Title: Time of arrival during disease progression and humidity additively influence *Salmonella enterica* colonization of lettuce.

Megan H. Dixon[a,b], Dharshita Nellore[a], Sonia C. Zaacks[a], and Jeri D. Barak[a]

[a]Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin, USA;

[b]Microbiology Doctoral Training Program, University of Wisconsin, Madison, Wisconsin, USA

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Address correspondence to Jeri D. Barak, jeri.barak@wisc.edu

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ABSTRACT

The interplay between plant host, phytopathogenic bacteria, and enteric human pathogens in the phyllosphere have consequences for human health. *Salmonella enterica* has been known to take advantage of phytobacterial infection, but there is little knowledge of additional factors that may influence the relationship between enteric pathogen and plant disease. In this study, we investigated the role of humidity and the extent of disease progression on *S. enterica* colonization of plants. We found that high humidity was necessary for replication of *S. enterica* on diseased lettuce, but not required for *S. enterica* ingress into the UV-protected apoplast. Additionally, the *Xanthomonas vitians*-infected lettuce host was found to be a relatively hostile environment for *S. enterica* when it arrived prior to the development of watersoaking or following necrosis onset, supporting the existence of an ideal window during *X. vitians* infection progress that maximizes *S. enterica* survival. Lastly, proximity of *S. enterica* to *X. vitians* cells may allow *S. enterica* to benefit from cross-feeding during plant infection. Overall, this study emphasizes the role of phytobacterial disease as a driver of *S. enterica* success in the phyllosphere, demonstrates how time of arrival during disease progress can influence *S. enterica's* fate in the apoplast, and highlights the potential for humidity to transform an infected apoplast into a growth-promoting environment for bacterial colonizers.

Importance: Bacterial leaf spot of lettuce caused by *X. vitians* is a common threat to leafy green production. The global impact caused by phytopathogens, including *X. vitians*, is likely to increase with climate change. We found that even under a scenario where increased humidity did not enhance plant disease, high humidity had a substantial effect on facilitating *S. enterica*

growth on *Xanthomonas*-infected plants. High humidity climates may directly contribute to the survival of human enteric pathogens in crop fields or indirectly affect bacterial survival via changes to the phyllosphere brought on by phytopathogen disease.

INTRODUCTION

Agricultural fields serve as habitats for diverse communities of microorganisms, including bacteria. Although the surface of a leaf may seem innocuous, bacteria that colonize the phyllosphere must adapt to hostile conditions including nutrient scarcity, extreme fluctuations in moisture availability, UV irradiation, and the host immune response (1, 2). To increase survival, some bacteria abandon the leaf surface and enter the apoplast where they may evade such stresses, so long as they can evade or suppress the plant immune response. Phytobacterial pathogens overpower host defenses and destructively alter the leaf environment, creating an aqueous niche that supports bacterial replication (3–8). For example, *Xanthomonas hortorum* pv. vitians (hereafter, *X. vitians*) causes bacterial leaf spot of lettuce, which is characterized by symptom development of dark, water-soaked leaf lesions (9, 10). Phytobacterial disease causes yield loss (11), which devastates agricultural industries worldwide and is already a major cause for concern on its own. However, growing evidence supports that phytobacterial disease may be a risk factor for another issue: food-borne illness.

Salmonella enterica is an environmental microbe that effectively colonizes both mammalian and environmental hosts (12), relying on its ability to adapt and survive in diverse niches (13). Irrigation water, soils, and plant surfaces are all examples of places where *S. enterica* can persist for long periods of time (14–16). *S. enterica* is one of several enteric pathogens recognized as colonizers of plants (17), and salmonellosis outbreaks have been linked to consumption of raw produce (18). Given the prevalence of phytobacterial disease on agricultural crops, *S. enterica* is likely to interact with pathogenic phytobacteria when *S. enterica* is present in a field. Considering the public health threat posed by *S. enterica* colonization of

crop plants, studying biotic and abiotic factors that influence its ability to survive in the environment is a critical area of research.

