Xanthomonas infection transforms the apoplast into an accessible and habitable niche for *Salmonella enterica*

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ABSTRACT

The physiology of plant hosts can be dramatically altered by phytopathogens. *Xanthomonas hortorum* pv. *gardneri* is one such pathogen that creates an aqueous niche within the leaf apoplast by manipulating the plant via the transcription activator-like effector AvrHah1. Simultaneous immigration of *X. gardneri* and *Salmonella enterica* to healthy tomato leaves results in increased survival of *S. enterica* as *Xanthomonas* infection progresses. However, the fate of *S. enterica* following arrival on actively infected leaves has not been examined. We hypothesized that the water-soaking caused by *X. gardneri* could facilitate the ingression of *S. enterica* into the apoplast, and that this environment would be conducive for growth. We found that a water-soaked apoplast, abiotically or *Xanthomonas*-infected, enabled surface *S. enterica* to passively localize to the protective apoplast and facilitated migration of *S. enterica* to distal sites within the aqueous apoplast. *AvrHah1* contributed to the protection and migration of *S. enterica* early in *X. gardneri* infection. *Xanthomonas*-infected apoplasts facilitated prolonged survival and promoted *S. enterica* replication compared to healthy apoplasts. Access to an aqueous apoplast in general protects *S. enterica* from immediate exposure to irradiation whereas, the altered environment created by *Xanthomonas* infection provides growth-conducive conditions for *S. enterica*. Overall, we have characterized an ecological relationship in which host infection converts an unreachable niche to a habitable environment.

Importance: Bacterial spot disease caused by *Xanthomonas* species devastates tomato production worldwide. Salmonellosis outbreaks from consumption of raw produce have been linked to the arrival of *Salmonella enterica* on crop plants in the field via contaminated irrigation water. Considering that *Xanthomonas* is difficult to eradicate, it is highly likely that *S. enterica*

arrives on leaves pre-colonized by *Xanthomonas* with infection underway. Our study demonstrates that infection and disease fundamentally alter the leaf, resulting in redistribution and change in abundance of a phyllosphere bacterial member. These findings contribute to our understanding of how *S. enterica* manages to persist on leaf tissue despite lacking the ability to liberate nutrients from plant cells. More broadly, this study reveals a mechanism by which physiochemical changes to a host environment imposed by a plant pathogen can convert an uninhabitable leaf environment into a hospitable niche for select epiphytic microbes.

INTRODUCTION

Within an environment as dynamic and complex as the phyllosphere, water is one of the most critical factors for microorganism survival (1). The waxy cuticle limits the amount of moisture available to bacteria that colonize leaves. However, surface bacteria have been known to colonize particularly leaky areas of the cuticle such as leaf veins, cell junctions, and the base of glandular trichomes (2–4). Some phytopathogenic bacteria have adapted to the water scarcity issue by creating their own aqueous environment within the apoplast of leaf tissues (5–7). Infection relies on first entering the leaf through stomata and then colonizing the extracellular internal space, or apoplast, within the leaf. *Xanthomonas hortorum* pv. *gardneri* (hereafter, *X. gardneri*), a causal agent of bacterial spot disease of tomato and pepper, is one such plant pathogen that infects leaves. Once *X. gardneri* has actively migrated to the apoplast, it can deploy its type-III secretion system to manipulate the host into leaking cellular constituents into this space (6), creating an aqueous environment. These physiochemical modifications greatly benefit *X. gardneri* but devastate its host. Bacterial spot disease often results in necrotic lesions on leaves and fruit and defoliation (8) which are detrimental to the health of the plant.

Diseased plants are hosts to a diverse community of microbes, which can be influenced by the drastic changes to their environment caused by phytopathogen infection (9–11). Microbes that are non-pathogenic to plants can benefit from disease. For example, enteric human pathogens such as *Salmonella enterica* lack enzymes that would enable the liberation of nutrients directly from plant cells (12, 13) and are typically forced to scavenge scant resources on the leaf surface. On healthy plant tissues, *S. enterica* populations decline over time (14, 15). However, plant pathogens and even insect pests that exist in agricultural fields have been shown to enhance *S. enterica* populations on plant leaves, and such organisms that can promote *S. enterica*

persistence and/or replication have been coined “biomultipliers” (15–19). Bacterial pathogens including *Xanthomonas* spp. and *Pectobacterium carotovorum* have been implicated as biomultipliers for *S. enterica* on leaves (15–17, 20, 21). The mechanisms explaining how these organisms benefit *S. enterica* are not well understood, posing ecological questions with important agricultural and human disease epidemiological implications.

The prevalence of food-borne illness outbreaks linked to consumption of raw produce has climbed in the past few decades (22). Such outbreaks have been linked to pre-harvest contamination of crops in the field via irrigation water (23, 24). The presence of enteric pathogens within internal plant tissues has been a concern of food safety researchers, especially considering efforts to mitigate outbreaks through post-harvest sanitation of produce. Various studies investigated *S. enterica* colonization of the leaf apoplast, and the extent to which *S. enterica* can colonize this protective niche varies based on host and method. The flagella-dependent localization of *S. enterica* to internal tissue in response to light has been demonstrated via confocal microscopy of lettuce leaf pieces submerged in *S. enterica* inoculum (25). However, tomato leaves have been shown to be impermeable to *S. enterica* internalization when compared with lettuce leaves under comparable conditions (26, 27). Whether or not phytopathogen infection could enhance the permeability of leaves to enteric pathogens was not addressed by these studies.

Xanthomonas infection promotes *S. enterica* survival when both the plant and human pathogen arrive on healthy tomato leaves simultaneously (15, 16, 21), however, the fate of *S. enterica* on leaves actively infected with *Xanthomonas* spp. has not been described. *X. gardneri* infection can facilitate the migration of surface *X. gardneri* cells to distal tissue via absorption of *X. gardneri* suspensions into needle-damaged tissue (6), suggesting that water-soaking may

promote the ingression of bacteria into the apoplast of leaves when external water is present. Water-soaking has been demonstrated to be enhanced by *X. gardneri* type-III effector AvrHah1 (6, 28). *AvrHah1* encodes a transcription activator-like effector that binds host DNA and upregulates the activity of a host pectate lyase (6), forcing the host to degrade the pectin "cement" that joins adjacent plant cells, resulting in an aqueous apoplast (29). Data from our lab has shown that *avrHah1* expression by *X. gardneri* is necessary for enhancing the persistence of *S. enterica* on tomato leaves when co-inoculated with *X. gardneri* (21). Based on these observations, we hypothesized that *avrHah1*-dependent water-soaking and aqueous apoplasts in general could serve as a mechanism for epiphytic *S. enterica* to enter protected sites of leaves.

The goal of our study was to examine the fate of epiphytic *S. enterica* following arrival on the surface of infected leaves. We examined whether an abiotically water-soaked or *Xanthomonas*-infected apoplast could facilitate the relocation of *S. enterica* to protected niches, particularly in the absence of leaf wounding. *S. enterica* populations were considered protected if they survived UV irradiation applied to leaves. We also identified the roles of *avrHah1*, *Xanthomonas* infection progress, and *S. enterica* motility on *S. enterica* entry into the apoplast and subsequent migration beyond the arrival site. Lastly, we investigated whether the aqueous niche created by *Xanthomonas* species enhanced the prolonged survival of *S. enterica* within the apoplast to determine whether factors aside from water availability contribute to *S. enterica* survival in the apoplast. Identifying host and microbial factors that influence the redistribution and survival of a model epiphyte will broaden our understanding of microbe-facilitated adaptations by plant-associated bacteria.

METHODS

Bacterial Strains, Media, and Culture Conditions

Bacterial strains used in this study are described in Table 1. *Xanthomonas* cultures were prepared by inoculating nutrient broth (NB) with frozen cells and incubating at 28°C with shaking at 200 rpm for two days. *S. enterica* cultures were prepared similarly but grown in lysogeny broth (LB) and incubated overnight. Cultures were prepared to desired cell concentrations via dilution in sterile water as described below.

Plant Infiltration

For all experiments, *Solanum lycopersicum* (tomato) cultivar Moneymaker seedlings were cultivated in growth chambers with a 16 hr photoperiod at 24°C. Inoculated plants were maintained in plastic bins at a relative humidity (RH) > 90% once experiments were in progress. For *Xanthomonas* infiltration experiments, 5-week-old tomato plants were infiltrated with *Xanthomonas* and inoculated with droplets of *S. enterica* as described previously (6) with modifications (Figure 1). *Xanthomonas* cultures were prepared to an optical density at 600 nm (OD_{600}) = 0.3, 0.25, or 0.2 (10^8 CFU/mL) for *X. gardneri* WT, *X. gardneri avrHah1*$^{\Delta DBD}$, and *X. vesicatoria*, respectively, and infiltrated into two leaflets per plant on the third true leaf via needleless syringe. Infiltration medium was gently forced into the leaf tissue through the abaxial leaf surface until the infiltration zone was approximately 2 cm long, and then the infiltration zone was outlined with permanent marker. When applicable, a subset of plants was infiltrated with sterile water as a control. For *S. enterica* infiltration, similar methods were applied, although the culture used for infiltration was prepared to a concentration of 10^7 CFU/mL and infiltrations were performed in a biosafety cabinet. Following infiltration, plants were then incubated in bins

with lids on for 1-2 days prior to subsequent *S. enterica* application. For some experiments, infiltration with sterile water was delayed for a subset of plants until immediately prior to *S. enterica* inoculation to simulate arrival of *S. enterica* on healthy water-soaked tissue. These plants were incubated alongside the already infiltrated plants prior to their infiltration such that environmental conditions would be consistent among all plants within an experiment.

