Visualization of Localized Pathogen-Induced pH Modulation in Almond Tissues Infected by *Colletotrichum acutatum* Using Confocal Scanning Laser Microscopy

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ABSTRACT

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Modulation of pH within the host during infection of almond by the anthracnose pathogen *Colletotrichum acutatum* was studied using confocal scanning laser microscopy and the dual emission fluorescence indicator SNARF-1. This highly sensitive method allowed visualization of the spatial distribution of localized pathogen-induced pH modulation within and in proximity to fungal infection structures in host tissue at the cellular level. Ratiometric measurement of fluorescence at two emission wavelengths and in situ calibration allowed the quantification of pH ranges. After incubation of leaf epidermal tissue with SNARF-1, distinct alkaline (pH 8 to ≥ 9), red-spectrum (650 nm wave length) fluorescent zones developed as partial or complete halos around many fungal appressoria and in infection vesicles at 24 to 36 h after inoculation. In samples taken after 48 to 72 h, colonizing hyphae in the biotrophic phase and subse-

quently in the necrotrophic phase were also emitting the red fluorescence that extended into the surrounding host tissue, as also verified by depth analyses. Host epidermal cells were intact and apparently alive during the fungal alkalization process, with no visible disruption of cell structure. Generally, the pH of epidermal cells in noninoculated samples or in areas away from the infection in inoculated samples was lower than pH 7 with green (i.e., 500 to 550 nm wave length) fluorescence detected. Using standard electrodes, a significant increase in pH and ammonia concentration in leaf and fruit tissue was also measured but only at advanced stages of disease. In contrast, hyphae of the pathogen *Alternaria alternata* were mostly acidic and no change in fluorescence was found inside invaded host cells. The sequence of events in the *C. acutatum*-almond interaction includes penetration, production of ammonia by *C. acutatum*, and subsequent pH modulation within almond epidermal tissue to an alkaline environment that leads to further colonization of the host.

Additional keywords: host-pathogen interactions, histology, cellular pH shifts.

Species of the genus *Colletotrichum* cause economically important diseases on a wide range of crops worldwide (17). These fungi successfully colonize their hosts due to their ability to secrete pathogenicity factors such as host cell wall-degrading enzymes (14,19,21). Ambient pH can affect the expression and activity of these enzymes (2,10,13,21) and, based on acidification or alkalization of the host tissue during infection, plant pathogenic fungi of several genera have been categorized (21). A number of biochemical, physiological, and molecular studies on the influence of pH on fungal infection have also been done with *Colletotrichum* species such as *C. acutatum* on apple, *C. coccodes* on tomato, and *C. gloeosporioides* on avocado (20,21). Thus, the expression of the pectin degrading enzyme pectate lyase was shown to increase as the ambient pH increased from pH 4.2 to 6.3 (8,20,27). The local increase in pH was found to be correlated with secretion of ammonia by the pathogen (20). Thus, several species of *Colletotrichum* are able to locally modify or modulate their infection sites by secreting ammonia and increasing the pH of the host tissues that subsequently results in increased pectolytic enzyme activity and increased virulence.

Based on these studies, considerable progress has been made in understanding the impact of pH shifts in the infection processes

of *Colletotrichum* species. Still, changes in the microenvironment were only detected on the tissue level by microelectrode pH measurements and by determining ammonia concentrations in tissue homogenates. Information on changes at the cellular level and their relationship to the formation of specific infection structures by the fungus is lacking. A sequence of developmental structures has been described during infection processes of *Colletotrichum* spp. (18). Conidia adhere to the host surface, germinate, and germ tubes either grow to develop a mycelium that produces secondary conidia or appressoria that form penetration pegs that directly penetrate the cuticle and host cell wall leading to colonization of the host (4). Two basic colonization strategies have been described in *Colletotrichum* spp. based on colonization and host interaction: intracellular hemibiotrophy and subcuticular-intramural necrotrophy (4,16,18). For *C. acutatum* J. H. Simmonds infecting almond (*Prunus dulcis* [Mill.] D. A. Webb) tissues, early infection structures have been characterized and a combination of the two infection strategies has been described (6,7). In this latter pathosystem, under favorable environments, the pathogen is known to infect blossoms, leaves, and fruit at all stages of development and subsequently colonizes woody tissues causing dieback of the host (1,9). The fungus initially invades host tissues as a biotroph and then transforms into a necrotroph after some time interval that depends on the tissue being colonized. For blossom petals and immature leaves, the transition from the biotrophic to the necrotrophic host interaction is short, whereas for developing fruit and mature leaves the duration may be more extended. *C. acutatum* produces subcuticular infection vesicles, as