Phytobacterial disease promotes the survival of *S. enterica* on plants (7, 19–25). A recently published study from our lab showed that tomato leaves infected by *X. hortorum* pv. gardneri enabled *S. enterica* cells that arrive on the leaf surface to become internalized within the leaf apoplast and replicate (24). After completing this work, we questioned whether *S. enterica* would benefit from any bacterial leaf spot disease. Lettuce has been implicated in food-borne illness outbreaks caused by *S. enterica*, and lettuce is susceptible to the bacterial spot disease pathogen, *X. vitians* (26–28). Because lettuce is commonly eaten raw, without food safety intervention strategies, we determined that studying the romaine lettuce-*X. vitians* pathosystem would be key to expanding our knowledge of factors that influence *S. enterica* population size on plants.

The goal of our study was to examine how *X. vitians* disease and environmental factors may impact the fate of *S. enterica* on lettuce plants. We asked how the combination of disease and high humidity would impact *S. enterica* growth and colonization of the apoplast. Additionally, we investigated how *S. enterica's* success on *X. vitians*-infected lettuce could be influenced by how early or late during infection that *S. enterica* arrives, effectively using *S. enterica* as a biological reporter for host changes during phytobacterial infection and disease progress. Lastly, we measured how *S. enterica* growth in a sucrose-rich environment is affected by *X. vitians in vitro* to determine a mechanism by which *S. enterica* replicates in the presence of *X. vitians* infection.

METHODS

Bacterial strains, media, and culture conditions

Bacterial strains used in this study are listed in Table 1. *Xanthomonas vitians* cultures were prepared by inoculating 3 mL of nutrient broth with frozen cells and incubating at 28°C with shaking at 200 rpm for 2 days. *Salmonella enterica* cultures were prepared in a similar manner but incubated in lysogeny broth (LB) overnight. Cultures were then diluted in sterile water and prepared to the desired cell concentration. Nutrient agar (NA) was used for enumeration of *X. vitians*, and LB agar amended with 20 µg/mL of nalidixic acid was used for enumerating *S. enterica*. Agar plates were incubated for ~48 hrs at 28°C or 16-24 hr at 37°C for *X. vitians* and *S. enterica*, respectively.

A fluorescently labeled *X. vitians* strain was produced for this study by transforming a wild-type *X. vitians* L7 strain with high-copy plasmid pNO-mCherry (gifted from Z. Gitai lab; modified version of plasmid described in (29)) derived from pTH18Cr (30), which carries a chloramphenicol resistance cassette and constitutively expresses mCherry via $P_{Ltet\mathrm{O}\text{-}1}$ (31) promoter with *tet* repressor removed. Plasmid DNA was isolated from *Escherichia coli* using a Spin MiniPrep kit (Qiagen) and transferred to *X. vitians* via electroporation.

Bacterial inoculation of lettuce

Lactuca sativa (romaine lettuce) seedlings were cultivated in growth chambers with a 16-h photoperiod at 24°C. Lettuce plants (4-5 weeks old) were infiltrated with *Xanthomonas vitians* inoculum prepared to an OD_{600} = 0.2 (~3 x 10^8 CFU/mL) or sterile deionized H_2O as a control. *Xanthomonas* infiltration was performed via needleless syringe as described previously (32) and

adapted for lettuce leaves. “Middle leaves” (the 3rd or 4th oldest true leaves) were selected for infiltration, which were fully open and relatively flat. The approximate volume infiltrated into each infiltration zone was 15 μL, which produced infiltration zones 1-1.5 cm long.

