Ammonium Secretion by *Colletotrichum coccodes* Activates Host NADPH Oxidase Activity Enhancing Host Cell Death and Fungal Virulence in Tomato Fruits

Noam Alkan,¹ Olga Davydov,² Moshe Sagi,³ Robert Fluhr,² and Dov Prusky¹

¹Department of Postharvest Science of Fresh Produce, Agricultural Research Organization, the Volcani Center, Bet Dagan 50250, Israel; ²Department of Plant Sciences, Weizmann Institute of Science, Rehovot, Israel; ³Department of Dryland Biotechnologies, Jacob Blaustein Institutes for Desert Research, Ben-Gurion University, Sede Boqer Campus, Midreshet Ben-Gurion 84990, Israel

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Colletotrichum pathogens of fruit and leaves are known ammonium secretors. Here, we show that *Colletotrichum coccodes* virulence, as measured by tomato (*Solanum lycopersicum* cv. Motelle) fruit tissue necrosis, correlates with the amount of ammonium secreted. Ammonium application to fruit tissue induced hydrogen peroxide (H₂O₂) accumulation. To examine whether the tomato NADPH oxidase, SIRBOH, is a source for the ammonium-induced H₂O₂, wild-type and antisense lines abrogated for SIRBOH (SIRBOH-AS) were examined. Wild-type lines produced 7.5-fold more reactive oxygen species when exposed to exogenous ammonium than did SIRBOH-AS lines. *C. coccodes* colonization of wild-type tomato lines resulted in higher H₂O₂ production and faster fungal growth rate compared with colonization in the SIRBOH-AS mutant, although the amount of ammonium secreted by the fungi was similar in both cases. Enhanced ion leakage and cell death of fruit tissue were correlated with H₂O₂ accumulation, and treatment with the reactive oxygen scavenger *N*-acetyl-L-cysteine decreased H₂O₂ production, ion leakage, and cell death. Importantly, the activation of reactive oxygen species production by ammonium was positively affected by an extracellular pH increase from 4 to 9, implying that ammonium exerts its control via membrane penetration. Our results show that *C. coccodes* activates host reactive oxygen species and H₂O₂ production through ammonium secretion. The resultant enhancement in host tissue decay is an important step in the activation of the necrotrophic process needed for colonization.

Colletotrichum coccodes is a hemibiotrophic pathogen that colonizes unripe, living-host fruit tissue symptomlessly for long periods of time (quiescent biotrophic stage) and activates its colonization during fruit ripening and senescence (active necrotrophic stage) (Prusky 1996; Latunde-Dada 2001). Ammonium accumulation has been detected in many *Colletotrichum* spp., including *C. gloeosporioides*, *C. acutatum*, and *C. coccodes* (Prusky et al. 2001). The ammonium secreted by *Colletotrichum* spp. enhances alkalization of the host tissue.

The increase in pH is an important signal for the induction of fungal gene expression, including the secretion of virulence factors such as pectate lyase (Yakoby et al. 2000; Prusky et al. 2001). The level of ammonium in the infection court is high and can reach approximately 3.5 mM, as has been found in avocado and tomato fruits (Prusky et al. 2001; Alkan et al. 2008). Ammonium production by these fungi appears to begin with the activation of fungal proteases and concomitant deamination of the resultant amino acids (Prusky and Yakoby 2003). Pathogenicity of *Colletotrichum* spp. in different hosts is thought to be related to ammonium accumulation (Prusky et al. 2001; Alkan et al. 2008). Interestingly, the initial acidic pH of the fruit has been shown to be conducive to the enhanced ammonium secretion and host-tissue alkalization that facilitate fungal virulence. The isolation of *C. coccodes* mutants with reduced ammonium production has provided direct evidence for the necessity of ammonium accumulation during *Colletotrichum* pathogenicity (Alkan et al. 2008), indicating that ammonium accumulation is a critical factor contributing to *Colletotrichum* spp. necrotrophic development in ripening fruit (Prusky and Yakoby 2003).