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well as subcuticular and broad, intracellular primary hyphae during the biotrophic colonization. Later, narrow secondary hyphae develop intercellularly during the necrotrophic phase (6,7).

The goal of this study was to localize microenvironmental pH conditions during the infection of almond leaves by *C. acutatum* at the cellular level using ratiometric imaging in confocal microscopy. In ratiometric imaging, protonation shifts the dual-emission spectra of pH-sensitive fluorophores, and the ratio of intensities obtained at two wavelengths can be used to quantify pH changes (26). The pH-sensitive fluorophore probe 5(6)-carboxy-seminaphthorhodafluor-1 (SNARF-1) has been utilized in a number of confocal imaging studies to measure intracellular pH levels in plant (3) or animal (15,23) cells, as well as in bacterial biofilms (11). Studies with fungi are limited to in vitro germ tube development of *Magnaporthe grisea* (12) and intracellular alkalinization in *Saccharomyces cerevisiae* after exposure to acid (28).

Thus, we explored the use of this technology to obtain new insights in pH modulation during plant-pathogen interactions. Specific objectives included the (i) confirmation of an increase in pH and the production of ammonia in almond leaves infected by *C. acutatum*, a host-pathogen system that has not been evaluated previously; (ii) microscopic visualization and quantification of pH changes in infected leaf tissues, as well as their spatial and temporal relation to pathogen infection structures; (iii) quantification of pH changes in relation to tissue depth; and (iv) comparison of these microscopic observations using almond leaves infected by *Alternaria alternata* (Fr.:Fr.) Keissler, another important pathogen of this crop.

MATERIALS AND METHODS

Preparation of inoculum and almond tissue inoculation. An isolate of *C. acutatum* from almond of the predominant 'pink' phenotype in California (9) was grown on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) for 10 to 14 days at 22 to 25°C. An isolate of *A. alternata* from almond was cultured on 5% PDA (22,25). Conidial suspensions (10^6 conidia/ml) were prepared in sterile distilled water. Leaves of potted cv. NePlus Ultra almond plants grown in a greenhouse at 22 to 27°C and immature fruit (approximately 3 months after bloom) of cv. Carmel obtained from an orchard were used in the experiments. Detached mature leaves (leaves 5 to 15 from the apex of each shoot) were inoculated on the upper surface with 12 to 15 10- μ l droplets of the conidial suspensions and droplets were spaced 5 mm apart. Inoculated leaves were incubated on moist filter paper in petri dishes at 20°C, and the ends of the petioles were sealed with Parafilm to minimize water loss. Almond fruit were surface sterilized for 1 min in sodium hypochlorite (400 mg of HOCl/liter), rinsed with water, and air dried. Fruit were then spray-inoculated with the conidial suspension of *C. acutatum* using an air-nozzle sprayer (DeVilbiss 15-RD, Sunrise Medical, Inc., Somerset, PA) and incubated in sealed plastic boxes at 20°C. Leaves inoculated with *C. acutatum* were used in all studies listed below, leaves inoculated with *A. alternata* were only used in confocal microscopy, and fruit inoculated with *C. acutatum* were used for determining the pH of infected tissue surfaces and for measurement of ammonia concentrations.

