Direct Estimation of Local pH Change at Infection Sites of Fungi in Potato Tubers

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ABSTRACT

Fungi can modify the pH in or around the infected site via alkalization or acidification, and pH monitoring may provide valuable information on host–fungus interactions. The objective of the present study was to examine the ability of two fungi, *Colletotrichum coccodes* and *Helminthosporium solani*, to modify the pH of potato tubers during artificial inoculation in situ. Both fungi cause blemishes on potato tubers, which downgrades tuber quality and yield. Direct visualization and estimation of pH changes near the inoculation area were achieved using pH indicators and image analysis.

The results showed that the pH of the area infected by either fungus increased from potato native pH of approximately 6.0 to 7.4 to 8.0. By performing simple analysis of the images, it was also possible to derive the growth curve of each fungus and estimate the lag phase of the radial growth: 10 days for *C. coccodes* and 17 days *H. solani*. In addition, a distinctive halo (an edge area with increased pH) was observed only during the lag phase of *H. solani* infection. pH modulation is a major factor in pathogen–host interaction and the proposed method offers a simple and rapid way to monitor these changes.

Tubers of the potato plant (*Solanum tuberosum*) carry many seed- and soilborne pathogens that affect quality and yield (Tsrer (Lahkim) et al. 1999). Silver scurf, caused by *Helminthosporium solani*, and black dot, caused by *Colletotrichum coccodes*, produce tuber blemishes and affect processing and fresh-market trade (Mattupalli et al. 2013). According to the British Potato Council estimations, direct annual losses associated with these diseases is up to £5 million in the United Kingdom alone (Lees and Hilton. 2003). The pathogens can penetrate the tubers during growth or during harvest and handling. Although *C. coccodes* can colonize all underground parts (daughter tubers, stolons, and roots), basal stems, and foliage (Tsrer (Lahkim) and Peretz-Alon 2004), *H. solani* is considered to be a storage disease and the symptoms appear only on the tuber surface, localized to the periderm (Errampalli et al. 2001). Symptoms of silver scurf and black dot are frequently confused due to the similarity of blemishes caused on the periderm, and because they may be present simultaneously on the same tuber (Mattupalli et al. 2013).

Examination of tubers with a hand lens or microscope is required to observe the characteristic black microsclerotia of *C. coccodes*, or typical conidiophores and conidia of *H. solani* (Errampalli et al. 2001; Tsrer (Lahkim) and Peretz-Alon 2004). However, these structures are not always present, and additional diagnostic methods must be applied (isolation on selective media, serology, or molecular techniques).

Environmental pH is one of the key physiochemical factors that determine survival and control growth and development in fungi (Bassil et al. 2012; Wasantha Kumara and Rawal 2008; Prusky et al. 2001). Enzymes essential for fungal infection are mainly extracellular, and pH regulation ensures that extracellular enzymes, including amylase, cellulase, lipase, pectinase, and protease, are secreted under optimal conditions for their activity (Abe et al. 2015; Cotton et al. 2003; Drori et al. 2003; Maccheroni et al. 2004). For example, pectate lyase

is secreted by *C. gloeosporioides* at pH values higher than 5.8, even though it is already expressed at pH values of 5.1 (Prusky et al. 2001).

Fungi can, to some extent, modify the pH in or around the infected site via alkalization or acidification. For instance, *Colletotrichum* and *Alternaria* genera alkalize their environment, whereas *Penicillium* spp. and *Sclerotinia* and *Botrytis* genera acidify it (Davidzon et al. 2010). Alkalinization by fungi has been reported in a number of crops: in avocado infected by *C. gloeosporioides* and in tomato and apple fruit infected by *Colletotrichum* genera (Alkan et al. 2008). In contrast, colonization of mango and grape by *Phomopsis mangiferae* was accompanied by local acidification of the host tissue, from 5.1 to 3.8 in mango and from 4.1 to 2.5 in grape (Davidzon et al. 2010). It has been suggested that ammonification and alkalization are correlated (Alkan et al. 2008). Ammonification of the infected site affects various processes such as enzyme secretion and virulence increase (Prusky and Lichter 2007), activation of appressorium formation and differentiation (Miyara et al. 2010), and activation of quiescent infections in postharvest pathogens (Prusky et al. 2013).

pH alkalization at the cellular level, using a pH-sensitive fluorophore in confocal scanning microscopy, was observed within and in proximity to fungal infection structures of *C. acutatum* in almond tissue (Diéguez-Urbeondo et al. 2008). Furthermore, Bi et al. (2016) reported that differential pH modulation by fruit fungal pathogens is a host-dependent mechanism that is affected by host sugar content, which modulates environmental pH and enhances fruit colonization.