Nevertheless, how ammonium contributes to pathogenicity remains an open question. Ammonium may have pleiotropic toxic effects on the host, such as activating the transport of various solutes, changing the concentration of cytosolic protons, or initiating transduction signals that may result in metabolic activation of different plant processes (Mathieu et al. 1994). Direct toxicity symptoms may include effects on the biochemical pH-stat mechanisms in cells, causing differences in the internal H⁺ balance and in membrane flux processes (Britto and Kronzucker 2002). Elucidating the nature of the toxicity involved in fungus–fruit interactions is fundamental to understanding *Colletotrichum* biology. The lifestyle of pathogenic fungi can be broadly divided into necrotrophs, hemibiotrophs, and biotrophs, depending upon the type of interaction between the fungus and host tissue. Much knowledge about plant–pathogen interactions has been garnered from studies on leaf pathogens. The production of reactive oxygen species (ROS), which precedes a hypersensitive response (HR), has been shown to be associated with increased resistance to biotrophs but can also increase susceptibility to necrotrophs (Greenberg 1997; Gilchrist 1998; Glazebrook 2005; Kliebenstein and Rowe 2008; Shetty et al. 2008). A key element responsible for controlled ROS production in innate immune processes is the evolutionarily highly conserved NADPH oxidases, called respiratory burst oxidase homologs

Corresponding author: Dov Prusky; Telephone 972-3-9693880; Fax: 972-3-9683622; E-mail: dovprusky@agri.gov.il

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(RBOH) in plants, which produce superoxides that are subsequently converted into hydrogen peroxide (H_2O_2) (Sagi and Fluhr 2006). *Arabidopsis* AtRBOHD and AtRBOHF mutants show differential effects on disease (Torres et al. 2002) whereas tomato lines with reduced RBOH levels show reduced wound responses (Sagi et al. 2004).

It has been suggested that the leaf HR is triggered by a signal generated by the balance between nitric oxide and superoxide production (Delledonne et al. 1998). Diverting this signal via superoxide induction and, thus, the subsequent tissue HR has been shown to be important in facilitating infection by the necrotroph *Botrytis cinerea*, although how the ROS were induced is not known (Govrin and Levine 2000). Tissue colonization by *B. fuckeliana* was also reported to be facilitated by ROS production, in this case brought about by changes in the host's (tomato) antioxidant capacity (Kuzniak and Sklodowska 2005). Thus, a well-established cell stress response in many organisms is ROS accumulation followed by localized cell death (D'Autreaux and Toledano 2007).

Successful colonization of necrotrophic pathogens in fruit has not been followed in the context of pathogenicity facilitation by ROS. The synchronic infection court system developed for studying *C. coccodes* mutants represents a good system for such an analysis (Alkan et al. 2008). We examined whether ROS play a role in contributing to fruit tissue decay as they does by enhancing leaf HR and hypothesized that ammonium could trigger this process. Using tomato (*Solanum lycopersicum* cv. Motelle) lines with downregulated expression of the tomato NADPH oxidase gene, SIRBOH-antisense (SIRBOH-AS), we demonstrate a role for ammonium secretion in the induction of host ROS accumulation. We show that ROS are a key factor in promoting cell death in tomato fruit at the leading edge of the colonizing hyphae. Thus, *C. coccodes* fungi activate a host-based innate immunity-signaling system to enhance their own colonization.

RESULTS

Ammonium accumulation affects *C. coccodes* pathogenicity and host RBOH.

A striking feature of *C. coccodes*'s lifestyle is the secretion of ammonium. To examine the relationship between ammonium accumulation and fungal colonization of tomato fruit, we compiled the results of seven experiments (41 data points) covering a wide variety of experimental conditions with wild-type and ammonia secretion mutants of *C. coccodes* (Alkan et al. 2008) and under different pH conditions in the infection court (Fig. 1). A 1-cm epidermal region was excised from the fruit and inoculated with *C. coccodes* and the growth in decay diameter was examined after 48 h. Concentrations of ammonium were found to range from 0.5 to 5 mM and the decay diameter from 0.1 to 2 cm beyond the initial inoculation-site diameter. Decay development increased in direct relation to the amount of ammonium accumulation ($R^2 = 0.865$; $P < 0.01$).