Measurement of pH and of ammonia concentrations in almond tissues. Measurements were done using leaves 4, 7, and 14 days after inoculation and fruit 21 days after inoculation. Healthy, noninoculated leaves and fruit were used as controls. For leaves, the pH was measured directly on the surface, whereas for fruit, a thin layer of the epicarp tissue was removed using a razor blade before measurement. For both types of tissues, 20 μ l of distilled water was pipetted onto the area of interest, a flat surface combination electrode (Corning, Inc., New York, NY) attached to a pH meter (Orion 5 Star; Thermo Fisher Scientific, Inc.,

Waltham, MA) was gently pressed against the tissue, and readings were recorded.

For determining the ammonia concentration in plant tissues, 1.2 g fresh weight of tissue (i.e., whole leaf or half fruit samples) was homogenized in 8 ml of distilled water for 30 s using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA). The suspension was filtered through two layers of cheesecloth and the volume was adjusted to 100 ml with distilled water. Measurements of ammonia concentration were conducted using an ammonia combination electrode (Corning, Inc) that is designed for the accurate determination of ammonia in aqueous solutions. Following the manufacturer's instructions, the electrode was calibrated with 1, 10, and 100 mg/liter ammonia (prepared from NH_4Cl) before measurement of the samples. Results were expressed in micromole NH_4 per gram fresh weight of sample.

For both pH and ammonia measurements, experiments were done three times using three samples for each of the tissue types and sampling times. Bartlett's test for homogeneity of variances was performed for repeated experiments and data sets with homogeneous variances ($P < 0.05$) were combined. Data were then analyzed using general linear model and least significant difference mean separation procedures of SAS version 9.1 (SAS Institute, Cary, NC).

Sample preparation, dye loading, and confocal scanning laser microscopy (CSLM). Due to the difficulty to observe structures on the trichome-covered almond fruit surface, only leaf tissues were used in microscopic observations. Epidermal peels (approximately 3 mm $\times$ 3 mm) were prepared using a fine forceps from noninoculated leaves and from leaves 24, 36, 48, or 72 h after inoculation with conidia of *C. acutatum*. For observation of *A. alternata* infections, leaf pieces (3 mm $\times$ 3 mm) were sampled 96 h after inoculation. Epidermal peels were floated with the cuticle side up on a 50- μ l drop of a solution of 8 μM SNARF-1 (5-[and-6]-chloromethyl SNARF-1 acetate; Molecular Probes, Inc., Eugene, OR), whereas leaf pieces were submerged in the solution. This SNARF-1 solution was prepared by adding 24 μ l of 25 mM MES buffer (pH 6) and 1 μ l of the dispersion agent Pluronic F-127 (20%, wt/vol, in DMSO; Molecular Probes, Inc.) to 50 μ g of the probe. Of this solution, 12.5 μ l was then added to 12.5 ml of distilled water, resulting in the 8- μM working solution. Samples were incubated for 15 to 30 min at 25°C, washed with distilled water, mounted in distilled water, and observed by CSLM. Data were obtained from three independent experiments.

Samples were observed under a Zeiss LSM510 confocal microscope (Carl Zeiss, Germany) using bright field microscopy (incandescent light source) and CSLM with an argon laser at 488 nm for excitation and dual emission channel sensors at 500 to 550 nm for the acidic form of the probe and at 650 nm for the alkaline form of the probe. Water immersion lenses (25 $\times$ /0.8 Plan-Neofluar and 63 $\times$ /1.2 C-Apochromat) and an oil immersion lens (100 $\times$ /1.3 Plan-Neofluar) were used to collect images at 1,024 by 1,024 frame scans in the x,y and x,z planes. Z-step scanning was done at 1- μ m intervals.