The average pH value of potato tubers has been estimated to be 6.0, and long storage generally did not have a significant effect on pH of the tubers of wild species (Kiszonas and Bamberg 2010). Nitzan and Tsrer (Lahkim) (2003) reported that the optimal pH value for *C. coccodes* isolates in vitro growth ranged from 6.0 and 7.0, depending on the isolate. This fact can explain the necessity of ammonia secretion into the local surroundings of the fungus during infection (Alkan et al. 2008).

The available methods, either direct or indirect, for measuring pH in fruit and vegetables include potentiometric (pH electrode) and optical methods (pH indicator and pH test strips). The optical methods are based on visual and photometric analysis of pH-dependent color

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changes, which are evaluated by visual comparison of color against a reference color scale. However, its precision is only sufficient to provide a rough estimate. The most common method for accurate pH measurement is by pH electrodes (Kurkdjian and Guern.1989). Unfortunately, insertion of conventional pH electrodes (either “flat” or “spear”) and microelectrodes is not feasible in all fruit and vegetables, due to their diversity in texture and structure. In particular, insertion of microelectrodes into potato tubers is practically impossible due to their rigid structure. Consequently, at present, there is no available method for measuring pH in potato tuber flesh which does not require a primary preparation procedure that destroys the tuber tissue (i.e., indirect homogenization method) (Alkan et al. 2008.)

Tuber wounds or skin injuries may accelerate the infection process. The inner tissues of the injured tubers, exposed to the external environment, may become desiccated or infected by pathogens,

creating lesions within days or weeks (Barth et al. 2010). The main defense mechanism of potato tubers against water loss, mechanical damage, and pathogens consists of a specialized suberin-rich layer, called the native periderm (Ginzberg 2008).

The objective of the present study was to develop a method that enables direct visualization and estimation of pH change on the potato tuber surface during artificial inoculation (in situ), based on pH indicator and image analysis.

MATERIALS AND METHODS

In order to study the difference between the effect of fungal infection and skin injuries on pH change, and to rule out the possibility that observed pH changes were associated with the wound-periderm formation and suberization process itself rather than with the pathogen infection, the experiments were divided into two pathways: one addressing biotic infection (artificial fungal inoculation) and a second addressing abiotic injury, as further described below. The study was conducted with two fungi which have similar visual symptoms (namely, *C. coccodes* and *H. solani*) originally isolated from potato tubers with typical symptoms of each disease.

Tuber inoculation, treatment, and infection. *Preliminary preparation of potato tubers.* All experiments were conducted with ‘Winston’ potato. Tubers were stored at 4 to 8°C and transferred to room temperature prior to the experiments. Tubers were surface disinfected with a sterilant chlorox solution (0.15% [vol/vol]) for 10 min, washed twice with sterile deionized water, air dried for 1 h in a petri dish, cut with a sterile scalpel to equally sized slices 1 to 1.5 cm thick, and further air dried in a petri dish.

Artificial inoculation with C. coccodes and H. solani. Our efforts to inoculate a whole tuber did not yield consistent results; therefore, we adapted the “hyphal mat infection method” (Alkan et al. 2008) to inoculate the tuber flesh, and established a homogenous system with consistent and reproducible conditions. Potato slices were prepared on day zero prior to inoculation. The inoculation with *C. coccodes* or *H. solani* was done by placing a plug (2 by 2 mm) of 3- to 5-day-old culture fungal mycelium (grown on potato dextrose agar) (Oxoid) on the potato disc. The inoculated discs were incubated in sterile petri dishes at 25°C for the entire experimental period. Noninoculated

Fig. 1. RGB value intensity versus pH in 0.01% phenol red agar plates. Error bars correspond to the standard deviation within the 300-by-300-pixel region of interest.

Fig. 2. Visualization of pH change in the area inoculated with *Colletotrichum coccodes* at A, 3; B, 10; C, 21; and D, 32 days after inoculations. Images labeled “1” are the original images with phenol red indicator, and images labeled “2” are the corresponding image after analysis in which white areas correspond to pH > 7.4.

potato discs (controls) were incubated under similar conditions. Examination of the inoculated potato discs was carried out twice a week. At each sampling date, 10 slices from each treatment were used for examination: 9 samples for each pH indicator treatment (3 triplicates per pH indicator) and 1 slice for control (noninoculated). For each experiment, a different set of indicators was used, as explained below.