Droplet Inoculation

Infiltrated leaflets were marked again with permanent marker on the *S. enterica* arrival day to designate the site of *S. enterica* droplet placement on the infiltration zone. *S. enterica* cultures were prepared to an OD_{600}= 0.1 (10^8 CFU/mL) and 15 μL droplets of *S. enterica* were applied to the adaxial surface within the part of the infiltration zone closest to the leaf margin. Note that droplets were applied such that they did not overlap with any minor tissue damage caused by the needleless syringe infiltration from the abaxial surface. Plants inoculated with *S. enterica* remained inside plastic bins, and the lids were kept off (RH ~70%) until droplets were no longer visible (i.e., absorbed or evaporated). Visual observations of droplet absorption (complete, partial, or none) were recorded at 2.5 hours after droplet application. When specified, a micropipette was used for removing each remaining droplet and measuring their volume. Leaf tissue was then sampled destructively at the indicated time post-*S. enterica* arrival to quantify bacterial populations.

Bacterial Population Sampling

Whole leaflets were removed at the petiolule and when specified, treated with 254 nm UV radiation (Stratalinker® UV Crosslinker 1800) at 600,000 μJ/cm^2 with the adaxial leaf surface

facing upwards towards the bulb. Leaflets irradiated with UV were chosen at random between the two leaflets sampled per plant and the second leaflet was left non-irradiated as a control. Bacterial populations were measured as described previously (18). Unless noted otherwise, all inoculated leaflets were sampled regardless of observations made at the droplet absorption stage. Briefly, leaf tissue was excised using a sterile 1 cm diameter tissue corer and homogenized in 500 μL of sterile water using a hand Dremel. For *S. enterica* droplet-inoculated tissue, the site of *S. enterica* arrival was sampled by centering the tissue corer between the marker guidelines. When indicated, tissue was also sampled distal from the arrival site by excising a second tissue core 1-2 mm away from the first leaf disk overlapping the infiltration zone. Leaf tissue homogenate was dilution-plated on LB agar amended with 50 μg/mL kanamycin for enumeration of *S. enterica* populations. Colonies were counted after 1 day of incubation at 37°C.

Statistical Analyses

R Studio version 2021.09.1 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria [http://www.R-project.org]) was utilized for all statistical analyses and plot generation. Each experiment was repeated three times, and each boxplot represents the aggregation of three independent experiments found to be statistically similar ($P > 0.05$), unless noted otherwise in the figure legend. Within each experiment, the number of technical replicates N = 4-8 leaflets. For comparing *S. enterica* populations among treatment groups at the arrival tissue site, the ANOVA and post-hoc Tukey tests were used. When two leaflets were inoculated per plant, the data were fit to a linear mixed effects model using the lme4 package in which individual plants were modeled as a random effect. For comparing *S. enterica* populations among treatment groups at the distal tissue site, the non-parametric Kruskal-Wallis and post-hoc

Wilcox tests were used. In addition, the proportion of samples with detectable bacterial populations at the distal tissue site were compared using the Fisher's Exact Test to compare the incidence of *S. enterica* colonization of distal tissue. When applicable, the limit of detection minus one (i.e., 49 CFU/leaf disk) was substituted for non-detects. The significance threshold for all comparisons was $P < 0.05$.

RESULTS

Abiotically water-soaked apoplasts enable *S. enterica* to migrate via stomata to a UV-protected niche. To characterize the role of water in the apoplast in the migration of *S. enterica* to a protected niche and migration to sites distal to the arrival site, we developed a method involving infiltration of intact tomato leaflets, application of droplets of *S. enterica* cell suspensions, and measurement of UV-protected *S. enterica* populations (Fig. 1). Our methods were inspired by work previously published by Schwartz et al. (6), with a few key differences that included applying bacterial droplets to intact (not detached) leaflets onto the adaxial surface, refraining from wounding the leaves at the droplet application site, and utilizing UV irradiation to enrich for apoplastic *S. enterica* populations. Additionally, we manipulated the timing of *S. enterica* arrival on water-infiltrated leaves to observe how the presence of water in the apoplast influences the relocation of *S. enterica*.

Infiltration of water into the apoplast simulated abiotic water-soaking that may occur during periods of high precipitation (30). Under the tested conditions (~70% RH), water infiltration created an obvious darkening or "wet" appearance to the tissue that lasted for a few hours (data not shown). *S. enterica* droplets were applied to two treatments: leaves with a (i) "healthy/dry" apoplast where the tissue had become macroscopically dry at time of *S. enterica*

application at 2 day post-infiltration (DPI) or (ii) “healthy/wet” apoplast where tissue was still visibly dark and wet in appearance when *S. enterica* was applied within 10 minutes of water infiltration (0 DPI). A third treatment was included in which we infiltrated *S. enterica* directly into the apoplast to determine the relative amount of UV protection provided to apoplastic *S. enterica* cells.

Within a few hours of *S. enterica* arrival on the leaf, we observed the disappearance of the droplets specifically into healthy/wet tissue in the absence of any applied epidermal wounding, suggesting that the *S. enterica* suspension had been absorbed through stomata. Leaflets were observed for a duration of 2.5 hours post-application of *S. enterica*, and absorption of the entire 15 μL suspension could be observed within a minimum of 30 minutes. The success rate for complete absorption of the droplet for healthy/wet tissue varied between 50-83% among three experimental replicates, although droplets that failed to be completely absorbed within the 2.5 timeframe had visibly reduced in size, suggesting that they were partially absorbed. By contrast, healthy/dry leaflets had an absorption rate of 0%. Beyond the observation period, after several hours had passed, all droplets disappeared either by absorption, evaporation, or both. *S. enterica* populations were enumerated at 1 day post-arrival, and among the three treatments, UV irradiation resulted in the lowest *S. enterica* populations when *S. enterica* was applied to leaflets with a healthy/dry apoplast (Fig. 2). These surface-limited populations were reduced ~2.5 logs by UV irradiation. Arrival on healthy/wet tissue and direct infiltration into the apoplast resulted in equivalent UV-protected populations and relatively small population reductions of ~0.9 and ~0.7 logs, respectively, in response to UV irradiation (Fig. 2). The increased UV protection conferred to *S. enterica* when applied to healthy water-soaked tissue suggests that an aqueous apoplast provides a gateway for surface bacteria to move inside the leaf.

Next, we investigated whether movement of surface bacteria to the apoplast was limited to the immediate substomatal chamber or extended beyond. We observed distinct differences in the ability of *S. enterica* to migrate from the arrival site to tissue non-overlapping the droplet application site depending on the physical state of the apoplast. A healthy/dry apoplast did not readily facilitate lateral migration of *S. enterica* to areas within the apoplast distal from the arrival site whereas, a healthy/wet apoplast enabled recovery of significantly higher *S. enterica* populations at distal sites (Fig. 2). Distal sites within the apoplast provided *S. enterica* with a protected niche if the apoplast was an aqueous environment at time of *S. enterica* arrival, as the population reduction in response to UV irradiation was only ~0.5 logs (Fig. 2). These findings support the conclusion that an aqueous apoplast permits surface bacteria to migrate beyond the initial arrival site.

***Xanthomonas* water-soaking promotes *S. enterica* entry to the apoplast and facilitates migration to distal tissue.** *X. gardneri* is known to cause water-soaking (6, 28), and thus we examined whether *X. gardneri* infection could confer protection to *S. enterica* against UV irradiation and facilitate its migration to infected sites distal from the initial site similarly to a healthy/wet apoplast. We found that *X. gardneri*-infected tissue enabled complete absorption of *S. enterica* droplets into the leaves at 1 DPI (Fig. 3a) and 2 DPI (images not shown) within 0.5-2.5 hours. To assess the protection of absorbed *S. enterica* cells against UV, we infiltrated an excess number of plants with *X. gardneri*, and the first 50% of plants that completely absorbed the droplets were specifically selected for *S. enterica* population sampling at 1 day post-*S. enterica* arrival. *S. enterica* cells were found to be more protected against UV irradiation following droplet absorption into *X. gardneri*-infected tissue at 2 DPI compared to *S. enterica* cells that were limited to the surface of healthy/dry tissue (Fig. 3b). Like a healthy/wet apoplast,

a *X. gardneri*-infected apoplast facilitated robust colonization of *S. enterica* cells to sites in the apoplast beyond the initial arrival site, where *S. enterica* was protected from UV irradiation (Fig. 3b).

***AvrHah1* facilitates *S. enterica* localization to the apoplast and lateral migration early in infection**. Knowing that an aqueous apoplast facilitates the migration of *S. enterica* to a protected niche within a day of arrival, we examined whether protection against UV irradiation exists as early as 2.5 hours post-arrival, a time point at which *S. enterica* droplet suspensions were often completely absorbed. Also, we compared the fate of *S. enterica* following arrival on tissue infected by one of three *Xanthomonas* strains including *X. gardneri* WT, *X. gardneri avrHah1*$^{\Delta DBD}$, and *X. vesicatoria*. *X. vesicatoria* is a pathogen within the bacterial spot disease complex that lacks *avrHah1* and has been shown in co-inoculation experiments with *S. enterica* to not promote *S. enterica* growth on tomato leaves, in contrast to *X. gardneri*, which does facilitate *S. enterica* replication (15, 21).

The duration of infection with *Xanthomonas spp.* was found to affect visible water-soaking, absorption of the *S. enterica* suspension, and *S. enterica* protection from UV irradiation (Fig. 4). Tissue infected with *Xanthomonas* spp. was generally water-soaked at 1 and 2 DPI, except for leaflets infected with the *X. gardneri avrHah1*$^{\Delta DBD}$ strain at 1 DPI (Fig. 4a). Leaflets infected with *X. vesicatoria* became water-soaked at a similar rate as *X. gardneri* WT-infected leaflets despite lacking the *avrHah1* gene. As for effects on *S. enterica*, tissue that was macroscopically dry at *S. enterica* arrival, which included the healthy/dry and *X. gardneri avrHah1*$^{\Delta DBD}$ 1 DPI treatments, did not absorb the *S. enterica* suspension, resulting in the highest droplet recovery volumes at 2.5 hours post *S. enterica* droplet application (Fig. 4b). In contrast to dry tissue, the recovered droplet volumes were lower for water-soaked tissue with healthy/wet,

X. gardneri WT-infected, or *X. vesicatoria*-infected leaves, and these tissues often absorbed the *S. enterica* suspension completely (Fig. 4b). Congruently, *S. enterica* arrival on water-soaked tissues resulted in the greatest protection, as evident by smaller reductions in population in response to UV (Fig. 4c). Overall, infection progress had a significant effect on *S. enterica* protection in our model.

