurements. **B,** pH and ammonia concentration in the juice of the fruit. Measurements were performed 5 days postinoculation. Results are from one of four experiments. DC, decay center; DE, decay edge; OE, outside healthy edge; OH, healthy tissue.

significantly lower concentrations of the antifungal diene were still resistant to *C. gloeosporioides* (Prusky 1996). The absence of fungal colonization in fruit pericarp and PL expression at pH values lower than 5.8 suggested that host environmental regulation of pH affects PL secretion and virulence (Prusky 1996). When *Colletotrichum* species were grown on solid medium containing yeast extract at pH 4.2, the pH of the colony area increased to values of 7.7 and the area of pH levels of >6.0 expanded out from the colony. Presumably, this indicates that the metabolic activity at the leading hyphal edge of the fungus grown on yeast extract, including oxidative deamination of amino acids, leads to ammonia accumulation, diffusion (Jennings 1989), and a correspondent pH change. Analysis of ammonia levels produced by *Colletotrichum* species and the *C. gloeosporioides pel*B gene-disrupted mutant in the presence of a concentrated protein source and pH increase indicates that ammonia was produced during growth at levels sufficient to account for alkalinization of the medium. Ammonia was reported to be produced by several fungi such as *Neurospora crasa*, *Aspergillus fumigatus*, *Metarhizium anisopliae* (St. Leger et al. 1998), and *Candida albicans* (De Bernardis et al. 1998). Ammonia production by fungi during growth on protein as the sole carbon source is believed to result from dissimilation of amino nitrogen produced in excess of that required for growth (Jennings 1989). *N. crassa* and *A. fumigatus*, however, also produced ammonia in minimal medium, suggesting that the source may be internal nitrogenous reserves. The production of ammonia by *Colletotrichum* species and the decay of fruits such as avocado, tomato, and apple suggests that *Colletotrichum* species efficiently utilize the nitrogen sources present in the fruits.

pH increases in liquid medium exhibit a two-stage process. During the first stage, an increase of 6,000 µmol of ammonia per g of dry weight of *Colletotrichum* spp. hyphae increase the pH in the medium by 1 U, whereas in the second stage, only 1,500 µmol of ammonia per g of dry weight was needed to increase the pH in the medium by 1 U. This suggests that buffer capacity affects the pH increase in the medium. The importance of buffer capacity in the medium was shown when the reduction (from 50 to 5 µM) of the concentration of the phthalate buffer, pH 4.2, led to a significant increase in ammonia accumulation. When the fungus was grown in 50 µM buffer at pH 6.2, however, rapid ammonia secretion occurred, comparable to 5 µM buffer at pH 4.2. This suggests that low pH is not a signal for the initiation of ammonia secretion; rather, the combination of a proper pH and buffer strength regulates ammonia secretion and, consequently, gives an increase in pH.

Previous studies by Yakoby et al. (2000) with avocado fruits indicate that pH increases in ripening fruits can lead to PL secretion and consequent decay development. In the present report, apples, tomatoes, and avocados with a pH of 3.5, 4.2, and 6.5, respectively, had an increase of local pH to 7.2 or above when inoculated with compatible *Colletotrichum* spp. pathogens. In all three fruit–pathogen interactions, a significant increase in ammonia concentration also was detected locally in the decayed tissue. The amount of ammonia accumulated in *C. acutatum*-decayed apples was approximately one-tenth (250 µM) of that in tomato and avocado. These results suggest that there are different initial levels and, possibly, sources of nitrogen in each fruit, which also may contribute to the presence of different pH values in healthy fruit

(Hulme 1970). It may be possible that the higher initial pH values of avocado and tomato enable the fungus to grow rapidly and produce more ammonia, similar to the growth in liquid medium at higher pH. In nonhost interactions where *C. gloeosporioides* and *C. coccodes* caused only reduced or minor infections on apples, the ammonia levels in infected tissue were similar to those in the healthy part of the fruit.