These results implied that ammonium is correlated with the success of fungal colonization. To examine for a possible causal relationship between ammonium accumulation by *Colletotrichum* spp. and tomato fruit tissue necrosis, we analyzed the effect of ammonium on the production of ROS in fruit from wild-type and SIRBOH-AS tomato lines. Wild-type tomato fruit slices treated with 5 and 20 mM NH_4Cl at pH 7.0 showed a 2.9- and 7.5-fold increase in ROS production within 15 min of treatment, as detected by staining with the fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCF) (Fig. 2A and D) and confocal microscopy (Fig. 2E). Similar treatment of fruit tissue from two independent SIRBOH-AS lines (lines 9 and 12) showed only minor changes in ROS production compared with

the untreated control (Fig. 2B and C). The applied ammonium rapidly dissipated over the course of the experiment to 5% of its initial level (data not shown). Hence, the levels of ammonium used here were physiologically relevant to the infection court as measured in Figure 1. At the lower and higher ammonium concentrations (1 and 50 mM), a 1.6- and 9.4-fold increase in ROS, respectively, was obtained 15 min after treatment of the wild-type tomato pericarp (results not shown). The induction was not a result of osmotic changes, because the phosphate-buffered saline (PBS) itself contains 140 mM NaCl. These results showed that SIRBOH-dependent ROS accumulation in tomato fruit tissue is induced by physiologically relevant levels of ammonium. Moreover, confocal microscope pictures taken 30 min after ammonia application showed ROS accumulation in the cells (Fig. 2E).

Effect of ammonium on membrane stability and cell death.

Our results indicated that fungal necrotrophic development can be enhanced by local cell death induced by ROS production. To monitor early insidious aspects of cell death, cellular ion leakage was determined as a measure of membrane stability in ammonium-treated wild-type and SIRBOH-AS tomato fruit. Ion leakage from the tissue membrane was detected by conductivity measurements and monitored simultaneously with H_2O_2 production in treated fruit disks transferred to water. Pretreatment of tomato disks with 20 mM NH_4Cl increased ROS production and membrane leakage by 1.8- and 4.3-fold, respectively, in wild-type fruit (Fig. 3) compared with only 1.14- and 1.4-fold increases in SIRBOH-AS (line 9). Surprisingly, pretreatment of tomato fruit by direct application of H_2O_2 enhanced ROS and membrane leakage by 2.5- and 3.1-fold, respectively, compared with only 1.4- and 1.3-fold in the SIRBOH-AS lines (Fig. 3).

Fig. 1. Accumulation of ammonium in the infection court by *Colletotrichum coccodes* and decay diameter. Ammonium concentration and extent of the decay diameter beyond the initial hyphal mat inoculation were measured for 41 infection points in seven independent experiments according to the method of Alkan and associates (2008). Symbols: *, *areA*⁻ mutant strains with reduced ammonia secretion inoculated on ripe fruit; ●, *areA*⁻ mutant strains with reduced ammonia secretion inoculated on breaker stage fruit; ■, *nit1*⁻ and *nit3*⁻ mutants inoculated on ripe fruit; +, wild-type (WT) strain inoculated on fruit at various maturation stages (red, color break, and green); ◆, *nit1*⁻ and *nit3*⁻ mutants inoculated on breaker stage fruit treated with ammonia; ×, WT strain on ripe fruit treated with phthalate buffer pH 4.0 and 7.0; ▲, WT strain on breaker stage fruit treated with phthalate buffer pH 4.0, 4.5, 5.0, 6.0, 6.5, and 7.0.

This indicated that H_2O_2 can act as a trigger of RBOH. When tomato disks of the wild-type fruit were treated with the ROS scavenger *N*-acetyl-L-cysteine (NAC) prior to treatment with either ammonium or H_2O_2 , the increase in ROS was reduced by 63 to 91% and that of conductivity by 60 to 71%. Only minor effects of NAC were detected in the SIRBOH-AS fruit. NAC also inhibited the increases in ROS and conductivity caused by the water treatments (Fig. 3).