In addition to facilitating initial entry of *S. enterica* into the substomatal chamber, *avrHah1* was also found to contribute to the ability of *S. enterica* to migrate to distal tissue beyond the site of arrival early in infection (Fig. 5). Leaves infiltrated with *X. gardneri* *avrHah1*$^{\Delta DBD}$, which generally had a dry apoplast at 1 DPI (Fig. 4a) and did not result in visible absorption of the *S. enterica* suspension (Fig. 4b), exhibited reduced migration of *S. enterica* to distal tissue compared to leaves infiltrated with *X. gardneri* WT (Fig. 5). However, by 2 DPI, when the tissue was visibly water-soaked (Fig. 4a), *X. gardneri avrHah1*$^{\Delta DBD}$-infected tissue did facilitate colonization of distal tissue by *S. enterica* (Fig. 5). These findings demonstrate that the role of *avrHah1* in altering the apoplast into an aqueous environment occurs early in the disease progress, and these changes facilitate surface bacteria entry into the apoplast and subsequent lateral migration to other water-soaked areas. We also note that additional bacterial factors besides *avrHah1* contribute to water-soaking later in disease progression.

Active motility of *S. enterica* increases colonization of tissue beyond the arrival site as *X. gardneri* infection progresses. To test the hypothesis that active *S. enterica* motility contributes to migration away from the surface, bacterial populations for a *S. enterica* Δ*motA* mutant with non-functional flagella were compared to a wild-type *S. enterica* strain. At 2.5 hours post-*S. enterica* arrival, the Δ*motA* mutant colonized UV-protected sites of water-soaked leaves equivalently to the wild-type *S. enterica* strain (Supp. Fig. 1), indicating that motility does not

affect *S. enterica's* ability to enter the aqueous apoplast. We also compared *S. enterica* WT and Δ*motA* populations at tissue distal from the arrival site at 1 day post-*S. enterica* arrival. Motility did not affect *S. enterica* colonization of distal tissue infected with *X. gardneri* when *S. enterica* arrived at 1 DPI, however, at 2 DPI, *S. enterica* populations at distal sites were enhanced by motility (Fig. 5). Deletion of *motA* did not eliminate *S. enterica* colonization of distal tissue nor affect the proportion of distal sites with detectable *S. enterica* populations, indicating that motility was useful but not required for lateral migration of *S. enterica*. Overall, motility was not required for entry into the apoplast or migration beyond the initial arrival site, supporting a model of passive, rather than active, *S. enterica* relocation within water-soaked tissue.

Prolonged survival of *S. enterica* in a protected niche is increased by *Xanthomonas* infection. Having established that aqueous apoplasts result in similar migration of *S. enterica* to UV-protected niches regardless of whether the apoplast is abiotically water-soaked or water-soaked via *Xanthomonas* infection, we then tested the hypothesis that the apoplast of *Xanthomonas* infected leaves would support higher *S. enterica* populations than healthy leaves following *S. enterica* application at 1 DPI. We found that *S. enterica* arrival on *X. gardneri* WT or *X. vesicatoria*-infected tissue resulted in the highest UV-protected *S. enterica* populations at three days post-arrival (Fig. 6). A healthy/wet apoplast facilitated equal populations of *S. enterica* to the substomatal chamber compared to *X. gardneri* WT and *X. vesicatoria* infected tissue (Fig. 4C; 2.5 hours post-*S. enterica* arrival; HPA) yet supported relatively lower *S. enterica* populations at the arrival and distal sites (Fig. 6; 3 DPA). *X. gardneri avrHah1*$^{\Delta DBD}$-infected plants (1 DPI) did not permit droplet absorption (Table S1), yielded intermediate UV-protected *S. enterica* populations at the arrival and distal sites (Fig. 6), and resulted in the most variable *S. enterica* populations by 3 DPA (Fig. 6). Healthy/dry tissue also did not absorb the

droplets (Table S1) and supported the lowest *S. enterica* populations at the arrival and distal sites (Fig 6). The higher apoplastic *S. enterica* populations observed in infected tissue substantiates the conclusion that prolonged survival in the apoplast by surface bacteria is supported by an aqueous environment resulting from phytobacterial infection, which is distinct from a healthy, watery apoplast.

DISCUSSION

In this study, we determined the fate of *S. enterica* following arrival on *Xanthomonas*-infected tissue, specifically regarding ingression into the apoplast and migration to distal sites. We hypothesized that the modifications to the host caused by *Xanthomonas* infection could facilitate ingression of *S. enterica* into the apoplast, where it could migrate to distal sites and thrive. *S. enterica* was applied to leaves as a droplet to model splash dispersal of bacteria in the field, which can occur during irrigation or precipitation events. Various host and microbial factors including the presence of water in the apoplast, flagellar function by *S. enterica,* and *avrHah1* expression by *X. gardneri* were manipulated to understand how alterations to the infection court, the apoplast, impact plant-associated surface bacteria.

Colonization of protected niches such as the apoplast is essential to the lifestyle of various plant-associated bacteria. To determine whether *S. enterica* could localize to protected niches of leaves, UV irradiation at 254 nm, which is an FDA-approved method for decontamination of food surfaces (31) and has been applied in previous plant bacterial endophyte studies (32, 33), was utilized as a surface sterilant. The use of UV irradiation additionally models the UV exposure that plant-associated microbes are routinely exposed to, though it’s worth noting UV wavelengths less than 300 nm are typically absorbed by our atmosphere and do not reach Earth (34). Initially, we tested whether *S. enterica* cells that were infiltrated directly into

the apoplast of leaves would be protected from UV irradiation applied to the adaxial surface, and we hypothesized that the palisade cells, upper epidermal cells, and cuticle would occlude UV penetration to the apoplastic *S. enterica*. We determined that *S. enterica* cells localized to the apoplast were more protected from UV compared to *S. enterica* on the surface, although UV irradiation reduced the apoplastic *S. enterica* populations a small but statistically significant amount (Fig. 2). Leaves must permit light to penetrate leaf tissue for photosynthesis and act as "light traps (35)," which may explain why a subset of apoplastic *S. enterica* cells were vulnerable to UV. The duration of UV exposure influenced the magnitude of *S. enterica* population reduction (data not shown), and the exposure level of 0.6 J/cm^2 selected for these experiments resulted in >2.0 log reduction of surface *S. enterica* populations with minimal effects on the apoplastic populations. As for the surface-localized *S. enterica* cells, the small subpopulations that survived UV irradiation likely resisted sterilization by being canopied by leaf surface structures, such as trichomes, or wedged within the grooves between adjacent cells (36–38).

S. enterica successfully colonized UV-protected niches of abiotically water-soaked or *Xanthomonas*-infected tissue. We rationalized that *S. enterica* had accessed the leaf apoplast based on a few lines of evidence. First, the extent to which *S. enterica* was protected from UV irradiation following arrival on abiotically water-soaked or *X. gardneri*-infected apoplast was similar to cells infiltrated directly into the apoplast (Fig. 2). Second, *S. enterica* droplets were absorbed by abiotically water-soaked or infected tissue, as evident by rapid disappearance of the droplets in as little as 30 minutes. Healthy tissue is typically impermeable to spontaneous absorption of surface water because the surface tension of water is greater than the critical surface tension of the leaf surface (39). However, the relatively rapid disappearance of droplets

into water-soaked leaves indicated that the wettability of the leaf surface had changed. Considering that the leaf tissue was deliberately not wounded, open stomata likely served as entry points for external water and suspended *S. enterica* cells to enter the apoplast. We theorize that an aqueous apoplast enabled a watery film to extend upwards through the stomatal pores, providing a continuous "watery highway" that could facilitate movement of *S. enterica* into the substomatal space. Lastly, *S. enterica* was consistently isolated at high levels from abiotically water-soaked (Fig. 2) and infected (Fig. 3) tissue ~1 cm distal from the arrival site when the apoplast was water-soaked at *S. enterica* arrival, but not when it was dry. Schwartz et al. found that *X. gardneri*-infected leaves facilitated migration of surface *X. gardneri* cells to the apoplast (6), and here we have confirmed that other bacteria besides the phytopathogen *X. gardneri* could be transported by water-soaking. To verify that *S. enterica* colonization of distal tissue was not driven by migration across the surface but by migration through the apoplast, the adaxial surface of leaf tissue disks were pressed to agar plates following UV exposure. *S. enterica* was rarely isolated from the surface of leaves distal to the arrival site with a healthy/dry apoplasts (data not shown). Overall, *S. enterica* was found to relocate from the surface of leaves into the substomatal chamber and beyond. These observations posed the question of whether *S. enterica* motility is necessary for the redistribution of cells following *S. enterica* arrival.

Flagellar motility is key to survival on the leaf surface for recent immigrants (40), but we found that the relocation of *S. enterica* from the leaf surface to the aqueous apoplast can occur independent of functional flagella (Fig. 5), thus favoring a model of passive *S. enterica* relocation in the presence of a water-soaked apoplast. However, active motility was found to provide *S. enterica* with an advantage in colonizing sites beyond the initial arrive site at 2 days post-*X. gardneri* infection, but not earlier (Fig. 5). *In planta* microscopy of *Pseudomonas*

syringae colonization has revealed that distinct bacterial subpopulations exist within infected tissue, suggesting that the apoplast is a heterogeneous environment (41). Later in *Xanthomonas* infection when more nutrients may be available in the apoplast, motile *S. enterica* cells may employ chemotaxis to colonize nutritional micro-oases within the apoplast. Therefore, as the *Xanthomonas* infection court dynamically changes over time, the importance of specific traits possessed by plant-associated microbes also changes.