In situ calibration. Almond leaf peels were incubated in 50 mM potassium phosphate buffer at pH 6, 7, 8, or 9 with the addition of 25 μM nigericin (Molecular Probes, Inc.) to facilitate equilibration of the intracellular pH with the extracellular medium. After 1 to 2 h, samples were transferred to the 8- μM SNARF-1 solution, incubated for 30 min, and mounted in distilled water for microscopic observation. Fluorescence intensities at the two emission channels were measured over a distance equivalent to 1,024 data points. For each pH, five locations were selected within the cell lumen and five within the cell wall. Data obtained in the Zeiss LSM 510 Image Browser software were averaged for each pH value. The ratios of fluorescence intensities in the alkaline and acidic channels were calculated. Subsequently, pH values were determined using the following equation (Technical sheet MP 01270, Molecular Probes, Inc.):

$\text{pH} = \text{pK}_a - \log[(R - R_B/R_A - R) \times (F_{B(\lambda_2)}/F_{A(\lambda_2)})]$, where R is the ratio of $F_{\lambda_1}/F_{\lambda_2}$ of fluorescence intensities (F) measured at two wavelengths λ_1 (i.e., 500 to 550 nm) and λ_2 (i.e., 650 nm). The subscripts A and B represent the limiting values at the acidic and alkaline endpoints of the calibration range, respectively. The factor $F_{B(\lambda_2)}/F_{A(\lambda_2)}$ is for normalization of pH values for pH-dependent wavelengths of SNARF-1 (pK_a value = 7.5).

Image analysis of localized pH modulation in leaves infected by *C. acutatum* or *A. alternata*. SNARF-1 dual emissions were analyzed with the Zeiss LSM 510 Image Browser by selecting regions of interest in the micrographs including infection vesicles, intercellular and intracellular hyphae, and adjacent host tissues (e.g., under appressoria), as well as cell lumen and cell walls of host tissues not affected by the pathogen. A total of 1,024 measurement points were taken for each line profile that was approximately 5 μm in length when using the 100 $\times$ objective or 12 μm in length when using the 63 $\times$ and 25 $\times$ objectives. Average emission ratios were calculated for each line profile and corresponding pH values were determined based on the calibration range used. Each image in the results section is displayed in five modes: bright field view, SNARF-1 emission in the alkaline (red) channel at 650 nm, SNARF-1 emission in the acidic (green) channel at 500 to 550 nm, an overlay or composite of these three images, and a pseudo-colored ratiometric image indicating pH distributions.

Depth analysis of localized pH modulation at penetration sites of *C. acutatum*. Depth analyses were done on localized pH modulations under appressoria. For this, a series of 10 to 15 sequential images in the z plane in 1- μm intervals were analyzed for fluorescence emissions to localize the source of the emissions within the penetration site. The Zeiss LSM 510 Image Browser was used to generate depth information and to visualize z -axis data in relation to fungal structures and pH modulation within host tissue.

RESULTS

pH and ammonia concentrations in almond tissues after inoculation with *C. acutatum*. Tissues used in these studies included the noninoculated controls, leaves sampled 4, 7, and 14 days after inoculation, and fruit sampled 21 days after inoculation. At these timings, leaves had no symptoms, early symptoms (small water-soaked lesions), and advanced symptoms (large water-soaked lesions and fungal sporulation), respectively. Fruit sampled 21 days after inoculation showed advanced symptoms with sunken lesions that were dark brown under the epicarp and extended into the mesocarp. Significant increases in pH values as compared to the noninoculated controls were observed with both leaves and fruit when tissues with advanced disease symptoms were evaluated, but not when nonsymptomatic leaves or leaves with early symptoms were analyzed (Fig. 1A and B). Values for healthy leaves and leaves with advanced symptoms were 5.78 and 7.60, respectively, whereas for healthy and diseased fruit they were 5.12 and 6.48, respectively. These increases in pH were correlated with increases in ammonia concentrations in tissue homogenates. Healthy, noninoculated leaves and leaves with advanced symptoms contained 3.2 and 22.7 $\mu\text{mol NH}_4/\text{g}$ fresh weight, respectively (Fig. 1B). Concentrations in nonsymptomatic, inoculated leaves were not different from those of the control, but a significant increase to 7.3 $\mu\text{mol NH}_4/\text{g}$ fresh weight was detected in leaves with early symptoms. For almond fruit, an approximately 10-fold increase was observed between noninoculated fruit (5.2 $\mu\text{mol NH}_4/\text{g}$ fresh weight) and fruit with advanced symptoms (54.4 $\mu\text{mol NH}_4/\text{g}$ fresh weight) (Fig. 1B).