The increased conductivity after NH_4Cl treatment was reflected in the ensuing cell death, as measured by Evans blue staining. Cell death increased 5- and 14.4-fold in the wild-type

fruit 24 and 48 h posttreatment, respectively, whereas no increase was detected during the same period in SIRBOH-AS line 9 (Fig. 4A and 4B). The increase in cell death in the wild-type fruit after NH_4Cl treatment was abrogated by pretreatment with the ROS scavenger NAC (Fig. 4D). Because an initial addition of 50 mM NH_4Cl may be perceived as high, another set of fruit was treated with 5 mM NH_4Cl reapplied every 6 h for 24 and 48 h: here, too, significant levels of cell death were induced (Fig. 4C). The results indicated that ammonium treatment induces SIRBOH-dependent cell death which can be reduced in the presence of ROS scavengers.

Fig. 2. Effect of ammonium on reactive oxygen species accumulation as measured by 2',7'-dichlorodihydrofluorescein diacetate fluorescence in **A** and **D**, wild-type (WT) and **B** and **C**, SIRBOH-AS tomato fruit tissue. **A**, Fluorescence of WT tomato fruit tissue slices after application of: 5 mM NH_4Cl in phosphate-buffered saline (PBS) (▲), 20 mM NH_4Cl in PBS (◆), or PBS (■). All treatments were at pH 7.0. **B** and **C**, Treatments and fluorescence measurements were carried out as in **A** with SIRBOH-AS line 9 and 12 tomato fruit tissue, respectively. **D**, Fluorescence binocular pictures were taken 0, 15, 30, and 60 min after 20 mM ammonia application on WT tomato fruit tissue. **E**, Confocal microscope pictures were taken 0 and 30 min after 20 mM ammonia application on tomato fruit tissue of the WT (upper two) and SIRBOH-AS (lower two).

Contribution of the host response to *C. coccodes* infection.

To determine the effect of host ROS production and its contribution to the pathogenicity and necrotrophic development of *C. coccodes* on tomato, we inoculated wild-type and SIRBOH-AS tomato fruit and followed its colonization pattern (Fig. 5). Both cultivars showed similar ammonium amounts and pH levels in the colonized tissue (Fig. 5A and B). However, the wild-type tomato fruit showed a significantly higher amount of ROS accumulation as detected by DCF fluorescence (Fig. 5C). Furthermore, wild-type tissue was more susceptible than the SIRBOH-AS strain to fungal colonization, as reflected by the larger decay diameter (Fig. 5D).

Activation of RBOH by ammonium is dependent upon pH.

Our previous data has shown that ammonium secretion is induced in *C. coccodes* at the ambient low pH present in fruit (Alkan et al. 2008). However, additional pathogenicity factors are activated when the host environment pH increases (Prusky et al. 2001). Therefore, it was of interest to study ammonium-induced ROS production as a function of pH. To this end, fruit tissue was exposed to PBS alone, or amended with 10 mM NH_4Cl , at various pHs. Ammonium induction of ROS was strongly enhanced with an increase in pH from 4 to 8 (Fig. 6B). The observed increase in fluorescence was likely the result of DCF oxidation and not of changes in pH, because DCF varies less than 30% over a pH range of 5.2 to 8 (Ge and Chen 2008). The increased ROS as a function of pH could be

Fig. 3. H_2O_2 accumulation and electrolytic leakage in tomato fruit disks. Disks were pretreated with H_2O_2 , NH_4Cl , or water, with or without N-acetyl-L-cysteine (NAC). Treatment was for 30 min and then disks were washed and incubated for 2 h. The experiments were carried out on wild-type (black column) and SIRBOH-AS (white column) tomato fruit disks. H_2O_2 was detected by Amplex red 15 min after pretreatment and incubation in phosphate-buffered saline (upper box). Electrolyte leakage was detected after 2 h by measuring conductivity (lower box). Numbers are expressed as percentage of the water treatment (double-distilled water) for each tomato line. Experiments are an average of four replicates, three disks per replicate.