In addition to determining how *S. enterica* redistribution is affected by one of its own genetic traits, we also examined the role of *X. gardneri* type-III effector AvrHah1 to identify mechanisms by which phytopathogen infection influences the fate of *S. enterica. AvrHah1* contributed to *S. enterica's* ability to access the protective apoplast (Fig. 4) and migrate to distal tissue in the early stage of an ongoing *X. gardneri* infection (Fig. 5). AvrHah1 is the only *Xanthomonas* effector that has been directly linked to the water-soaking phenotype, despite water-soaking being a characteristic symptom of bacterial spot disease. Other Xanthomonads outside of the bacterial spot complex that infect diverse hosts, including leafy greens, cause water-soaking (42, 43), yet no other water-soaking effectors have been identified within the *Xanthomonas* genus. Among the various *Xanthomonas* species that cause bacterial spot disease, which includes *X. vesicatoria, X. euvesicatoria, and X. perforans, avrHah1* was originally specific to *X. gardneri* (44, 45). Recently, however, *X. perforans* strains carrying *avrHah1* have been identified in the southeastern United States (46). *X. vesicatoria,* which lacks *avrHah1,* induced visible water-soaking of tomato leaves as rapidly as *X. gardneri* and promoted protection and survival of *S. enterica* equal to or better than *X. gardneri.* These results contrast with previous studies documenting that *X. vesicatoria* infection does not promote *S. enterica* persistence on tomato leaf tissue following co-arrival of *X. vesicatoria* and *S. enterica* on healthy

plants (15, 21). However, a key difference between those studies and the study presented here is the physiological state of the leaf when *S. enterica* arrives: healthy leaves versus infected leaves. Infected tissue can permit *S. enterica* to enter the apoplast, and therefore, the benefits conferred to *S. enterica* by *X. vesicatoria* infection may be conditional on *S. enterica* entry. Overall, the ability of *avrHah1*-lacking *Xanthomonas* strains to facilitate water-soaking mediated ingression of *S. enterica* into the apoplast and the observed delay, not elimination, of water-soaking in *X. gardneri avrHah1*$^{\Delta DBD}$ suggest that other factors within the *Xanthomonas* genome independent of *avrHah1* contribute to creating an aqueous niche within the tomato leaf apoplast. Identifying these bacterial factors in future studies would improve our understanding of phytopathogen-mediated water-soaking.

Leaf surfaces are harsh environments that require plant-associated bacteria to flexibly adapt to extreme and dynamic conditions, including desiccation, heavy rainfall, UV irradiation, and sporadic nutrient availability (47–50). Many phytopathogenic bacteria have evolved to infect the apoplast of leaves where they can replicate to high population levels whilst exploiting host resources (51). The apoplast can be protective and provide bacteria with increased nutrient availability, however, this environment is exclusive to microbes that can successfully migrate there and tolerate the host immune response. Tomato leaves are relatively restrictive to *S. enterica* ingression (26, 27), yet we found that *S. enterica* can access an apoplast that is water-soaked either by *Xanthomonas* infection or abiotically. Additionally, comparison of *S. enterica* populations between experiments sampled at 2.5 hours post-arrival and 3 days post-arrival indicates that *S. enterica* populations are stable within a healthy apoplast and increase within an infected one. An infected tomato apoplast relieves suppression of *S. enterica* replication, suggesting that *Xanthomonas* infection aids in overcoming the host immune response and/or

liberates nutrients inaccessible to *S. enterica*. *S. enterica* is known to elicit the plant immune response (52) upon recognition of microbe-associated molecular markers such as flg22 (53, 54), although some evidence suggests that the host response provoked by *S. enterica* is relatively weak (55, 56). If modulation of the immune response by *Xanthomonas* does enhance *S. enterica* fitness in the apoplast, it is likely that this is not the only mechanism involved. The replication of *S. enterica* in an infected apoplast also suggests that nutrients have been made bioavailable by *Xanthomonas* infection. *Xanthomonas spp.* presumably liberate nutrients from leaf tissue, as supported by their own replication to populations above 10^8 CFU/cm^2 (21) and increased electrolyte leakage of tomato leaf tissue (15). One hypothesis is that an infected apoplast is enriched in carbohydrates, and *S. enterica* metabolizes these nutrients that it can't access on its own. *S. enterica* can utilize many different sugars as sole carbon sources (57), and although only 10% of *Salmonella* species can metabolize sucrose (58), one of the most prevalent carbohydrates available in plant tissues (51), other sugars such as glucose and fructose (59, 60) could be available to *S. enterica*. Future studies will be informative in describing *avrHah1*-independent and -dependent mechanisms by which *Xanthomonas* releases host nutrients and identifying resources scavenged by *S. enterica* within infected leaves.

Overall, we have identified a common phytobacterial disease symptom of leaf pathogens, water-soaking, as a mechanism by which *S. enterica* can be passively relocated to the apoplast of leaves, resulting in access to protected niches including the substomatal chamber directly below the initial arrival site and distal sites of the apoplast. Our results show that *avrHah1* expression by *X. gardneri* influences the fate of *S. enterica*, and considering evidence that *avrHah1* is being transferred between *Xanthomonas* species, gene flow within a plant pathogen complex may have dire effects on the persistence of enteric human pathogens in crop plants that eventually will be

consumed raw. Altogether, our findings illustrate a model for how enteric bacterial human pathogens are dispersed in agricultural fields via contaminated irrigation water and subsets of the bacterial population can relocate from the hostile leaf surface to a habitable niche within the apoplast (Fig. 7). As phytopathogen populations evolve and respond to global environmental changes, understanding their effects on the redistribution and survival of other plant-associated microbes will be critical.

REFERENCES

1. Beattie GA. 2011. Water Relations in the Interaction of Foliar Bacterial Pathogens with Plants. Annu Rev Phytopathol 49:533–555.
2. Schonherr J. 2006. Characterization of aqueous pores in plant cuticles and permeation of ionic solutes. J Exp Bot 57:2471–2491.
3. Monier J, Lindow SE. 2004. Frequency , Size , and Localization of Bacterial Aggregates on Bean Leaf Surfaces. Appl Environ Microbiol 70:346–355.
4. Barak JD, Kramer LC, Hao L-Y. 2011. Colonization of Tomato Plants by Salmonella enterica Is Cultivar Dependent, and Type 1 Trichomes Are Preferred Colonization Sites. Appl Environ Microbiol 77:498–504.
5. Xin XF, Nomura K, Aung K, Velásquez AC, Yao J, Boutrot F, Chang JH, Zipfel C, He SY. 2016. Bacteria establish an aqueous living space in plants crucial for virulence. Nature 539:524–529.
6. Schwartz AR, Morbitzer R, Lahaye T, Staskawicz BJ. 2017. TALE-induced bHLH transcription factors that activate a pectate lyase contribute to water soaking in bacterial spot of tomato. Proc Natl Acad Sci 114:E897–E903.
7. Roper MC, Burbank LP, Williams K, Viravathana P, Tien H-Y, von Bodman S. 2015. A Large Repetitive RTX-Like Protein Mediates Water-Soaked Lesion Development, Leakage of Plant Cell Content and Host Colonization in the Pantoea stewartii subsp. stewartii Pathosystem. Mol Plant-Microbe Interact 28:1374–1382.
8. Goode MJ, Sasser M. 1980. Prevention - the Key to Controlling Bacterial Spot and Bacterial Speck of Tomato. Plant Dis 64:831–834.
9. Ginnan NA, Dang T, Bodaghi S, Ruegger PM, McCollum G, England G, Vidalakis G,

Borneman J, Rolshausen PE, Caroline Roper M. 2020. Disease-induced microbial shifts in citrus indicate microbiome-derived responses to huanglongbing across the disease severity spectrum. Phytobiomes J 4:375–387.

10. Gao M, Xiong C, Gao C, Tsui CKM, Wang MM, Zhou X, Zhang AM, Cai L. 2021. Disease-induced changes in plant microbiome assembly and functional adaptation. Microbiome 9:1–18.

11. Yang F, Zhang J, Zhang H, Ji G, Zeng L, Li Y, Yu C, Fernando WGD, Chen W. 2020. Bacterial Blight Induced Shifts in Endophytic Microbiome of Rice Leaves and the Enrichment of Specific Bacterial Strains With Pathogen Antagonism. Front Plant Sci 11:1–14.

12. Teplitski M, Barak JD, Schneider KR. 2009. Human enteric pathogens in produce : un-answered ecological questions with direct implications for food safety. Curr Opin Biotechnol 20:166–171.

13. Abbott DW, Boraston AB. 2008. Structural Biology of Pectin Degradation by Enterobacteriaceae. Microbiol Mol Biol Rev 72:301–316.

14. Islam M, Morgan J, Doyle MP, Phatak SC, Millner P, Jiang X. 2004. Fate of Salmonella enterica Serovar Typhimurium on Carrots and Radishes Grown in Fields Treated with Contaminated Manure Composts or Irrigation Water. Appl Environ Microbiol 70:2497–2502.

15. Potnis N, Colee J, Jones JB, Barak JD. 2015. Plant Pathogen-Induced Water-Soaking Promotes Salmonella enterica Growth on Tomato Leaves. Appl Environ Microbiol 81:8126–8134.

16. Potnis N, Soto-Arias JP, Cowles KN, van Bruggen AHC, Jones JB, Barak JD. 2014.

Xanthomonas perforans Colonization Influences Salmonella enterica in the Tomato Phyllosphere. Appl Environ Microbiol 80:3173–3180.

17. Kwan G, Charkowski AO, Barak JD. 2013. Salmonella enterica Suppresses Pectobacterium carotovorum subsp. carotovorum Population and Soft Rot Progression by Acidifying the Microaerophilic Environment. MBio 4:1–9.

18. Cowles KN, Groves RL, Barak JD. 2018. Leafhopper-Induced Activation of the Jasmonic Acid Response Benefits Salmonella enterica in a Flagellum-Dependent Manner. Front Microbiol 9:1–15.

19. Harrod VL, Groves RL, Maurice MA, Barak JD. 2021. Frankliniella occidentalis facilitate Salmonella enterica survival in the phyllosphere. PLoS One 16:1–16.