It is difficult to conclude that ammonia production directly enhances the pathogenicity of *Colletotrichum* species because it affects virulence by increasing the environmental pH needed for full pathogenicity. It is clear, however, that *pelB* expression is not dependent on the secretion and accumulation of ammonia because PL was not produced when the mutant was grown in media-inducing ammonia formation (Yakoby et al. 2001). When ammonia-inducing or -releasing compounds were added to the infection point, however, colonization by *C. gloeosporioides* (a nonhost pathogen in apples) increased in a similar manner as the compatible *C. acutatum*. This effect did not occur when ammonia-inducing compounds were added to *C. coccodes* spores before apple inoculation, suggesting that factors other than the presence of ammonia may be necessary to enhance decay development of *C. coccodes* in apples. In a bacterial system, an increase in intercellular pH from 5.5 to 7.5 was found when *Pseudomonas syringae* pathovars infected *Phaseolus vulgaris*. This pH change appears to promote bacterial population growth. In this case, sucrose efflux was suggested as the causal factor for bacterial growth. It should be noted that the effect of nitrogen-containing compounds on the pH change in this bacterial system was not investigated (Atkinson and Baker 1987).

The importance of pH-responsive gene regulation during pathogen attack was not always considered a pathogenicity factor. Rollins and Dickman (2001) and earlier work suggests that pathogenicity of *Sclerotinia sclerotiorum* is regu-
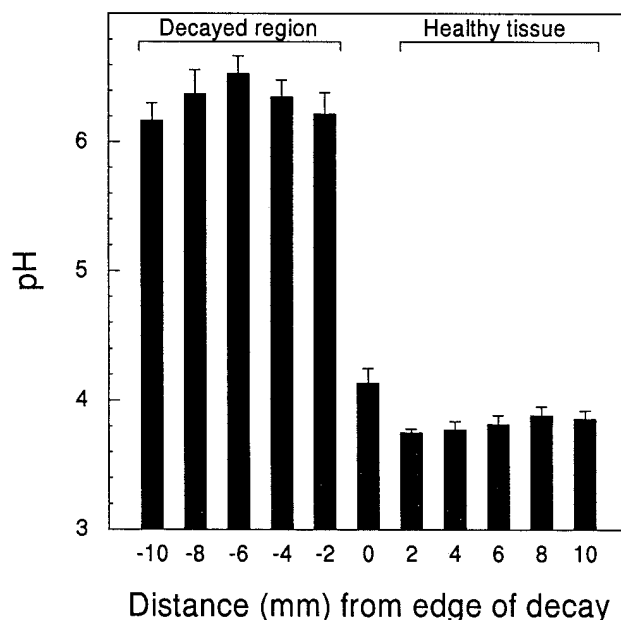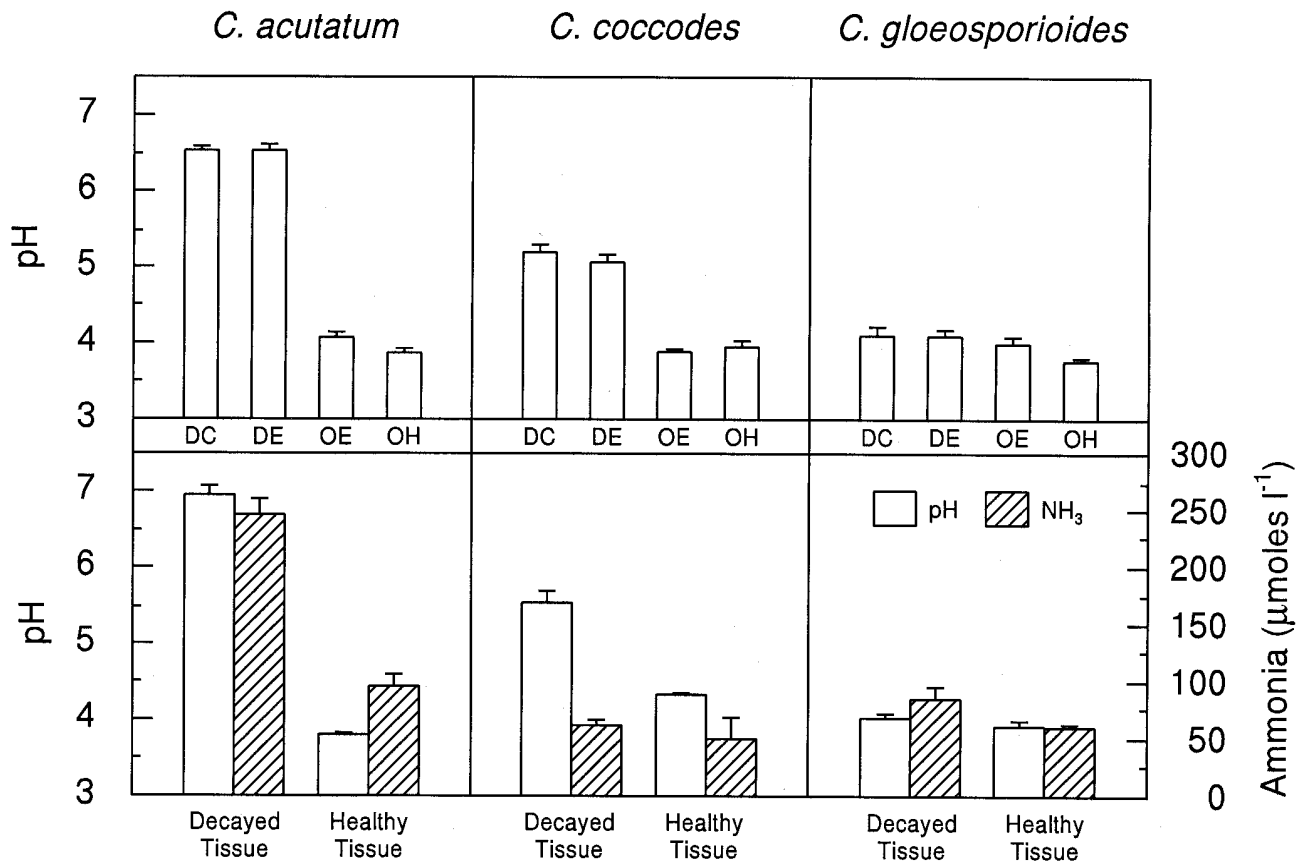