the result of differential penetration of the ammonium solution into the tomato parenchyma cells at each particular pH. Indeed, it is expected that, at higher pH, more neutral ammonium NH_3 species will be present, and membrane permeability to the noncharged species is higher than that to the ionated NH_4^+ species. The penetration of ammonium was estimated by measuring the degree of cytosol alkalization, using the fluorescent probe 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF, AM), in the presence of ammonium as a function of increasing pH from 4 to 7. The cytosolic pH rose rapidly at 10 mM ammonium at pH 7.0 (Fig. 6A), the rise in cytosolic pH is likely the result of hydrogen ion uptake in the cytosol by the permeating NH_3 form, which results in cytosol alkalization. These results suggested that internalization of ammonium and cytosolic pH changes both function in SIRBOH activation. Interestingly, when inorganic phosphate buffer was used at high concentrations (50 mM; i.e., 5.7-fold that found in PBS), a significant ROS response was also obtained concomitant with alkalization of the cytosol (Fig. 6C). Phosphate species likely penetrate via membrane phosphate transporters (Eckardt 2005; Gonzalez et al. 2005). In contrast, the nonpermeable phthalate buffer at 50 mM and pH 7.0 did not induce ROS or cause cytosolic alkalization (Fig. 6C).

DISCUSSION

Our observations established a direct correlation between ammonium accumulation and size of the *C. coccodes* infection court. Ammonium application by itself was found to directly activate ROS production in tomato tissue and the source of this ROS, as established by the use of SIRBOH antisense lines, was the tomato NADPH oxidase. Moreover, confocal observation (Fig. 2D) further established that the ROS activated by ammo-

Fig. 4. Effect of ammonium on cell death in whole tomato fruit from wild-type and SIRBOH-AS lines. **A**, **B**, and **D**, Fruit was injected with water, ammonium, or simultaneously with N-acetyl-L-cysteine (NAC) and ammonium, respectively. Cell death at the injection site was detected 24 and 48 h after 50 mM NH_4Cl had been injected into the pericarp. Cell death was monitored by Evans blue staining. Treatments were **A**, **C**, and **D**, wild type and **B**, SIRBOH-AS. **C**, 5 mM NH_4Cl was applied after removal of a 1-cm-diameter epidermal disk and reapplied every 4 h.

nium can be detected within the cell. Although RBOH protein has been detected on the plasmalemma (Keller et al. 1998; Sagi and Fluhr 2001; Kobayashi et al. 2006) and, in that case, would catalyze superoxide production outside of the cell, the DCF reporter can report only internal accumulation of H_2O_2 (Allan and Fluhr 1997; Ashtamker et al. 2007). Apparently, either the superoxide or its dismutated form (H_2O_2) enters the cell.

Fig. 5. Ammonium level, reactive oxygen species (ROS) accumulation, pH changes, and colonization diameter during *Colletotrichum coccodes* growth on wild-type (WT) and SIRBOH-AS tomato fruit. Spores of *C. coccodes* were injected into tomato mesocarp. **A** and **B**, Ammonium and pH, respectively, measured 48 h postinoculation. **C**, ROS accumulation measured by 2',7'-dichlorodihydrofluorescein diacetate fluorescence 48 h post-inoculation. **D**, Colonization diameter measured over the course of 6 days post-inoculation. Micrographs of the treated area were analyzed by SigmaScan software.

Due to the lack of a full genomic sequence in tomato, the exact member of the SIRBOH multigene family that is involved is not known. However, a specific and direct reduction in total SIRBOH polypeptides (Wf1 and SIRBOH1), total ROS, and wound-signal transduction was observed previously in SIRBOH-AS lines (Sagi et al. 2004). ROS accumulation was part of the signal for induced cell death, because this induction was abrogated by the addition of the ROS scavenger, NAC. Ammonium affected ion leakage of tomato cells (as determined by measuring conductivity) and cell death (as detected by Evans blue staining). The simplest scenario consistent with these observations is that ammonium secretion serves a dual purpose in *C. coccodes* colonization: as a signal for the synchronous induction of pathogenicity genes in the fungus and as an inducer of host ROS accumulation. In tomato fruit with reduced SIRBOH activity, these dual functions are separated: while the fungus continues to secrete ammonium and modify the infection court pH, the mutant host tissue is less responsive in terms of ROS production and subsequent induction of cell death. Importantly, ammonium accumulation by the pathogen is independent of ROS accumulation and the fungus uses fruit pH as a cue for ammonium production (Prusky and Yakoby 2003).