20. Wells JM, Butterfield JE. 1997. Salmonella contamination associated with bacterial soft rot of fresh fruits and vegetables in the marketplace. Plant Dis 81:867–872.

21. Cowles KN, Block AK, Barak JD. Xanthomonas hortorum pv. gardneri TAL effector AvrHah1 is neessary and sufficient for increased persistence of Salmonella enterica on tomato leaves. Res Sq https://doi.org/10.21203/rs.3.rs-1229600/v1.

22. Hoagland L, Ximenes E, Ku S, Ladisch M. 2018. Foodborne pathogens in horticultural production systems : Ecology and mitigation. Sci Hortic (Amsterdam) 236:192–206.

23. Centers for Disease Control and Prevention (CDC). 2008. Outbreak of Salmonella Serotype Saintpaul Infections Associated with Multiple Raw Produce Items --- United States, 2008. Morb Mortal Wkly Rep 57:929–934.

24. Centers for Disease Control and Prevention (CDC). 2018. Multistate Outbreak of E. coli O157:H7 Infections Linked to Romaine Lettuce (Final Update).

25. Kroupitski Y, Golberg D, Belausov E, Pinto R, Swartzberg D, Granot D, Sela S. 2009.

Internalization of Salmonella enterica in Leaves Is Induced by Light and Involves Chemotaxis and Penetration through Open Stomata □ †. Appl Environ Microbiol 75:6076–6086.

26. Golberg D, Kroupitski Y, Belausov E, Pinto R, Sela S. 2011. Salmonella Typhimurium internalization is variable in leafy vegetables and fresh herbs. Int J Food Microbiol 145:250–257.

27. Chahar M, Kroupitski Y, Gollop R, Belausov E, Melotto M, Sela-Saldinger S. 2021. Determination of Salmonella enterica Leaf Internalization Varies Substantially According to the Method and Conditions Used to Assess Bacterial Localization. Front Microbiol 12:1–12.

28. Schornack S, Minsavage G V, Stall RE, Jones JB, Lahaye T, Jones JB. 2006. Characterization of AvrHah1 , a novel AvrBs3-like effector from Xanthomonas gardneri with virulence and avirulence activity. New Phytol 179:546–556.

29. Collmer A, Keen NK. 1986. The Role of Pectic Enzymes in Plant Pathogenesis. Annu Rev Phytopathol 24:383–409.

30. Clayton E. 1936. Water-Soaking of leaves in relation to development of the wildfire disease of tobacco. J Agric Res 52:239–269.

31. Sommers CH, Sites JE, Musgrove M. 2010. Ultraviolet light (254 nm) inactivation of pathogens on foods and stainless steel surfaces. J Food Saf 30:470–479.

32. Wilson M, Hirano SS, Lindow SE. 1999. Location and survival of leaf-associated bacteria in relation to pathogenicity and potential for growth within the leaf. Appl Environ Microbiol 65:1435–1443.

33. Kennedy BW, Ercolani GL. 1978. Soybean Primary Leaves as a Site for Epiphytic

Multiplication of Pseudomonas glycinea. Ecol Epidemiol 68:1196–1201.

34. Döhring T. 2017. Critical discussion on the UV absorption properties of Earth's atmosphere.Proceedings of SPIE 10453.

35. Vogelmann TC, Bjorn LO. 1986. Plants as light traps. Physiol Plantarium 68:704–708.

36. Doan HK, Ngassam VN, Gilmore SF, Tecon R, Parikh AN, Leveau JHJ. 2020. Topography-driven shape, spread, and retention of leaf surface water impacts microbial dispersion and activity in the phyllosphere. Phytobiomes J 4:268–280.

37. Fino VR, Kniel KE. 2008. UV light inactivation of hepatitis A virus, aichi virus, and feline calicivirus on strawberries, green onions, and lettuce. J Food Prot 71:908–913.

38. Huang R, Chen H. 2019. Comparison of Water-Assisted Decontamination Systems of Pulsed Light and Ultraviolet for Salmonella Inactivation on Blueberry, Tomato, and Lettuce. J Food Sci 84:1145–1150.

39. Schonherr J. 1972. Penetration of Stomata by Liquids 813–819.

40. Haefele DM, Lindow SE. 1987. Flagellar Motility Confers Epiphytic Fitness Advantages Pseudomonas syringae. Appl Environ Microbiol 53:2528–2533.

41. Rufian J, Sanchez-Romero M, Lopez-Marquez D, Macho A, Mansfield J, Arnold D, Ruiz-Albert J, Casadesus J, Beuzon C. 2016. Pseudomonas syringae differentiates into phenotypically distinct subpopulations during colonization of a plant host. Environ Microbiol 18:3593–3605.

42. Sahin F, Miller SA. 1997. Identification of the bacterial leaf spot pathogen of lettuce, Xanthomonas campestris pv. vitians, in Ohio, and assessment of cultivar resistance and seed treatment. Plant Dis 81:1443–1446.

43. Dia NC, Morinière L, Cottyn B, Bernal E, Jacobs JM, Koebnik R, Osdaghi E, Potnis N,

Pothier JF. 2022. Xanthomonas hortorum – beyond gardens: Current taxonomy, genomics, and virulence repertoires . Mol Plant Pathol 1–25.

44. Potnis N, Krasileva K, Chow V, Almeida NF, Patil PB, Ryan RP, Sharlach M, Behlau F, Dow JM, Momol MT, White FF, Preston JF, Vinatzer BA, Koebnik R, Setubal JC, Norman DJ, Staskawicz BJ, Jones JB. 2011. Comparative genomics reveals diversity among xanthomonads infecting tomato and pepper. BMC Genomics 12:1–23.

45. Schwartz AR, Potnis N, Timilsina S, Wilson M, Patane J, Martins J, Minsavage G V., Dahlbeck D, Akhunova A, Almeida N, Vallad GE, Barak JD, White FF, Miller SA, Ritchie D, Goss E, Bart RS, Setubal JC, Jones JB, Staskawicz BJ. 2015. Phylogenomics of Xanthomonas field strains infecting pepper and tomato reveals diversity in effector repertoires and identifies determinants of host specificity. Front Microbiol 6:1–17.

46. Newberry EA, Bhandari R, Minsavage G V., Timilsina S, Jibrin MO, Kemble J, Sikora EJ, Jones JB, Potnis N. 2019. Independent Evolution with the Gene Flux Originating from Multiple Xanthomonas Species Explains Genomic Heterogeneity in Xanthomonas perforans. Appl Environ Microbiol 85:1–17.

47. Beattie GA, Lindow SE. 1995. The secret life of foliar bacterial pathogens on leaves. Annu Rev Phytopathol 33:145–172.

48. Pfeilmeier S, Caly DL, Malone JG. 2016. Bacterial pathogenesis of plants: future challenges from a microbial perspective. Mol Plant Pathol 7:1298–1313.

49. Monier J, Lindow SE. 2003. Pseudomonas syringae Responds to the Environment on Leaves by Cell Size Reduction 93:1209–1216.

50. Leveau JHJ, Lindow SE. 2001. Appetite of an epiphyte: Quantitative monitoring of bacterial sugar consumption in the phyllosphere. Proc Natl Acad Sci 98:3446–3453.

51. Fatima U, Senthil-Kumar M. 2015. Plant and pathogen nutrient acquisition strategies. Front Plant Sci 6:1–12.

52. Ferelli AMC, Bolten S, Szczesny B, Micallef SA. 2020. Salmonella enterica Elicits and Is Restricted by Nitric Oxide and Reactive Oxygen Species on Tomato. Front Microbiol 11:1–15.

53. Meng F, Altier C, Martin GB. 2013. Salmonella colonization activates the plant immune system and benefits from association with plant pathogenic bacteria. Environ Microbiol 15:2418–2430.

54. Garcia AV, Charrier A, Schikora A, Bigeard J, Pateyron S, de Tauzia-moreau M-L, Evrard A, Mithöfer A, Martin-magniette ML, Virlogeux-Payant I, Hirt H. 2014. Salmonella enterica Flagellin Is Recognized via FLS2 and Activates PAMP-Triggered Immunity in Arabidopsis thaliana. Mol Plant 7:657–674.

55. Wahlig TA, Bixler BJ, Valdés-López O, Mysore KS, Wen J, Ané JM, Kaspar CW. 2019. Salmonella enterica serovar Typhimurium ATCC 14028S is tolerant to plant defenses triggered by the flagellin receptor FLS2. FEMS Microbiol Lett 366:1–7.

56. Roy D, Panchal S, Rosa BA, Melotto M. 2013. Escherichia coli O157:H7 Induces Stronger Plant Immunity than Salmonella enterica Typhimurium SL1344. Phytopathology 103:326–332.

57. Tracy BS, Edwards KK, Eisenstark A. 2002. Carbon and nitrogen substrate utilization by archival Salmonella typhimurium LT2 cells. BMC Evol Biol 2:1–6.

58. Reid SJ, Abratt VR. 2005. Sucrose utilisation in bacteria: Genetic organisation and regulation. Appl Microbiol Biotechnol 67:312–321.

59. O'Leary BM, Neale HC, Geilfus CM, Jackson RW, Arnold DL, Preston GM. 2016. Early

changes in apoplast composition associated with defence and disease in interactions between Phaseolus vulgaris and the halo blight pathogen Pseudomonas syringae Pv. phaseolicola. Plant Cell Environ 39:2172–2184.

60. Courbier S, Grevink S, Sluijs E, Bonhomme PO, Kajala K, Van Wees SCM, Pierik R. 2020. Far-red light promotes Botrytis cinerea disease development in tomato leaves via jasmonate-dependent modulation of soluble sugars. Plant Cell Environ 43:2769–2781.

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Author Contributions: MHD, KNC, and JDB developed the experiments. MHD, KNC, SCZ, and INM performed the experiments. MHD and JDB wrote the manuscript, and all authors reviewed the manuscript. MHD created the original illustrations.