Fig. 7. pH changes induced by *Colletotrichum acutatum* on Golden Delicious apples. Direct pH measurements on decayed and healthy tissues were taken 10 days postinoculation when the decay diameter was 20 mm. Results are from one of five experiments conducted.

lated by the acidification of the environment as a result of oxalic acid secretion, which is necessary for the activity of polygalacturonases. These enzymes have been implicated as colonization and virulence factors in other plant-infecting fungi (Shieh et al. 1997; Ten Have et al. 1998). The present results, however, are what we believe to be the first report where local alkalinization, as a result of ammonia increase, is suggested to be a virulence factor. The ability of *Colletotrichum* spp. to modify the pH at the infection site results in enhanced virulence because ammonia secretion and the resulting pH increase leads to higher *pel*B expression and PL secretion (Yakoby et al. 2000). The finding that the *C. gloeosporioides pel*B gene-disrupted mutant produces amounts of ammonia similar to the wild-type *C. gloeosporioides* suggests that ammonia accumulation is independent of *pel*B expression. By secreting ammonia, the fungus ensures that PL is produced under optimal pH conditions because the protein has an apparent pH optimum of 8.9 (Wattad et al. 1994). The present results further emphasize the importance of PL secretion for pathogenicity because *pg* transcripts and PG protein secretion by *C. gloeosporioides* occurs at a lower pH (5.0). Activity of the PG protein has an optimum pH of 5.1, a condition under which avocado fruits are not susceptible (Prusky et al. 1989; Yakoby et al. 2000; Yakoby et al. 2001).

The pH effect on *pel*B expression suggests that an ambient pH signal transduction pathway exists in *C. gloeosporioides*. Such a pathway has been characterized in *Aspergillus nidulans* and *S. sclerotiorum*, and several components of this pathway, including the pH-dependent transcription regulator *pac*C, have been cloned and characterized (Rollins and Dickman 2001; Tilburn et al. 1995). The conservation of the zinc finger region among the various fungal homologs (Espeso et al. 1997; MacCabe et al. 1996; Suarez and Penalva, 1996) and the central role, which PacC plays in mediating the pH-dependent signaling, make the *pac*C gene an important gene for future testing during ambient pH induction of *pel*B expression and PL secretion of *C. gloeosporioides*.