Visualization and microscopic measurement of localized pH modulation in leaves infected by *C. acutatum*. The localized pH modulation was initially studied and quantified during fungal penetration and early colonization of *C. acutatum* in epidermal

tissue of almond leaves. At 24 h after inoculation, conidia of the fungus germinated and formed appressoria on the leaf surface (Fig. 2A). Approximately 20% of the appressoria were surrounded by an intense concentric ring of alkaline (red) fluorescence emission (Fig. 2B). No localized signal was observed in the acidic (green) emission channel (Fig. 2C). The two emission signal images in relation to fungal and plant structures are displayed in the composite ratiometric image of Figure 2D.

The red fluorescence emission around the appressoria was translated into an actual pH range based on an in situ calibration. In this calibration, as the pH of the buffer used to equilibrate the internal epidermal tissue increased from 6 to 9, emission intensities of SNARF-1 at 500 to 550 nm decreased whereas intensities at 650 nm increased. Using the pH calibration formula that was based on the ratios of fluorescence emissions in the acidic and alkaline channels for both the sample and standardized calibration endpoints, the line profile analysis of the appressorial ring within the epidermal tissue (see depth analysis) indicated that the pH was highly alkaline (i.e., pH 8.8). Line profile analyses for the epidermal cell lumen and the leaf surface showed that the pH was neutral (i.e., pH 7 to 7.2) and acidic (i.e., pH ≤ 6), respectively. A computerized pseudo-colored image of the composite ratiometric image was generated to map the pH distribution for the entire epidermal image (Fig. 2E). Appressoria showed low or no fluorescence emission signals from SNARF-1 due to their opacity and are shown as black structures.

Infection vesicles from appressoria were present in samples obtained 36 h after inoculation (Fig. 2F). An alkaline (Fig. 2G) but no acidic (Fig. 2H) fluorescence emission occurred within the

Fig. 1. pH and ammonia concentration in almond **A**, leaves and **B**, fruit non-inoculated or inoculated with conidia of *Colletotrichum acutatum*. Leaves were incubated for 4 (no symptoms), 7 (early disease), or 14 days (advanced disease), whereas fruit were incubated for 21 days (advanced disease) after inoculation and incubation at 20°C. Analyses of variances and least significant mean separation were done separately for pH and ammonia concentration. Bars followed by the same letter are not significantly different ($P > 0.05$) from each other.

vesicle. The two emission images were superimposed with the bright field image in the composite image of Figure 2I. The ratios of fluorescence emissions in the acidic and alkaline channels along a line profile across the infection vesicle indicated an alkaline pH of 7.6 to 9. The pseudo-colored image again shows the general acidic nature of the leaf epidermis and the alkaline pH

inside the vesicle adjacent to the no-emission appressorium of the fungus (Fig. 2J). The bright red to pink spherical structures within the epidermal cells are host cell organelles (perhaps mitochondria) or vesicles. Chloroplasts were absent from the epidermal cells.

Alkalization by intracellular and intercellular hyphae was visualized 48 and 72 h after inoculation of epidermal leaf tissue

Fig. 2. Bright-field (**A and F**) and confocal light microscopy fluorescence emission in the alkaline (red) channel at 650 nm (**B and G**) and in the acidic (green) channel at 500 to 550 nm (**C and H**) after excitation at 488 nm, ratiometric composite (**D and I**), and pseudo-colored ratiometric images (**E and J**) of SNARF-1-treated epidermal peels of almond leaves 24 h (**A to E**) and 36 h (**F to J**) after inoculation with conidia of *Colletotrichum acutatum* and incubation at 20°C. **A to E**, Appressoria (a) form from germinating conidia (c) 24 h after inoculation. Intense alkaline emission observed as a pink to red, alkaline ring (ar) is located around an appressorium. **F to J**, Infection vesicle (iv) developing from an appressorium (a) 36 h after inoculation with conidia (c). ec = epidermal cell. A calibration scale between pH ≤ 6 and ≥ 9 is shown in **E** and **J**. NS = no signal. Bars = 10 μ m.