TABLES

Table 1: Bacterial strains used in this study.

Strain Abbreviation	Description	Reference or Source
Xg WT	*Xanthomonas hortorum* pv. *gardneri* 444; Nal^R	(21)
Xg avr-	*Xanthomonas hortorum* pv. *gardneri* 444; *avrHah1*$^{\Delta DBD}$	received from J. Jones; (21)
Xv	*Xanthomonas vesicatoria* 1111; Nal^R	ATCC
Se WT	*Salmonella enterica* sv. Typhimurium 14028s; Kan^R	(21)
Se Δ*motA*	*Salmonella enterica* sv. Typhimurium 14028s; Δ*motA*::Kan	(18)

FIGURES

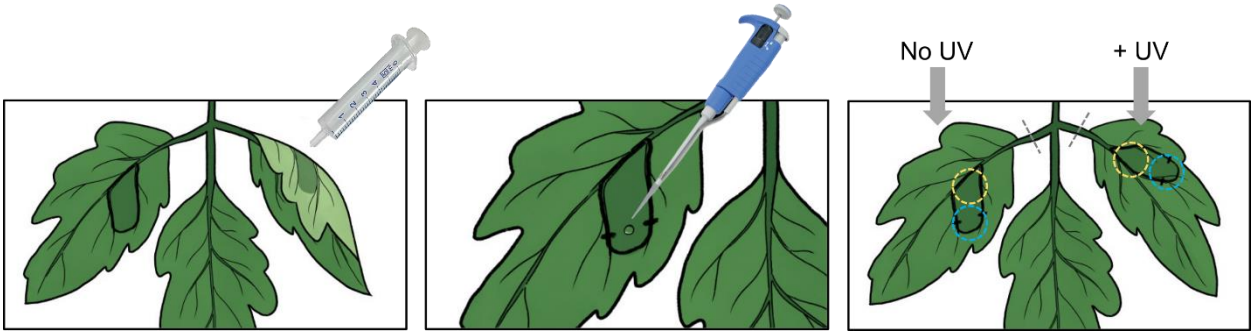

Figure 1: Infiltration and droplet inoculation assay. Two intact tomato plant leaflets per plant were infiltrated with water or bacteria through the abaxial surface, and these infiltration zones were outlined with permanent marker. After the indicated number of days post-infiltration (DPI), 15 µL *S. enterica* (*Se*) droplets were placed on the adaxial surface of each infiltration zone away from the previous syringe placement site. The arrival site was marked with permanent marker for future reference. Plants were incubated for the indicated time (HPA, hours post-*S. enterica* arrival; DPA, days post-*S. enterica* arrival) prior to sampling. Leaflets were detached at the petiolule, and designated leaflets were irradiated with UV. Tissue disks were sampled at the site of *S. enterica* arrival (blue dotted circles) and distal tissue sites (yellow dotted circles) for *S enterica* population enumeration.

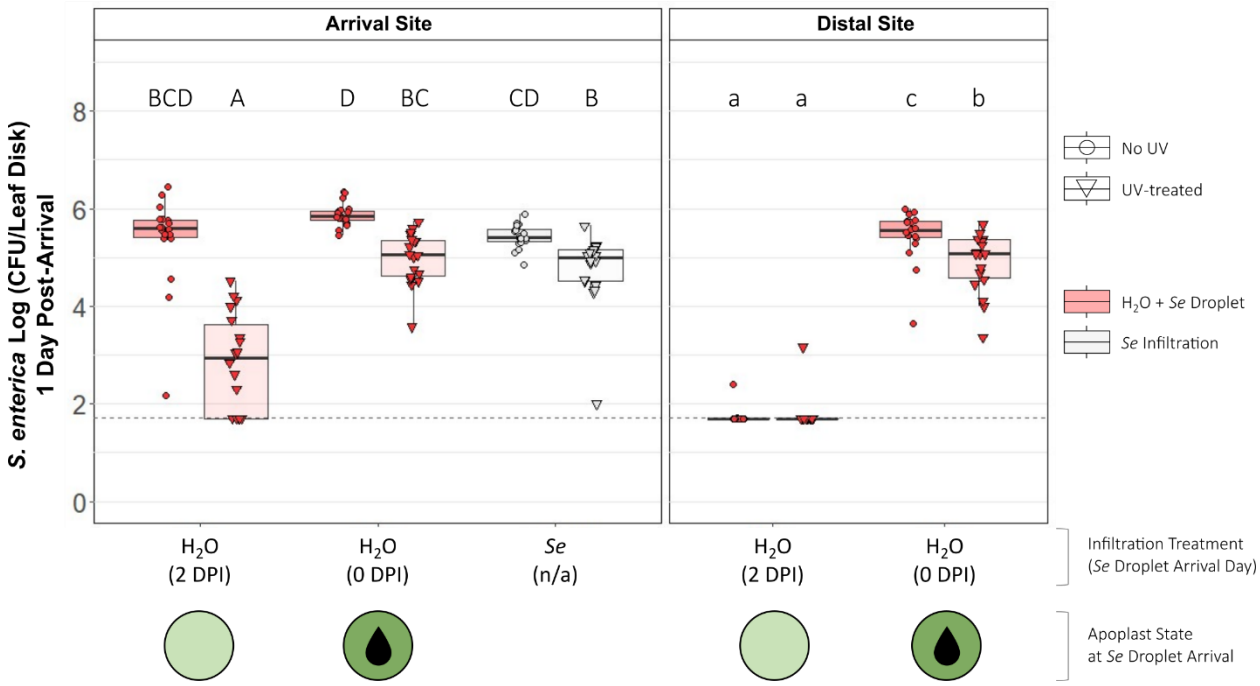

Figure 2: Healthy leaf tissue with a water-filled apoplast facilitates *S. enterica* localization to protected niche and migration to distal tissue. Tomato leaflets were inoculated with *S. enterica* via 15 μL surface droplet (10^6 CFU/leaflet; red) or infiltration (10^7 CFU/mL; white). *S. enterica* droplets were applied to leaflets infiltration with water at 2 or 0 DPI. Half of leaflets were treated with UV irradiation (600,000 μJ/cm^2; triangles) and the other half left non-irradiated (circles). Tissue was sampled for *S. enterica* population enumeration 1 day post-arrival at the site of *S. enterica* arrival and distal from the arrival site (latter for droplet-applied tissue). Uppercase letters above the boxplots denote significance among treatment groups within the arrival site based on a mixed-ANOVA and post-hoc Tukey test ($P < 0.05$) where shared letters indicate statistical similarity. Lowercase letters denote significance among treatment groups within the distal site based on a Kruskal-Wallis and post-hoc Wilcox test ($P < 0.05$). Dotted line indicates limit of detection. Green circles below the graph represent the relative state of the apoplast at time of *S. enterica* arrival (empty light green circles = healthy/dry apoplast, dark green circle with droplet = healthy/wet apoplast).