Infiltrated lettuce plants were maintained in large plastic bins at a moderate relative humidity (RH) of 50-70%. Lettuce plants were subsequently inoculated with *Salmonella enterica* cell suspensions at the given time post-infiltration with H_2O or *X. vitians* cells. *S. enterica* was diluted in sterile deionized water to a cell concentration ~10^6 CFU/mL. *S. enterica* cell suspension droplets (3 uL; ~3000 CFU/droplet) were pipetted onto the adaxial surface of each infiltration zone at the edge of the infiltration zone closest to the leaf margin, avoiding overlap between the droplet site and any damage caused by syringe infiltration. Leaves were then destructively sampled at given timepoints post-*S. enterica* arrival. When experiments required sampling beyond 1-day post-*S. enterica* arrival, lids were placed on the bins at 24 hours post-*S. enterica* arrival to raise the RH to >90%. When noted, lids were left off or removed following high RH exposure to achieve the lower RH level of 55-70%.

Bacterial population sampling

Lettuce leaves were cut from the plant and when applicable, UV irradiation was utilized to inactivate *S. enterica* cells colonizing the leaf surface and thus measure *S. enterica* colonizing the apoplast (32). Leaf tissue was irradiated with UV-C (254 nm) by placing samples adaxial side facing up inside a Stratalinker UV Crosslinker 1800 and dosing the tissue at an exposure level of 150,000 μJ/cm^2. For the experiment described in **Figure 1,** for each plant, one of two inoculated middle leaves was randomly selected for UV irradiation and the other leaf left non-irradiated as a control. For all other *in planta* experiments involving UV irradiation treatment, middle leaves

that had been inoculated with *S. enterica* cells on both sides of the midrib were split in half after sampling—one half of the leaf was randomly selected for UV irradiation and the half left non-irradiated. Similar to what was demonstrated previously with *S. enterica*-inoculated tomato leaves (32), UV irradiation treatment significantly reduced the populations of *S. enterica* applied to the lettuce leaf surface while leaving apoplastic *S. enterica* cell populations intact (data not shown).

When noted, measurement of surface *S. enterica* populations was determined by removing cells from sampled leaves using swabs dipped in Tween-20 as previously described (32). For all sampled leaves, bacterial colonization of leaf tissue was measured by excising 1-cm-diameter leaf disks from infiltration zones using a tissue corer and homogenizing the tissue in 500 uL of sterile water using a Dremel tool (33). Leaf homogenate was dilution-plated to enumerate *S. enterica* and *X. vitians* populations. The limit of detection (LOD) for *S. enterica* colonization was 5 CFU/leaf disk (or 10 CFU/sample for the summation of swab and leaf tissue *S. enterica* populations). The remaining leaf homogenate was enriched with LB containing nalidixic acid (20 μg/mL) to determine if *S. enterica* populations below the LOD were present in samples for which direct plating did not result in the growth of *S. enterica*. For non-detects, LB-enriched samples were plated ~24 hours after enrichment. *S. enterica* colonization datapoints plotted at zero were confirmed to be zero based on the absence of *S. enterica* in the LB-enriched sample. Data plotted halfway between zero and the limit of detection indicates absence of colonies via direct plating and a positive enrichment sample.

Digital photography

To capture time course images of *X. vitians*-infected leaves, infiltrated leaves of lettuce plants were photographed daily using a Canon PowerShot ELPH 190 IS digital camera. Plants were placed inside a light box with white backdrop for photographing to achieve consistent light conditions. Plants were maintained in bins under moderate RH (~50-70%). Images were cropped with no other corrections applied.

Electrolyte leakage measurements

When electrolyte leakage was measured, lettuce plants were prepared by infiltrating two adjacent middle leaves. The relatively older leaf was infiltrated four times, with two infiltration zones placed on each side of the leaf margin. One infiltration zone was randomly selected for bacterial population enumeration, and the remaining three infiltration zones were used for electrolyte leakage measurements. Electrolyte leakage was measured as performed previously (34, 35). In brief, for each biological replicate, three 1 cm diameter tissue disks were excised from a single leaf and pooled. Leaf tissue was placed in 12-well plates with wells containing 4 mL of water each. An EcTestr 11 (Eutech Instruments) conductivity tester was used to measure the leakage of electrolytes from the tissue into the water after 6 hours of incubation. Conductivity was reported by subtracting blank measurements taken immediately following a 45-minute wash step (time = 0 hours) from measurements taken at 6 hours.