Abiotic treatment (wound-periderm formation and suberization process). To evaluate whether the formation of wound periderm after skin injuries has an impact on pH, slices of potato tubers were incubated in sterile petri dishes at 25°C (the optimal temperature for

suberization) (Ginzberg 2008). The experiment was conducted for 14 days in order to fully complete the suberization process during wound periderm formation.

pH indicators. Base on previous reports only on the ability of *C. coccodes* to change ambient pH in other crops, three pH indicators were chosen to cover the whole range of pH values relevant to fungal infection: 0.01% (wt/vol) thymol blue solution (Sigma-Aldrich) (transition zone: 1.2 to 2.8 and 8.0 to 9.2), 0.01% (wt/vol) phenol red solution (Sigma-Aldrich) (transition zone: 6.8 to 8.2), and 0.01% (wt/vol) phenolphthalein solution (Sigma-Aldrich) (transition zone: 8.2 to 12). For abiotic injury experiments, the following indicators

Fig. 3. Visualization of pH change in the area inoculated with *Helminthosporium solani* at **A**, 3; **B**, 17; **C**, 32; and **D**, 45 days after inoculations. Images labeled “1” are the original images with phenol red indicator, and images labeled “2” are the corresponding image after analysis in which white areas correspond to pH > 7.4.

Fig. 4. Visualization of pH change in the area inoculated with **A** and **B**, *Colletotrichum coccodes* and **C** and **D**, *Helminthosporium solani*; 1 = nontreated, 2 = treated with thymol blue indicator, and 3 = treated with phenolphthalein indicator.

were used: 0.01% (wt/vol) bromocresol purple (Sigma-Aldrich) (transition zone: 5.2 to 6.8), 0.01% (wt/vol) phenol red solution (Sigma-Aldrich) (transition zone: 6.8 to 8.2), and 0.01% (wt/vol) Congo red solution (Sigma-Aldrich) (transition zone: pH 3.0 to 5.2).

pH visualization. In order to visualize pH shift in the infected area, pH indicator solution was placed on the entire potato disc using a sterile pipette and the sample was placed underneath an imaging system consisting of a CCD color camera (Basler Scout) equipped with an $f = 35$ mm/F1.4 Navitar lens. Each image included a pH measuring litmus paper previously soaked in distilled water, and the pixels of this region were used to verify that there was no significant fluctuation of the illumination. To establish a correlation between pH and apparent pixel color in the images, 0.01% phenol red agar plates (1.5% agar) with the following pH values were prepared: 6.9, 7.0, 7.3, 7.5, and 8.0. The plates were placed under the imaging system described above and several images of each plate were captured. In each image, the pixels of a region of interest (ROI) of 300 by 300 pixels were extracted and the mean and standard deviation of the three color components (RGB) were calculated.

Measurement of radial infected zone (decayed zone). Fungus growth is expressed in terms of the radius of a hypothetical circle which would have an area equal to that of the area of elevated pH.

RESULTS

pH visualization using the imaging system. A sharp decrease of the green and red components occurred in the ROI of the agar plates between pH 7.3 and 7.5 (Fig. 1). The blue component had much smaller values with relatively larger standard deviation;

Fig. 5. **A,** Noninoculated potato discs with phenol red indicator after 10 days of suberization. **B,** Image after analysis; the absence of white pixels in the right image indicates that the pH was below 7.4 in the whole area.

Fig. 6. Time evolution of the equivalent radius of the area inoculated with *Colletotrichum coccodes*. Error bars correspond to standard deviation of three samples.

therefore, this component was not used in the analysis. Based on the data presented in Figure 1, pixels with green value lower than 70 and blue value lower than 220 were considered to correspond to pH higher than 7.4 while the other pixels were considered to correspond to pH values lower than 7.4.

Visualization of pH change in the area inoculated with *C. coccodes* and *H. solani*. In order to detect the effect of growth on pH value at the infected area using pH indicators, the inoculated potato tuber discs were visualized using the imaging system, and the images were processed using the RGB thresholds determined above.

For both fungi, the pH shift stopped abruptly at the margin of the lesion (Figs. 2 and 3). A correlation between the morphological symptoms of the infected area and the visualized pH was observed. No color change was observed using thymol blue and phenolphthalein indicators (Fig. 4). Therefore, combining phenol red and thymol blue measurements showed that pH in the infected areas was between 7.4 and 8.0. No change in pH value was observed in the noninoculated control during the whole experimental period (Fig. 5).