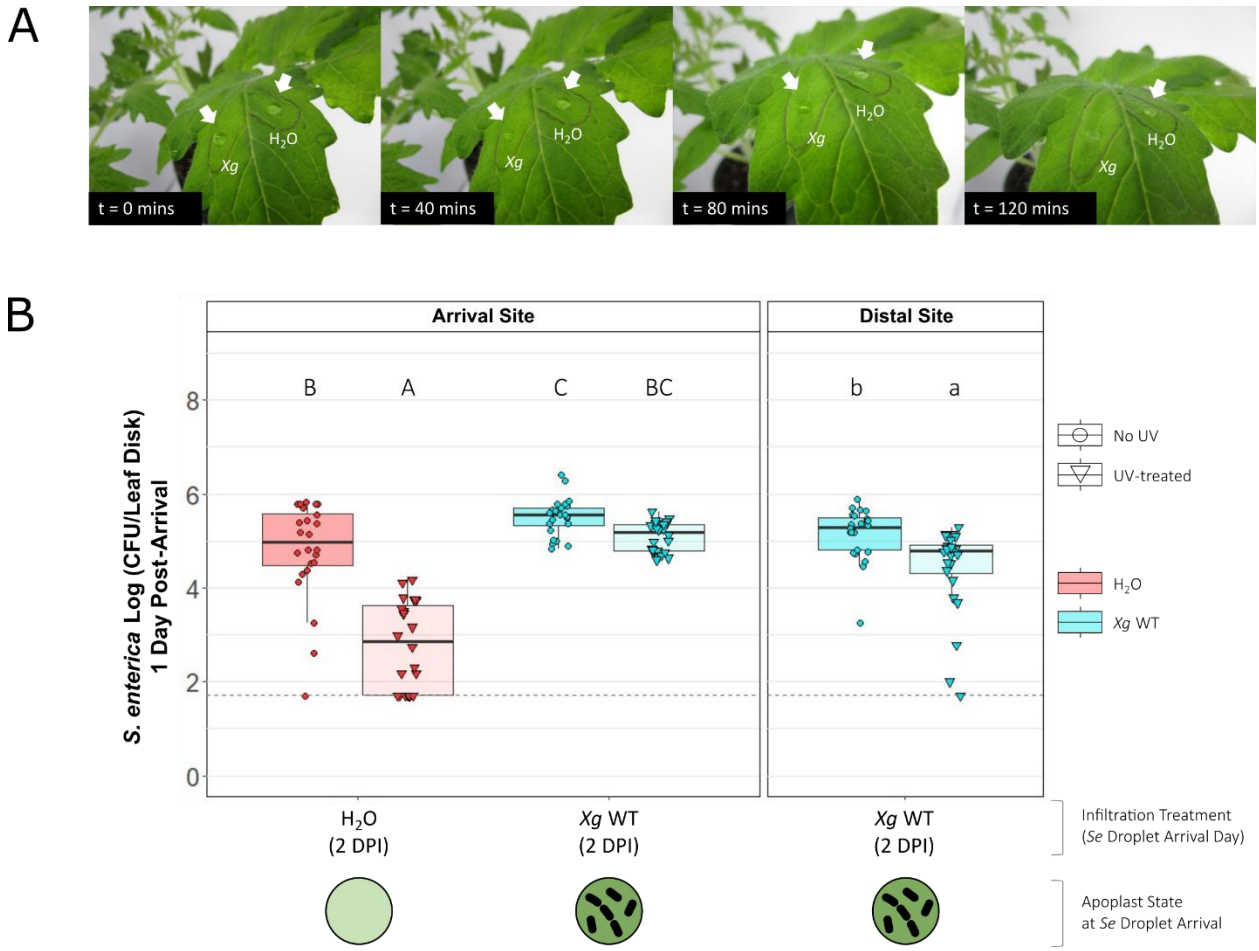

Figure 3: *X. gardneri* infection facilitates *S. enterica* localization to protected niche and migration beyond site of arrival. (a) Absorption of *S. enterica* droplet into tissue 1 DPI with *X. gardneri* (left infiltration zone) or water (right infiltration zone). For photography purposes only, *X. gardneri* and water infiltration treatments were performed on the same leaflet. White arrows mark visible droplets. (b) *S. enterica* populations 1 day post-arrival following arrival on tissue 1 DPI with water (red) or *X. gardneri* (turquoise). 15 µL droplets of *S. enterica* cells were applied to tomato leaflets (10^6 CFU/leaflet). Half of leaflets were treated with UV irradiation (600,000 µJ/cm^2; triangles) and the other half left non-irradiated (circles). Tissue was sampled for *S. enterica* population enumeration 1 day post-arrival (DPA) at the site of *S. enterica* arrival and distal from the arrival site (latter for droplet-applied tissue). Uppercase letters above the

boxplots denote significance among treatment groups within the arrival site based on a mixed-ANOVA and post-hoc Tukey test ($P < 0.05$) where shared letters indicate statistical similarity. Lowercase letters denote significance among treatment groups within the distal site based on a Kruskal-Wallis and post-hoc Wilcox test ($P < 0.05$). Dotted line indicates limit of detection. Green circles represent the relative state of the apoplast at time of *S. enterica* arrival (empty light green circles = healthy/dry apoplast, dark green circle with bacteria = infected/wet apoplast).

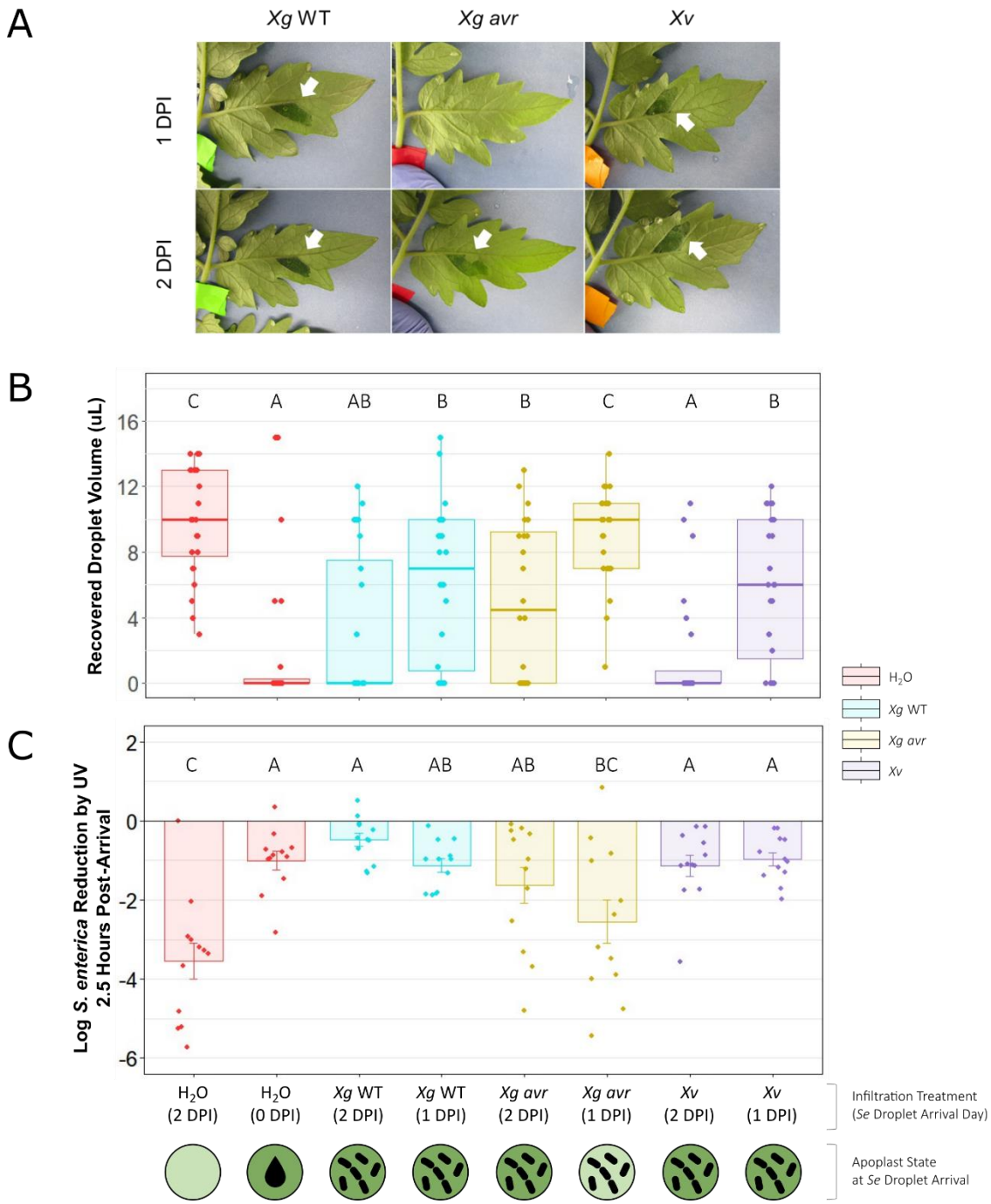

Figure 4: *S. enterica* access to protected niche within 2.5 hours of arrival on water-soaked tissue depends on *Xanthomonas* strain and duration of infection. Infiltration treatments include water (red points), *X. gardneri* WT (turquoise points), *X. gardneri avrHah1*$^{\Delta DBD}$ (gold points), and *X. vesicatoria* (purple points) (10^8 CFU/mL). (a) Representative images of leaflets infiltrated with

one of the three *Xanthomonas* strains at 1 and 2 DPI. White arrows mark visible water-soaking. (b) Remaining surface *S. enterica* droplet volumes measured 2.5 hours following application of 15 uL *S. enterica* droplets to leaflets. Differing letters above the boxplots denote significantly different treatment groups based on a Kruskal-wallis and post-hoc Wilcox multiple comparisons test ($P < 0.05$) (c) Reduction of *S. enterica* populations in response to UV (600,000 $\mu J/cm^2$) following arrival of *S. enterica* arrival (10^6 CFU/leaflet) at 0, 1, or 2 DPI with water or *Xanthomonas*. Each point represents the *S. enterica* populations for the UV-treated leaflet minus the non-irradiated leaflet for each plant. Tissue was sampled for *S. enterica* population enumeration 2.5 hours post-arrival at the site of *S. enterica* arrival. Letters above the boxplots denote significance among treatment groups based on a mixed-ANOVA and post-hoc Tukey test ($P < 0.05$) where shared letters indicate statistical similarity. Green circles represent the relative state of the apoplast at time of *S. enterica* arrival (empty light green circles = healthy/dry apoplast, dark green circle with droplet = healthy/wet apoplast, dark green circle with bacteria = infected/wet apoplast, light green circle with bacteria = infected/dry apoplast).

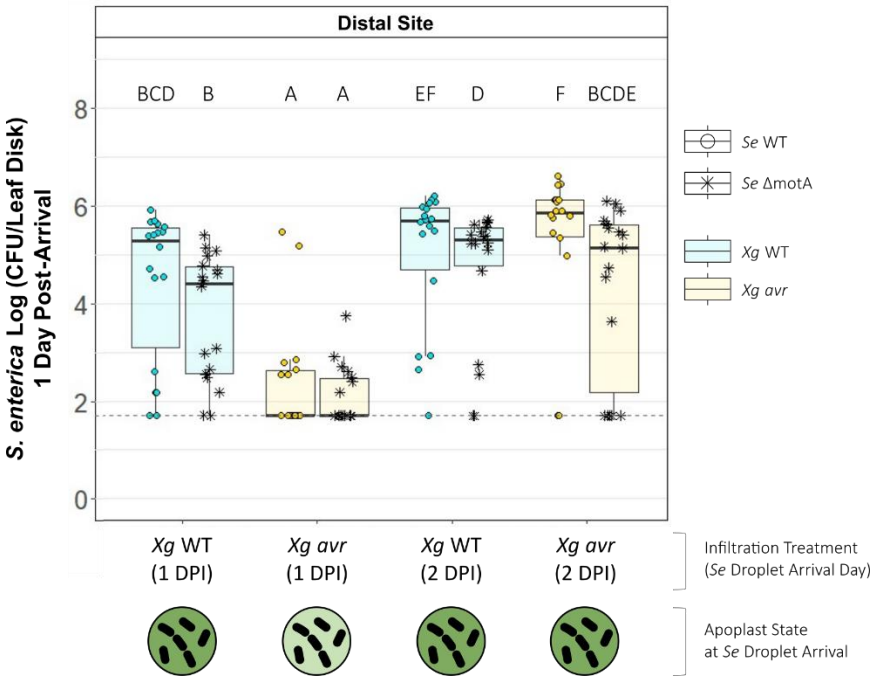

Figure 5: *AvrHah1* expression by *X. gardneri* enhances *S. enterica* migration during early infection and flagellar motility promotes *S. enterica* survival at distal tissue. *S. enterica* droplets (10^6 CFU/leaflet) were applied at 1 or 2 DPI with *X. gardneri* WT (turquoise) or *X. gardneri* *avrHah1*$^{\Delta DBD}$ (gold). Leaflets were treated with *S. enterica* WT (circle points) or *S. enterica* Δ*motA* (asterisk points). Tissue was sampled for *S. enterica* population enumeration at 1 day post-arrival. Letters above the boxplots denote significance among treatment groups within the distal site based on a Kruskal-Wallis and post-hoc Wilcox test ($P < 0.05$) where shared letters indicate statistical similarity. Dotted line indicates limit of detection. Green circles represent the relative state of the apoplast at time of *S. enterica* arrival (dark green circle with bacteria = infected/wet apoplast; light green circle with bacteria = infected/dry apoplast).