Confocal laser scanning microscopy

Lettuce plants were infiltrated with sterile deionized H_2O or *X. vitians* L7 + pNO-mCherry cells prepared to an OD_{600} = 0.2 and incubated in plastic bins under moderate RH (50-70%) as

described above. Droplets of *S. enterica* + pKT-Kan (GFP-labelled) cell suspensions containing ~10^5 CFU were applied to the surfaces of infiltrated zones at 4-days post-infiltration with H_2O and 1- or 4-days post-infiltration with *X. vitians*. Inoculated plants were then incubated for 2 days, with high humidity (RH >90%) applied for the final 24 hours prior to sampling. Whole leaves were excised from the plants and kept in petri plates containing a moist Kim-wipe prior to imaging. Slides were prepared immediately before imaging by excising 0.5 cm diameter tissue disks at the site of *S. enterica* application and wet mounting them between two glass cover slips.

Confocal laser scanning microscopy (CLSM) images were obtained using a Zeiss Elyra LSM780 inverted confocal microscope and 40x water immersion 1.10 C-Apochromat objective. The 488 and 561 nm lines of an argon laser were used for excitation of GFP, mCherry, and chlorophyll A autofluorescence, which were detected at 500-550 nm (GFP), 552-624 (mCherry), and 630-700 nm (chlorophyll A) using the Zen (Zeiss) software. GFP and chlorophyll A autofluorescence were detected using Track 1, and mCherry detected using Track 2, with track switching set to every line. Master gain for GFP, mCherry, and chlorophyll A was set to 700, 750, and 850, respectively. A transmitted light detector (with master gain set to 450) was used for capturing differential interference contrast (DIC) images, which are shown overlaid with confocal fluorescence imaging data. For each treatment, leaf samples from 2-3 plants were imaged and 9-15 image fields were captured at both the surface level and within the apoplast at a z-depth of 4.6-14.5 μm (mean = 8.7 μm) relative to the surface. Observations of the presence/absence of GFP-labelled *S. enterica* cells within the apoplast were recorded for every field.

For representative images shown, image processing via ImageJ was applied consistently across images, as performed previously (32). For DIC images, brightness and contrast were

linearly adjusted using the “reset” button within the “Brightness/Contrast” tool. To improve visibility of *S. enterica* and *X. vitians* cells, the “enhance contrast” tool was applied to the GFP and mCherry channels with level of saturated pixels set to 0.05% and “equalize histogram” box left unchecked. Chlorophyll A channel data was omitted for representative images.

Sucrose media co-culture assay

M9 minimal media containing sucrose or glucose as the sole carbon source (hereafter, M9 sucrose and M9 glucose) was prepared fresh for every experimental replicate with the following components diluted in de-ionized water: $MgSO_4$ (2.0 mM), D-glucose or D-sucrose (0.4% w/v), $CaCl_2$ (0.10 mM), Na_2HPO_4 (47.8 mM), KH_2PO_4 (22.0 mM), NaCl (8.6 mM), and NH_4Cl (18.7 mM).

Stationary phase *X. vitians* cells grown in NB were washed three times in 1X phosphate-buffered saline (PBS) and normalized to an OD = 0.2. 1.5 mL of the normalized culture was inoculated into 19.5 mL of M9 sucrose and incubated in 250 mL flasks at 28°C with shaking. Separate *X. vitians* culture flasks were incubated for either 24 or 48 hours in advance of *X. vitians* culture or cell-free supernatant (CFS) being introduced to *S. enterica.* CFS was prepared by successively filtering *X. vitians* culture through 0.8 μm and 0.2 μm filters. Sterility of CFS was confirmed via nutrient broth enrichment.