(Fig. 3A, B, D, and E). The ratios of fluorescence emissions of SNARF-1 for the acidic channel and alkaline channel (Fig. 3B and C) for line profiles inside hyphae and cell lumens or cell walls next to the hyphae corresponded to pH values of 8.7 and 9, respectively. The cell lumen of some noncolonized epidermal cells remained at pH 7 (right side of Fig. 3E). The lumen of other cells where intra- and intercellular hyphae were observed showed an alkaline pH of 8 to ≥ 9 (left side of Fig. 3E). Alkalization of host cells was less evident at the tips of the leading intercellular hyphae although internally, these hyphae were alkaline (Fig. 3B, D, and E).

To contrast pH modulation data obtained from infections caused by *C. acutatum*, we examined almond leaf tissue samples infected by *A. alternata*. The bright field image was dense due to the thickness of the leaf (Fig. 4A). Still, confocal microscope images of the alkaline (Fig. 4B) and acidic (Fig. 4C) channels and line profile analyses indicated that *A. alternata* hyphae at a depth of approximately 6 to 10 μm within the epidermis had a strong fluorescence emission in the green (acidic) channel (Fig. 4C, D, and E) with a pH of ca. 6, whereas the adjacent epidermal regions had a pH of 6 to 7. Still, some fluorescence emission in the red

to pink (alkaline) channel was detected in some regions (upper left Fig. 4E) and was prominent in one hyphal tip (Fig. 4B arrow). Because of the thickness of the leaf sample and its opacity, extended areas of the epidermal tissue provided no signal (Fig. 4E).

Depth analysis of localized pH modulation at penetration sites of *C. acutatum*. In order to determine the three-dimensional distribution of fluorescence emissions of SNARF-1 at fungal penetration sites, image depth analyses were done. The alkaline (red to pink) fluorescence emissions from infection structures of *C. acutatum* were found to be always localized below the leaf surface (Fig. 5). Thus, images of the penetration site studied at the cross lines of the x and y axes were taken at different focal planes (Fig. 5A, B, and C). Analysis of fluorescence localization in a z axis indicated that the alkaline (red to pink) fluorescence emissions were located 3 (Fig. 5D) to 7 μm (Fig. 5F) below the base of the appressorium and the cuticle of the leaf (indicated by horizontal blue lines in Fig. 5D, E, and F). The absence of emissions from the appressorium resulted in a dark or opaque region that extended through the center of the z-axis images (Fig. 5D, E, and F).

Fig. 3. Bright-field (A) and confocal light microscopy fluorescence emission in the alkaline (red) channel at 650 nm (B) and in the acidic (green) channel at 500 to 550 nm (C) after excitation at 488 nm, ratiometric composite (D), and pseudo-colored ratiometric image (E) of SNARF-1-treated epidermal peels of almond leaves 72 h after inoculation with conidia of *Colletotrichum acutatum* and incubation at 20°C. An intense alkaline emission is located in intra- (ih) and intercellular hyphae (eh) and inside some host epidermal cells (ec) next to the hyphae. Alkalization of host cells is less evident at the tips of the leading intercellular hyphae (arrowheads). A calibration scale between pH ≤ 6 and ≥ 9 is shown in E. NS = no signal. Bars = 10 μm .

DISCUSSION

Micro-environmental conditions within the plant host tissue including temperature, moisture, light, pH, and nutrition can directly affect the development of a plant pathogenic fungus, the host, and the subsequent host–pathogen interaction. The pH of the host environment is known to affect numerous regulatory processes in fungi, such as the activity of pathogenesis-related enzymes of the pathogen including those that degrade the host cell wall (5,10). Changes in pH can occur during normal aging,

ripening, or senescing processes of the host (21). Alternatively, in modulation of the host pH environment by plant pathogenic fungi, pathogens release either acidic or alkaline substances and thus, may create their own conducive micro-environment that allows infections to become established. These processes have been studied in several host–pathogen systems where changes in pH were detected in selected host tissues using pH electrodes of different types. Still, changes in pH were never directly associated with fungal structures during host penetration and colonization.