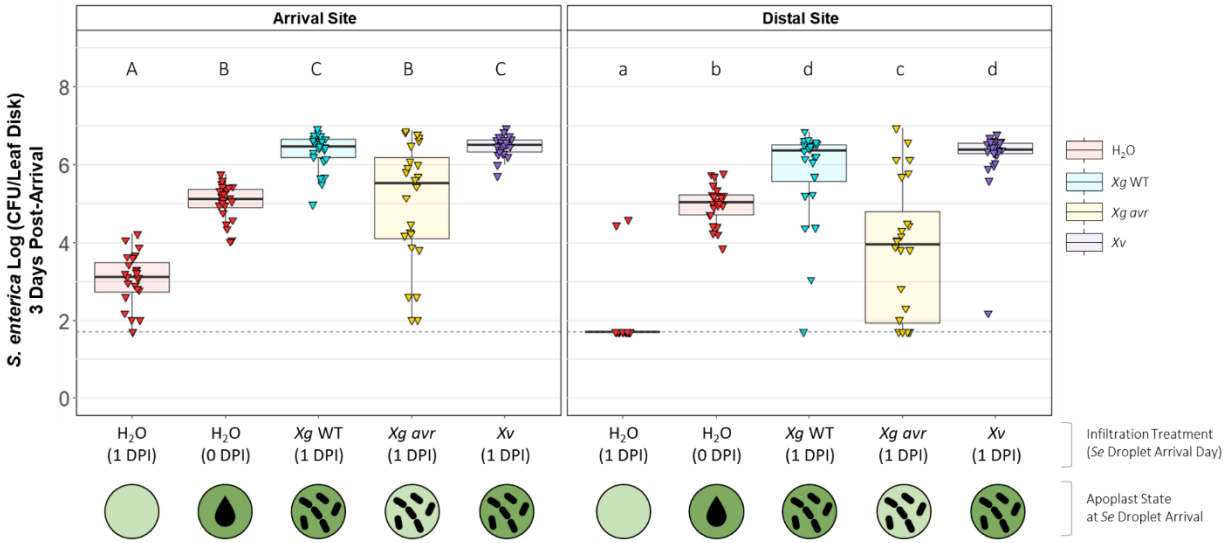

Figure 6: *Xanthomonas*-infected apoplast serves as a hospitable niche for *S. enterica* 3 days post-arrival. *S. enterica* droplets (10^6 CFU/leaflet) were applied at 0, or 1 DPI with water (red), *X. gardneri* WT (turquoise), *X. gardneri avrHah1*$^{\Delta DBD}$ (gold), and *X. vesicatoria* (purple). All leaflets were treated with UV irradiation (600,000 μJ/cm^2) prior to sampling. Tissue was sampled for *S. enterica* population enumeration at the site of *S. enterica* arrival and distal from the arrival site at 3 days post-arrival. Uppercase letters above the boxplots denote significance among treatment groups within the arrival site based on a mixed-ANOVA and post-hoc Tukey test ($P < 0.05$) where shared letters indicate statistical similarity. Lowercase letters denote significance among treatment groups within the distal site based on a Kruskal-Wallis and post-hoc Wilcox test ($P < 0.05$). Dotted line indicates limit of detection. Green circles represent the relative state of the apoplast at time of *S. enterica* arrival (empty light green circles = healthy/dry apoplast, dark green circle with droplet = healthy/wet apoplast, dark green circle with bacteria = infected/wet apoplast, light green circle with bacteria = infected/dry apoplast).

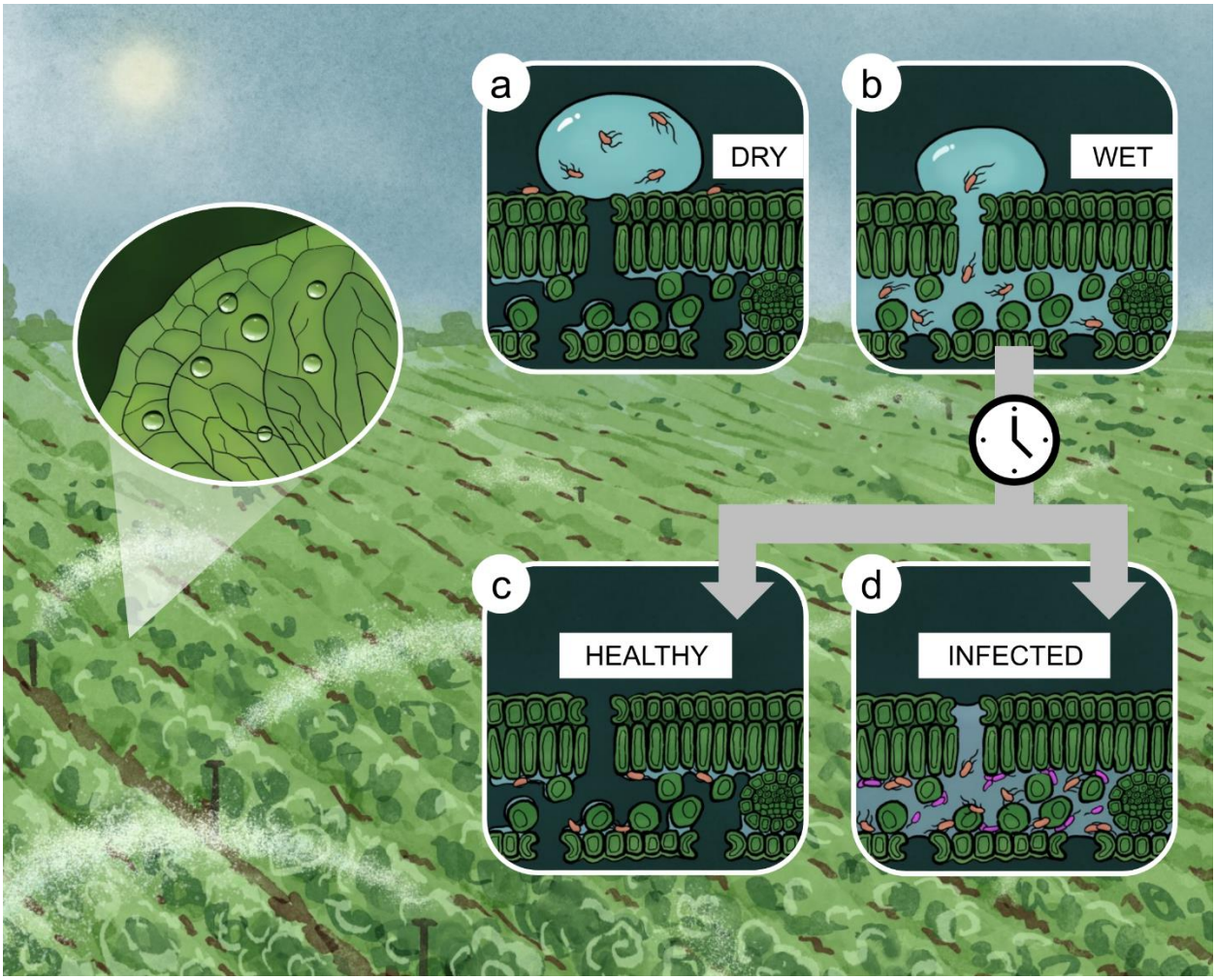

Figure 7: Model for *S. enterica* survival in a habitable apoplast. *S. enterica* can colonize crop plants via spray-irrigation of contaminated water. Macroscopically dry tissue (a) does not enable absorption of surface water, whereas tissue with an aqueous apoplast (b) facilitates the ingression of *S. enterica* into the apoplast. Healthy tissue that is abiotically water-soaked eventually dries out (c), creating an unfavorable niche for *S. enterica.* By contrast, an infected apoplast (d), which maintains water-soaking via phytopathogen effectors and is likely enriched with nutrients and other growth-promoting factors, facilitates *S. enterica* replication.

Descriptions for supplementary material

Figure S1: *S. enterica* motility does not affect ability to access protected apoplast. *S. enterica* droplets (10^6 CFU/leaflet) were applied at 0 or 2 DPI with water (red) or *X. gardneri* (blue). Leaflets were treated with *S. enterica* WT (circle points) or *S. enterica* Δ*motA* (asterisk points). All leaflets were treated with UV irradiation (600,000 μJ/cm^2) prior to sampling. Tissue was sampled for *S. enterica* population enumeration at the site of *S. enterica* arrival at 2.5 hours post-*S. enterica* application. Letters above the boxplots denote significance among treatment groups within the arrival site based on a mixed-ANOVA and post-hoc Tukey test ($P < 0.05$) where shared letters indicate statistical similarity. Dotted line indicates limit of detection. Green circles represent the relative state of the apoplast at time of *S. enterica* arrival (empty light green circles = healthy/dry apoplast, dark green circle with droplet = healthy/wet apoplast, dark green circle with bacteria = infected/wet apoplast). Data represents one representative replicate of the experiment.

Table S1: Incidence rate for absorption of *S. enterica* droplet suspension into H_2O or *Xanthomonas*-infiltrated leaves. *S. enterica* droplets were applied to H_2O-infiltrated leaves at 1 DPI or 0 DPI and *Xanthomonas*-infiltrated leaves at 1 DPI. Droplets were considered completely absorbed if no liquid remained on the surface by 2.5 hours post-*S. enterica* arrival. Letters in parentheses denote statistical similarities and differences among treatments based on the Fisher's Exact test ($P < 0.05$).