S. enterica cells grown overnight in LB were prepared by washing stationary phase cells three times in 1X PBS, normalizing to an OD = 0.1, and diluting 1000-fold in sterile water. Diluted *S. enterica* cells or sterile water (100 μL) was then combined with 1.4 mL of *X. vitians* cell cultures or CFS per well of a 12-well plate. *S. enterica* and *X. vitians* cell concentrations

were measured for triplicate wells immediately after 12-well plates were prepared at time = 0 hours and following 28 hours of incubation at 28°C.

Statistical analysis

Statistical analyses and plot generation were performed using R Studio version 2023.03.1 (R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria [http://www.R-project.org]). Data from three statistically similar and independent replicate experiments were aggregated, unless otherwise noted in the figure legend. The number of biological replicates for each replicate experiment was 6-8 lettuce leaves. For our *in vitro* experiment, the number of experimental (biological) replicates was 4, with each biological replicate calculated from the mean of three technical replicates. Bacterial population data were plotted following a $\log_{10}(x+1)$ transformation.

Several statistical tests were used to identify differences between treatment groups. Welch's analysis of variance (ANOVA) and post-hoc Games-Howell tests were used for comparing *in planta* bacterial populations among treatment groups. Compact letter displays were added above the boxplots to communicate statistical similarities and differences among treatment groups. Shared letters indicate statistically similar groups, whereas an absence of shared letters indicates statistically different groups. When comparing bacterial populations between timepoints, a Welch two-sample T-test was used, and statistical differences displayed above boxplots using an asterisk symbol (*). When *S. enterica in planta* populations were compared to the amount of *S. enterica* applied in the inoculum, a Wilcoxon one-sample test was used, and statistical differences indicated using a dagger symbol (†). Lastly, Fisher's exact test was used

for comparing proportions of *in planta* samples with *S. enterica* colonization that exceeds the inoculum. The significance threshold for all statistical comparisons was a *P* value of <0.05.

RESULTS

***X. vitians*-infected lettuce enables *S. enterica* growth and colonization of UV-protected niches, whereas healthy lettuce does not support *S. enterica* growth or UV protection.** To determine whether bacterial spot of lettuce, which is caused by *X. vitians*, can facilitate *S. enterica* growth and colonization of UV-protected niches, lettuce plants were infiltrated with H_2O or *X. vitians* cells and treated with *S. enterica* cells at 4-days post-infiltration (**Figure 1A**). Hence, *S. enterica* was applied to lettuce plants with either a healthy, macroscopically dry apoplast (for which most of the infiltrated water had since dissipated) or symptomatic plants with a *X. vitians*-infected, water-soaked apoplast at time of *S. enterica* arrival. For plants sampled as late as 3 days post-*S. enterica* arrival (dpa), high humidity (RH >90%) conditions were applied for 48 hours prior to sampling. Similarly to our previous study which utilized a tomato-*X. hortorum* pv. gardneri pathosystem (32), *S. enterica* cells were applied to the surface of non-wounded leaves, and UV irradiation was applied to leaves post-sampling to measure *S. enterica* colonization of the UV-protected apoplast. After leaves were destructively sampled at 1 or 3 dpa and half the leaves were UV-irradiated, cotton swabs dipped in 0.1% Tween-20 were used to measure *S. enterica* cells removable from the lettuce surface. Next, leaf tissue was homogenized to quantify *S. enterica* cells that were strongly attached to the leaf tissue or inside the apoplast. Viable *S. enterica* cells within the leaf tissue would not have been removed by surface swab or inactivated by UV irradiation.