Accumulation of ROS due to ammonium has been noted in other organisms. In human astrocytes, the application of ammonium was shown to activate NADPH oxidase (Reinehr et al. 2007). In yeast, an ammonium signal emitted by aging colonies triggered metabolic changes leading to yeast localized programmed cell death (Vachova and Palkova 2005). However, because yeast lack NADPH oxidases, the source of the ROS in that case must have been different (Kawahara et al. 2007).

Present observations, where ammonium activates NADPH oxidase and the accumulation of ROS, indicate a possible mechanism for understanding of the toxicity of ammonium to the host tissue (Medici et al. 2004). Presumably, as ammonium flows into the plant cell, increasing amounts of energy are diverted to its active removal. The resultant energy depletion is thought to be one agent of ammonium toxicity that can also have a negative effects on photosynthesis (Britto and Kronzucker 2006). Although energy depletion by ammonium may be a valid cause for toxicity at the whole-plant level during root solute interactions, it is unlikely to be the cause of cell death in tomato fruit; first, due to the rapidity of the reaction

Fig. 6. Effect of ammonium applied at different pHs on reactive oxygen species (ROS) induction. **A**, Fluorescence measurements of 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF, AM) after application of: 10 mM NH_4Cl at pH 4.0 (▲) and pH 7.0 (◆), 50 mM phosphate at pH 7.0 (×), 50 mM phthalate at pH 7.0 (◇), and H_2O (■). Results were recorded for 30 min after the treatments. **B**, 2',7'-Dichlorodihydrofluorescein diacetate (DCF) fluorescence measurements after application of 10 mM NH_4Cl at different pHs. **C**, DCF fluorescence measurements after application of 50 mM phosphate and phthalate buffers at pH 7.0. The results are averages of 500 parenchyma cells.

and, second, due to the fact that high concentrations of other ions, such as inorganic phosphate, also induce ROS in tomato tissue. Phosphate can penetrate plant cells and alkalize them in a manner similar to ammonium (Sakano 1990; Arif et al. 1995). We showed that ammonium alkalizes the cell cytosol more efficiently at higher pH and that the host cell reacts by accumulating more ROS. The ammonium ion import due to the extracellular excess can lead to perturbation of plasma-membrane membrane charge homeostasis, which promotes H⁺-ATPase activity to correct the changes in electronegativity (Pagliarini et al. 2008). Fluxes of this sort have been shown to precede RBOH activation.

Our data add to the increasingly complex picture of fruit-pathogen interactions. The increasing availability of amino acids during fruit ripening (Boggio et al. 2000) may ensure ammonium production by the pathogen and contribute to initiating its shift from the biotrophic-quiescent stage to the necrotrophic-colonization stage (Prusky and Lichter 2007). The ability of the pathogen to accumulate large amounts of ammonium in decaying tissue contributes to increased pH and virulence (Alkan et al. 2008). In this work, we show that host ROS production is a target for the accumulated ammonium. Our results suggest that ammonium secretion is a key factor modulating host physiology near the hyphae's leading edge by subverting host cellular signals to the benefit of *Colletotrichum* spp. necrotrophic growth. A similar conclusion was recently suggested by Asai and Yoshioka (2009), who reported the possible involvement of RBOH in host cell death resulting in the enhanced necrotrophic development of *B. cinerea*. Therefore, altering ammonium secretion, pH-regulated processes, or ROS scavenging could form the basis for a viable strategy to delay, reduce, or even prevent disease development by this plant-pathogenic fungus.

MATERIALS AND METHODS

Plant material, fungal strains, media, and growth conditions.

S. lycopersicum cv. Motelle wild-type and transgenic plants expressing *SIRBOH* (AAD25300) and *Wfil* (AF73124) fragments in sense and antisense orientation, respectively, were used to obtain SIRBOH-AS line 9 and line 12. They are independent isolates of the M3-type lines as described by Sagi and associates (2004). The plants were grown in pots filled with a peat-vermiculite (4:1, vol/vol) mixture containing slow-release high-N Multicote 4 with microelements (0.3% wt/wt; Haifa Chemicals, Haifa Bay, Israel). Average temperatures in the greenhouse during the growth period fluctuated from 18 to 25°C. All experiments were performed on *S. lycopersicum* cv. Motelle wild-type and transgenic plants SIRBOH-AS line 9 and line 12.