From binary images such as those shown in Figures 2 and 3, it is possible to estimate the radius equivalent to the area infected by the fungus or, more precisely, the area in which the pH is affected by the fungal activity (Figs. 6 and 7 for *C. coccodes* and *H. solani*, respectively). The lag phase of the radial growth for *C. coccodes* and *H. solani* lasted 10 and 17 days, respectively. Moreover, pH visualization revealed differences in the pathogen–host interaction between the two fungi during the initial lag phase of infection: *C. coccodes* affected pH in the whole infected area, whereas *H. solani* affected pH only at the edge of the infected area, creating a clearly defined halo (region with pH > 7.4). This phenomenon was observed during the first 17 days postinoculation (Fig. 3A and B).

Visualization of pH change during wound healing. In order to investigate whether the pH value of the wounded tissue was affected by the healing process, several pH indicators were used to cover acidic, neutral, and alkaline pH. No change in pH value was observed during the wound healing process (Fig. 8A, B, and C: phenol red, bromocresol purple, and Congo red, respectively).

DISCUSSION

pH monitoring provides valuable information on host–fungus interactions because pH is one of the main factors controlling fungal growth and has a crucial role in fungal establishment. The pH level prevailing in the host may cause activation from quiescence to an active infection (Prusky and Lichter 2007). These changes in host tissue activate transcription and secretion of fungal degradative

Fig. 7. Time evolution of the equivalent radius of the area inoculated with *Helminthosporium solani*. For the first four sampling dates, the inner radius of the halo is also indicated. Error bars correspond to the standard deviation of three samples.

Fig. 8. Noninoculated potato disc suberization treated with indicator after 10 days of suberization: **IA**, phenol red; **B**, bromocresol purple; and **C**, Congo red indicators.

enzymes. The ability of *C. coccodes* to cause alkalization has been previously reported, whereas no information about *H. solani* causing a shift in pH value at the infected site is available.

In the current study, pH visualization confirmed the ability of *C. coccodes* to alkalize potato tuber surroundings, as previously reported for other crops (Prusky et al. 2001), indicating that alkalization also is a crucial preliminary stage (alkalization was observed 3 days postinoculation) for fungal establishment on potato. The pH in the infected area shifted in more than 1.4 units from the potato native average pH (Fig. 1). A correlation between the morphological disease symptoms of the infected area and the visualized pH was observed (Figs. 2 and 3). This is the first report on the ability of *H. solani* to cause alkalization of the infected area, similarly to *C. coccodes*. No change was observed using thymol blue and phenolphthalein indicators for both fungi (Fig. 4). An interesting phenomenon was observed during the first 17 days after inoculation (Fig. 3A and B): a halo (region with pH > 7.4) appeared at the margin of the lesion formed after inoculation with *H. solani*. This halo was most probably formed due to extracellular enzymes and byproducts secreted into the infected area around the lesions. These extracellular enzymes released by the hyphal tips diffuse out ahead of the fungus in order to disintegrate compounds into nutrients needed for growth. Therefore, it can be suggested that alkalization of the tissue may be one of the factors that contributes to *H. solani* virulence and may serve as a biochemical activator.

Expressing the expansion of the infected area in terms of its equivalent radius (Figs. 6 and 7) enables a more quantitative examination of the pH change pattern over time. Although only three replicates were done for phenol red indicator, the pattern was clear and very consistent, with three main phases: lag, logarithmic, and stationary phase. We can conclude that, as disease symptoms progressed, potato tuber flesh became more alkaline for both investigated fungi. Wound periderm formation and the suberization process did not cause pH change in the noninoculated discs (Figs. 5 and 8); thus, it can be concluded that pH alteration was caused only by fungal activity. This agrees with the findings of Alkan et al. (2008), who did not observe pH change and ammonia accumulation in tomato fruit. Because, in the present study, fungal inoculation was done on tuber flesh rather than on tuber skin, at this stage we can only speculate that the fungi's ability to alkalinize their ambient pH will be preserved on the skin.

The proposed method could be applied to monitor pH changes caused by other microorganisms infecting potato tubers such as *Rhizoctonia solani*, the causal agent of black scurf (Gonzalez García et al. 2006) during disease establishment. This method could also be useful for screening disease-resistant cultivars, which suppress the development of silver scurf and black dot lesions.

This newly developed method is simple and does not require expensive equipment or chemicals, and can easily be applied to other pH ranges by selecting a pH indicator accordingly. It provides an alternative to (micro) electrodes-based measurements which cannot be performed in hard fruit and vegetables such as potato. However, its main limitation is that it provides only a rough estimate of pH and not the actual pH value (e.g., greater than or less than the

indicator transition value). Still, application of several indicators minimizes the range of uncertainty.

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