We found that at 1 dpa, the number of *S. enterica* cells attached to lettuce tissue was higher for *X. vitians*-infected lettuce than healthy lettuce, as demonstrated by the higher *S. enterica* populations measured on non-irradiated leaves following swab treatment (**Supplementary Figure 1B**). However, the amount of *S. enterica* cells recovered via surface swab was not influenced by *X. vitians* disease at 1 dpa (**Supplementary Figure 1A**). At 3 dpa, the population of *S. enterica* cells that were removable from the surface and those that were tightly associated with the lettuce tissue were significantly higher on *X. vitians*-infected leaves compared to healthy leaves (**Supplementary Figures 1A and 1B**). However, it's worth noting that at this later sampling timepoint, which was a total of 7 days post-*X. vitians* infiltration, *X. vitians*-infected leaves were more vulnerable to damage by swab application. Swab samples taken from *X. vitians*-infected leaves at late infection may have contained *S. enterica* cells that had been inside the infected leaves but were removed in the process of sampling the leaf surface by swab. We additionally found that UV irradiation significantly reduced the amount of *S. enterica* cells recoverable by swab for both healthy and *X. vitians*-infected leaves at both sampling timepoints (**Supplementary Figure 1A**), supporting the effectiveness of UV in inactivating *S. enterica* cells on the lettuce leaf surface.

X. vitians infection resulted in greater leaf tissue-associated *S. enterica* populations at both 1 and 3 dpa (**Supplementary Figure 1B)** compared to healthy leaves, indicating *S. enterica* benefits from *X. vitians* infection**.** To answer the question of whether *X. vitians* infection could promote *S. enterica* growth on lettuce, the number of *S. enterica* cells recovered from the swab and tissue for each leaf was summed to produce the total *S. enterica* colonization of the leaf sample. Through comparing *S. enterica* populations within each treatment group between both sampling timepoints, we found that total *S. enterica* populations only increased between 1 and 3

dpa for *X. vitians*-infected lettuce (**Figure 1B**), whereas healthy (H_2O-infiltrated) lettuce did not promote *S. enterica* growth. In addition, *S. enterica* populations on *X. vitians*-infected leaves significantly exceeded the inoculum dose on non-UV irradiated leaves at 3 dpa ($P < 0.05$, Wilcoxon one-sample test). *S. enterica* populations from both non-irradiated and UV-irradiated *X. vitians*-infected leaves increased over time (**Figure 1B)**, altogether supporting the conclusion that a *X. vitians*-infection supports *S. enterica* growth in the apoplast.

***S. enterica* growth on *X. vitians*-infected lettuce relies on high humidity, but high humidity is not required for *S. enterica* colonization of the apoplast of *X. vitians*-infected leaves.** After establishing that *X. vitians* infection promotes *S. enterica* growth and colonization of UV-protected niches, we investigated whether the combination of *X. vitians* infection and high humidity could additively benefit *S. enterica.* We tested the hypothesis that high humidity maximizes *S. enterica's* ability to grow on *X. vitians*-infected plants by measuring *S. enterica's* colonization of healthy and *X. vitians*-infected leaves at 4 days post-arrival. Prior to sampling, the lettuce plants were exposed to high humidity (RH > 90%) for variable amounts of time: 0, 24, or 72 hours (**Figure 2A**). In the absence of high humidity, plants experienced a moderate RH level ~50-70%.

We found that exposure to 72 hours of high humidity (Humidity Treatment III) resulted in greater *S. enterica* populations on *X. vitians*-infected plants compared to *S. enterica* populations on *X. vitians*-infected plants that did not experience high humidity (Humidity Treatment I, **Figure 2B**). In addition, a full 72 hours of exposure to high humidity resulted in greater *S. enterica* populations on *X. vitians*-infected lettuce compared to a fluctuating humidity treatment in which 24 hours of high humidity was followed by 48 hours of drier conditions (Humidity Treatment II). Furthermore, 72 hours of exposure to high humidity resulted in *S.*