Fungi can use a surprisingly diverse array of compounds as nitrogen sources and are capable of expressing, upon demand, the catabolic enzymes of many different pathways (Marzluf 1997). Few reports have suggested a relationship between nitrogen metabolic regulation and fungal pathogenesis. Nutritional limitations of various types, particularly nitrogen deprivation, appears to have a link to pathogenesis and other fungal morphogenetic switches (Lau and Hamer 1996; Talbot et al. 1993). Our results indicate that the accumulation of ammonia affects fungal attack. These findings, however, do not exclude that other factors such as nitrogen metabolism or the utiliza-

Fig. 8. pH changes and ammonia accumulation induced by *Colletotrichum acutatum, Colletotrichum gloeosporioides*, and *Colletotrichum coccodes* on Golden Delicious apples. **A,** Direct pH measurements. **B,** pH and ammonia concentration of the juice of the fruit. Measurements were performed 6–7 days postinoculation. Results are from one of five experiments conducted. DC, decay center; DE, decay edge; OE, outside healthy edge; OH, outside healthy tissue.

tion of host organic acid by the fungus may play a role in the increase of host pH. The activation of PL secretion, as a result of ammonia accumulation and elevated pH, seems to be a newly described mechanism through which nitrogen metabolism can affect pathogenicity. Because this mechanism was in three different hosts and occurred during the decay by three different *Colletotrichum* species, the effect of environmental pH modulation may have broad significance and could be used in plant-breeding programs to control decay occurring in other postharvest pathosystems.

MATERIALS AND METHODS

Strains, media, and growth conditions.

C. acutatum was isolated from decayed apples, *C. coccodes* from isolated decayed tomato, and *C. gloeosporioides* isolate Cg-14 was obtained from decayed avocado fruit. The *C. gloeosporioides pel*B gene-disrupted mutant was developed by Yakoby et al. (2001). Single-spore cultures were prepared from each isolate, and spores were stored in 10 mM Na phosphate buffer (pH 7.2) and 40% glycerol, at −80°C.

Spores taken from 10- to 20-day-old cultures were harvested from M_3S medium (Tu 1985). Erlenmeyer flasks (150 ml) containing 50 ml of medium, with 1% yeast extract (Sigma, St. Louis, MO, U.S.A.), at pH 4.0, were inoculated with the *C. acutatum* and Cg-14 isolates (1.0×10^6 spores per flask) and grown at 25°C on an orbital shaker (150 rpm). The resulting mycelium subsequently was filtered through Whatman No. 1 filter paper (Clifton, NJ, U.S.A.) and dried for 2 days at 40°C, prior to weighing. In vitro experiments were repeated four times. The results of one representative experiment are presented.

Fruits, inoculation conditions, and statistical analysis.

Golden Delicious apples were harvested in the preclimacteric stage and stored at 0°C for 2 to 3 months until used for experiments. Tomato fruit *Lycopersicum esculentum* cv. Roma and avocado fruits *Persea americana* cv. *drymifolia* cv. Hass were bought in a local supermarket.

Inoculation was carried out by wounding the fruits to a depth of 2 to 3 mm and placing 30 μl of spore suspension (10^6 spores per ml) at four points, two on each side of the longitudinal axis, of 15 fruits. The fruits were then incubated at 20°C, in 90% humidity, for 5 to 10 days. In some experiments, the inoculation medium was adjusted, to pH 6.2 and 4.2, with phthalate buffer of different molar concentrations.

In vivo experiments were repeated at least three times. The results of one representative experiment are presented. Standard errors of the means were calculated.

pH measurements.

pH was measured with a micro-combination pH electrode, Model 9810BN (Orion, Beverly, MA, U.S.A.), in 1- to 3-ml aliquots, sampled at different times after fungal inoculation. Pericarp pH was determined following a transverse cut through the infection site with a scalpel blade. pH measurements were taken by placing the micro pH electrode directly against the exposed tissue. The electrode can measure pH in a gel thickness of less than 1.5 mm. All measurements were repeated on 10 to 12 fruits at three different points (at least 30 measurements) on the transverse axis of the infection on each

fruit. The standard errors of the means of pH measurements were never higher than 2.5%. To test the hypothesis that direct pH measurement is a reliable indicator of the environment within the fruit, the direct measurement was compared with the pH determined by the common homogenization method (Lurie and Pesis 1992) in which 2 to 4 g of pericarp tissue was crushed with a plunger and filtered through a 4-ml, 0.45-μm centrifuge filter (Eppendorf Scientific, Westbury, NY, U.S.A.) at $4,000 \times g$. Direct and homogenate pH measurements were compared in all fruits used in the present work (30 measurements). The regression coefficient between the measurements was $r = 0.999$. pH also was evaluated with a pH indicator, Alizarin red S (Sigma), which shows red at a pH higher than 6.0 and yellow at pH values of 4.0 and less.