Fig. 4. Bright-field (A) and confocal light microscopy fluorescence emission in the alkaline (red) channel at 650 nm (B) and in the acidic (green) channel at 500 to 550 nm (C) after excitation at 488 nm, ratiometric composite (D), and pseudo-colored ratiometric image (E) of SNARF-1-treated almond leaf samples 96 h after inoculation with conidia of *Alternaria alternata* and incubation at 20°C. Emissions are mainly in the neutral to acidic range for colonizing hyphae (A, D, and E, arrowheads), however, some hyphal tips also show an alkaline emission (B, arrow). A calibration scale between pH ≤ 6 and ≥ 9 is shown in E. NS = no signal. Bars = 10 μ m.

Fig. 5. Depth analysis of localization of SNARF-1 fluorescence emissions adjacent to an appressorium of *Colletotrichum acutatum* inoculated on almond leaves. A to C, Red (x) and green (y) lines correspond to the x-y axes chosen for the analysis of a z series of images. D and E, The blue (z) lines indicate the focal plane depth in the z axis chosen for analysis. Images are ratiometric composites of bright field, alkaline, and acidic emission channels of an appressorium (a) with an intense alkaline (red) fluorescence emission. A and D, Images at the focal plane of the leaf surface with two conidia (c) and an appressorium. B and E, Images corresponding to a focal plane 3 μ m below the leaf surface. C and F, Images corresponding to a focal plane 7 μ m below the leaf surface. Note that the alkaline emission increases from image A to C and that this emission is most intense below the focal plane of the leaf surface (D to F). Bar = 10 μ m.

In the current study, we confirmed that an alkaline modulation of host tissues due to production of ammonia also occurs in a *Colletotrichum* species–host system not previously studied and, more importantly, using a pH sensitive fluorophore in CSLM we visualized pH shifts on the cellular level for the first time in any plant–pathogen system. Additionally, these pH shifts were detected from very early to later stages of pathogen colonization indicating that ammonia production and micro-localized pH shifts are nearly concurrent. The single excitation, dual emission fluorescence indicator SNARF-1 allowed us to probe pH environments within and in proximity to fungal infection structures. Due to the ratiometric measurement of fluorescence emissions with this indicator, pH determination is independent of fluorescence intensity, and thus, independent of variations in uptake of the probe. In situ calibration made it possible to quantify pH ranges in fungal structures and plant tissues. These ranges may not be absolutely accurate due to the possible insufficient equilibration of internal tissues from the innate buffering capacity of the plant and pathogen or due to non-pH related interactions of the SNARF-1 probe with intracellular components (11,24). Still, the general alkalization of host tissue and some fungal structures was clearly visualized in this host–pathogen interaction.