C. coccodes isolate 138, pathogenic on tomato fruit, was obtained from decayed potato tubers in Israel (supplied by L. Tzror, Agricultural Research Organization) (Nitzan et al. 2002). Single-spore cultures were maintained routinely on Mathur's medium (M3S) (Tu 1985) containing chloramphenicol at 250 mg/liter. Spores of the original single-spore culture were maintained in 40% glycerol at -80°C as a source of inoculum. *C. coccodes nit1*⁻ and *nit3*⁻ mutants were generated in the presence of potassium chlorate (Alkan et al. 2008). *C. coccodes areA*⁻ (strains 752, 787, 757, and ectopic 801) mutant lines were obtained by direct hygromycin insertion using the Gateway system (Alkan et al. 2008).

Fruit inoculation, fruit treatments, and infection parameters.

For inoculation with fungal hyphae, freshly harvested mature color-break tomato fruit of the same age and size (*S. lycopersicum* cv. Hazera 1402, susceptible to *C. coccodes*) were

used for inoculations. Fruit inoculation was carried out on the mesocarp. A 1-mm-thick, 10-mm-diameter piece of the peel pericarp tissue was removed from the fruit and replaced by a 1-mm-thick hyphal disk of *C. coccodes* mycelia showing different levels of reduction of ammonium secretion as wild type, *nit1*⁻ (nitrate reductase), *nit3*⁻ (nitrite reductase), and *areA*⁻ mutants (major nitrogen regulation factor) (Alkan et al. 2008). This hyphal disk was obtained after growing spores for 3 days on primary media and 1 day on secondary media (Alkan et al. 2008). Following inoculation, fruit were incubated at 22°C and 95% relative humidity in plastic containers. In some experiments, the inoculation sites were exogenously treated by pipetting 50 µl of phthalate at different pHs, or 10 mM KNO₃, glutamine, or ammonia. The diameter of decay development was measured 48 h after inoculation and reported in centimeters over the 1-cm diameter of infection with the hyphae mat. The ammonium concentration in the decayed tissue was measured 48 h after inoculation (as described below).

Fluorescence and staining methods.

Changes in tissue ROS and pH were detected using the fluorescent probes DCF D-399 and BCECF, AM B-1150, respectively (Molecular Probes, Invitrogen, Leiden, The Netherlands). Staining for ROS and pH evaluation in infected and noninfected tomato tissue were performed by exposing the tissue to 10 µM DCF or BCECF, AM, respectively, for 15 min in the dark at 24°C and then rinsing the sample twice in PBS. Sampling of the tissue for ROS and pH evaluation was performed by slicing 1-mm-thick by 20-mm-long parenchyma fruit strips from the tomato pericarp and mesocarp tissue diagonal to the surface area. The strips were viewed with a Leica fluorescence binocular or a laser-scanning confocal microscope (Olympus IX81). Intracellular fluorescence was excited at 488 nm and emission was detected at 520 nm. The picture was then analyzed by SigmaScan software (SYSTAT) to obtain the average green fluorescence for each strip for both pH and ROS measurements.

To examine the effect of ammonium on ROS accumulation, parenchyma strips from the wild type and SIRBOH-AS lines 9 and 12 were stained with DCF and rinsed in 5 or 20 mM NH₄Cl, or H₂O as a control (all solutions were at pH 7.0). The strips were viewed with a Leica fluorescence binocular and with a laser-scanning confocal microscope (Olympus IX81). To examine ROS induction and pH change following ammonium application at different pHs, the parenchyma strips were stained with DCF or BCECF, AM, respectively, and rinsed with 10 mM NH₄Cl at pH 4.0 to 9.0, or with 50 mM phthalate or phosphate buffered to pH 7.0. For DCF staining, pictures were taken after 60 min and, for BCECF, AM staining after 30 min, and then analyzed with SigmaScan software.