Detection of ammonia in the liquid media and in the tissue.

Ammonia concentration was determined in filtered culture medium or tissue homogenate. Ammonia was measured with an ammonia electrode, Model 95-12 (Orion), in 0.1- to 1-ml aliquots (depending upon the concentration), at pH 10.0, sampled at different times after inoculation. Different concentrations (7 to 700 μM) of NH_4Cl were used as standards. Experiments with three replications were repeated at least three times. The results of one representative experiment are presented. Standard errors of the means were calculated.

PL in liquid medium.

C. gloeosporioides hyphae was separated from the culture medium by centrifugation ($12,000 \times g$ for 10 min), at 4°C.
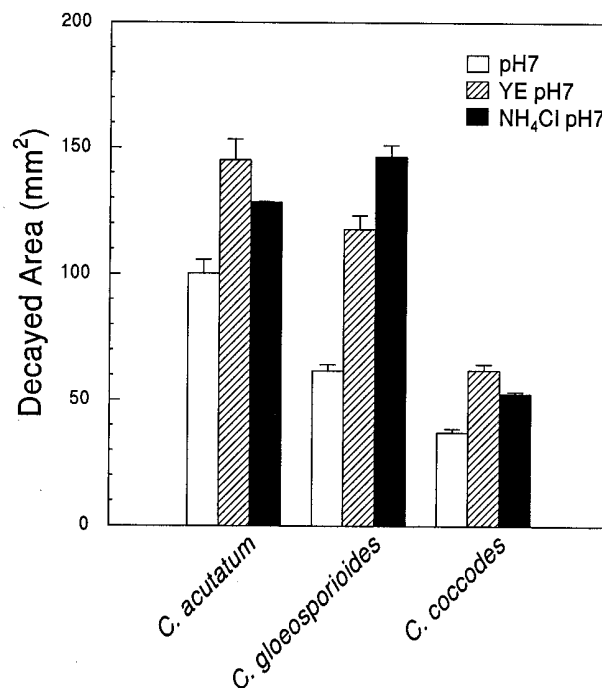
Fig 9. Enhancement of virulence of *Colletotrichum* species by ammonia releasing compounds in apple fruit cultivars Golden Delicious. Spores of *Colletotrichum gloeosporioides*, *Colletotrichum acutatum*, and *Colletotrichum coccodes* suspended in 0.05 M phthalate pH 7 or in the same buffer containing 1% yeast extract or 15 mM ammonium chloride. Spore suspension (50 μl; 10^6 spores per ml) in the different solutions was applied to the wounded apples.

The culture medium supernatant (50 ml) was freeze-dried and diluted in 5 ml of distilled water. The concentrated culture filtrate (Sigma) was dialyzed overnight (cut off at molecular weight of 6,000) against 2 liters of 50 mM Tris-HCl, pH 8.5, concentrated to 5 ml, as above; 5 μg per lane were used for Western blot analysis.

Each protein sample was quantified with Protein Assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.), with bovine serum albumin as the standard. Samples were boiled for 4 min in loading buffer, according to Sambrook et al. (1989), with 10% β-mercaptoethanol as a reducing agent. Samples were then loaded onto a 12.5% sodium dodecyl sulfate-polyacrylamide gel (Mini-Protean II; Bio-Rad Laboratories) and run for 1 h at a constant 150 V. Western blot analysis was performed with PL antibodies diluted to 1:500 (Wattad et al. 1997). Preimmune ascetic fluid was used as a control, and anti-mouse immunoglobulin G (IgG) alkaline phosphatase (AP) conjugate (Promega, Madison, WI, U.S.A.) was used as a secondary antibody. Nonimmune serum was used as a control. Anti-rabbit IgG AP conjugate was used as the secondary antibody. Both secondary antibodies were used at a dilution of 1:6,000.

ACKNOWLEDGMENTS

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