Studies were conducted with *C. acutatum* on almond (1), a host-pathogen system that has been studied in detail previously at the microscopic level (6,7). During epidemics, this pathogen is known to infect blossom, leaf, fruit, and woody tissues of the host. Although infections of fruit are generally of the highest economic importance, our microscopic studies were only conducted with leaves due to the difficulty of using fruits that have a dense layer of trichomes. A similar temporal sequence in formation of infection structures was observed as previously described (7). Conidia germinated on leaf surfaces and germ tubes produced appressoria. Mature appressoria, as characterized by the presence of an internal light spot (6,29), were generally observed 24 h after inoculation and incubation at 20°C, and infection vesicles were visible after 36 h. In subsequent stages of the hemibiotrophic colonization of the host, intracellular and intercellular hyphae were present. After incubation of leaf epidermal tissue with SNARF-1, distinct regions in and around these infection structures emitted fluorescence in the alkaline channel at 650 nm wave length and were visible as pink to red coloration zones. First, these zones appeared as partial or complete halos around appressoria (top views) and were associated with infection vesicles. Not all appressoria were surrounded by rings of alkaline environments. Thus, possibly host alkalization only occurs at specific stages during infection. More likely, however, the host sometimes may be successful to buffer the ammonia influx from the pathogen and this likely would prevent the establishment of an infection. In samples taken after 48 to 72 h, colonizing hyphae in the biotrophic phase and subsequently in the necrotrophic phase were also emitting the red spectrum and the emission extended beyond these colonizing hyphae into the surrounding host tissue. Alkalization of host cells, however, was less evident at the tips of the leading intercellular hyphae although internally, these hyphae were alkaline. Host epidermal cells were intact with no visible disruption of cell structure when viewed using bright-field microscopy and they were apparently alive during the fungal alkalization process. Generally, the pH of epidermal cells in non-inoculated samples was lower than pH 7 with green-spectrum fluorescence emissions detected by the acidic channel (i.e., 500 to 550 nm wave length). Depth analyses using confocal microscopy confirmed that the vesicles were subcuticular and colonizing hyphae were within the epidermal tissue, similar to previous studies that used image analysis of bright field micrographs (6,7), and that the fluorescence was located within the epidermis below the host cuticle and fungal appressoria.

The alkaline pH modulation from increased ammonia concentration within almond tissue infected by *C. acutatum* was con-

firmed by pH and ammonia measurements of leaves and fruit using pH and ammonia electrodes. Slight changes in ammonia concentration were observed during early stages of the disease in almond leaves. A significant increase in pH and ammonia of leaves and fruit, however, was only observed at advanced stages of the disease when extensive dark, watery lesions were present. Thus, as compared to pH measurements of tissue, SNARF-1 staining and CSLM was more sensitive in detecting early events of pH modulation. In comparison to our study where pH increased by 1.5 to 2 units in almond leaves and fruit, previous reports on avocado, tomato, and apple fruit infected by different species of *Colletotrichum* showed increases of 2 to 4 pH units (20).

Our findings in the current study on host pH modulation in the interaction of *C. acutatum* with almond tissues were in contrast to observations on leaves inoculated with *A. alternata*, another foliar pathogen commonly occurring in California almond production areas (25). In this latter interaction, fungal hyphae emitted fluorescence in the acidic pH range and no change in fluorescence was found inside invaded host cells. Thus, apparently *A. alternata* does not modify the pH of almond leaves. Because based on pH microelectrode measurements this fungus previously was shown to significantly increase the pH in several fruit hosts such as tomato, pepper, melon, cherry, and persimmon and ammonia production was demonstrated, it was grouped into the ‘alkaline fungi’ by Prusky and Yacoby (21). Still, increases in pH in these latter hosts were sometimes small (i.e., 0.2 pH units for persimmon) and thus, pH shifts are likely influenced by the specific host being colonized by *A. alternata*.

In summary, CSLM using the single-wavelength excitation, dual emission fluorophore SNARF-1 was highly sensitive in detecting and visualizing pH modulation spatially within the host tissue during early stages of pathogen penetration and colonization. By directly visualizing pH changes at the cellular level, we were able to demonstrate that pH modulation of almond by *C. acutatum* occurs in localized areas associated with specific fungal structures including appressoria, infection vesicles, and intra- and intercellular hyphae adjacent to and within host epidermal cells during successful colonization. Increases in ammonia concentration were also confirmed in this host–pathogen interaction, similar to other plant–*Colletotrichum* species interactions described by others. Thus, modulation of pH is an important factor involved in hemibiotrophic colonization due to its prevalence in our histological studies and may be critical in the transformation of the pathogen from a biotroph to a necrotroph within the host tissue. The sequence of events in the interaction of *C. acutatum* with almond includes host penetration, production of ammonia by *C. acutatum*, and subsequent pH modulation within almond leaf epidermal tissue to an alkaline environment that likely allows the increased expression and activity of fungal pectate lyase and possibly other enzymes leading to a transition from intra- to intercellular hyphal colonization of the host.

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