During the conductivity experiments, H₂O₂ concentration was determined 15 min after pretreatment with double-distilled water, ammonium, and H₂O₂ (described below). The level of H₂O₂ secreted from tomato fruit disks was measured by mixing 50 µl of the surrounding liquid with 50 µl of Amplex red (100 µM) in the presence of horseradish peroxidase at 0.2 U/ml (Invitrogen, Molecular Probes). The samples were measured using the Microplate Fluorescence Reader FL600 (BioTek Instruments, Winooski, VT, U.S.A.) by excitation at 530 nm and emission at 590 nm. Measurements were taken for 30 min every 3 min.

For cell-viability measurements, wild-type or SIRBOH-AS tomato fruit were injected with 50 µl of 0 or 50 mM NH₄Cl with or without 10 mM NAC (ROS scavenger). Alternatively, 50 µl of 5 mM NH₄Cl was applied every 6 h to the fruit mesocarp after a 1-mm-thick, 10-mm-diameter piece of pericarp tissue was removed from the fruit. At 24 or 48 h after treatment,

1-mm-thick strips were taken from the ammonia application site, diagonal to the surface. These strips were stained with 0.2% (wt/vol) Evans blue at room temperature for 5 min and destained by rinsing the strips several times in PBS. The strips were viewed with a Leica fluorescence binocular and the percentage of dead cells was calculated.

All experiments were repeated at least two times, with the results of a single representative experiment shown.

Conductivity measurements.

Wild-type or SIRBOH-AS tomato fruit pericarp disks (10 mm in diameter) were tested. The disks were cut to a 10-mm depth using a scalpel. Half of the disks were pretreated with 10 mM NAC (ROS scavenger) for 30 min and the other half were treated with PBS. Subsequently, all disks were treated with 5 ml of 20 mM NH_4Cl , 1 mM H_2O_2 , or PBS for 30 min with shaking at 50 rpm. For ROS measurements, three treated disks were placed in a 35-mm-diameter well containing 5 ml of PBS and, after 15 min, the surrounding liquid was checked for secreted H_2O_2 by Amplex red (as described above). The surrounding liquid was monitored for up to 2 h for conductivity by the auto-ranging conductivity meter TH-2300 (El-Hamma, Israel) with a Russell Thermo Electron Corp. (Russell/ThermoElectron, Auchtermuchty, Scotland) with a platinum conductivity electrode. Each treatment had four replicates. Experiments were repeated twice, with the results of a single representative experiment shown.

Fruit inoculation with *C. coccodes*.

Fruit inoculation was carried out by direct wound inoculation into the mesocarp and by the hyphal mat infection method described before (Alkan et al. 2008). The direct wound inoculation was carried out by injecting 5,000 spores of *C. coccodes* at a 2-mm depth onto the mesocarp of mature breaker-stage tomato fruit that had been surface sterilized in 0.3% (vol/vol) hypochlorite for 10 min and then rinsed thoroughly with water. For each experiment, 10 tomato fruit were inoculated. Fruit were inoculated on both sides and placed in 100% relative humidity in the dark at 25°C. Disease evaluation of inoculated tomato fruit included measurements of: i) decay diameter (in centimeters) for 6 days postinoculation; ii) ammonium, by ammonium test kit (Merck, Darmstadt, Germany); iii) pH, by Hamilton surface electrode; and iv) confocal microscope analysis after DCF staining (as described above) together with SigmaScan software (as described above). Ammonium, pH, and microscope measurements were taken 48 h postinoculation. Experiments were repeated three times, with the results of a single representative experiment shown.

Detection of ammonium and pH at the infection site.

Infected fruit tissue (1.5 g) was homogenized and then centrifuged at 10,000 rpm for 5 min; 50 μl of the supernatant was diluted to 5 ml and ammonium was measured using the photometric ammonium test kit (Ammonium Test; Merck) to determine the final concentration in the supernatant.

The pH at the center of the infection site was measured with a stinging-type electrode (Eutech, Singapore). Pericarp pH was determined by making an approximately 0.2-mm-deep cut with a scalpel. The pH measurements were made by placing the electrode directly against the exposed tissue. The depth of the pericarp cut was determined based on the localization of the infection.

Statistical analyses.

Linear regression and Pearson correlation were performed using SPSS statistical software to determine the *P* value of the regression, and the R^2 and significance of the correlation.

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LITERATURE CITED

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