enterica growth, based on comparison of *in planta S. enterica* populations to the amount of *S. enterica* cells applied in the inoculum (**Figure 2B**). Meanwhile, 24 hours of exposure to high humidity did not promote *S. enterica* growth but supported *S. enterica* survival at a population level similar to the initial inoculum level. For plants that were not exposed to high humidity at all (Humidity Treatment I), *X. vitians* infection resulted in greater *S. enterica* colonization compared to healthy leaves (**Figure 2B**), demonstrating that *X. vitians* infection influences *S. enterica* survival even in the absence of prolonged high humidity conditions. Contrastingly, *S. enterica* populations on healthy leaves were not influenced by high humidity, and *S. enterica* populations for all healthy plants regardless of humidity treatment were below the inoculum level at 4 days post-*S. enterica* arrival (**Figure 2B**). Additionally, fluctuating humidity (Humidity Treatment II) resulted in a reduction in *X. vitians* populations compared to constant moderate humidity conditions (**Supplementary Figure 2**). *X. vitians* and *S. enterica* populations were not found to be correlated ($P > 0.05$, Pearson correlation), demonstrating that *S. enterica's* response to increasing exposure to high humidity on *X. vitians*-infected leaves is attributed directly to humidity and not *X. vitians* population density. In fact, high humidity introduced during early infection at 3 dpi could stunt *X. vitians* disease progress, based on measurements of *X. vitians* populations and host electrolyte leakage (data not shown).

Having previously shown that water-congestion within the apoplast of tomato leaves is sufficient for *S. enterica* ingress into *Xanthomonas*-infected leaves (32), we hypothesized that water in the air, in the form of humidity, could influence *S. enterica* colonization of UV-protected niches of lettuce. To test this hypothesis, we applied *S. enterica* to lettuce plants infected with *X. vitians*, measured *S. enterica* colonization of leaf samples irradiated with UV after sampling, and compared *S. enterica in planta* populations to that of leaf halves that were

not treated with UV. Half of the lettuce plants were subjected to 24 hours of high humidity (RH > 90%) prior to sampling, whereas the other half were maintained at a lower humidity level for the duration of the experiment (RH ~55-70%, **Figure 3A**).

High humidity (Humidity Treatment ii) resulted in higher *S. enterica* populations at 2 days post-*S. enterica* arrival (**Figure 3B**). Meanwhile, *S. enterica* populations on plants that were not exposed to high humidity (Humidity Treatment i) were below the inoculum level at time of sampling. *S. enterica* populations of UV-irradiated leaves specifically were higher on plants exposed to high humidity (**Figure 3B**). *S. enterica* populations for UV-treated lettuce samples were not statistically different than populations on non-irradiated samples, regardless of whether *X. vitians*-infected lettuce plants were exposed to high humidity (**Figure 3B**). Additionally, the introduction of high humidity at 5 dpi was found to have no effect on *X. vitians* populations and host electrolyte leakage in this experiment (data not shown). These results demonstrate that high humidity prevents *S. enterica* population decline, as evident by *S. enterica* colonization dropping below the inoculum level only for plants that were not exposed to high humidity. High humidity exposure also supported higher levels of *S. enterica* colonization of UV-protected niches within *X. vitians*-infected leaves. However, high humidity was not found to be required for UV protection on *X. vitians*-infected leaves, given that *S. enterica* populations on plants that were not exposed to high humidity were not reduced by UV irradiation.

The state of disease progress at *S. enterica* arrival influences *S. enterica* ingress and survival within *X. vitians*-infected lettuce. We observed that *X. vitians* infection alters the host over time, with visible watersoaking onset occurring at 2 dpi (**Supplementary Figure 3**). During late infection (approximately 5 dpi), *X. vitians*-infected tissue began to appear drier, signaling the onset of tissue necrosis (**Supplementary Figure 3**). These observations prompted the question

of whether an ideal window during *X. vitians* infection exists that optimizes *S. enterica* growth and apoplast colonization. To test the hypothesis that *X. vitians* infection progress influences the fate of *S. enterica*, *S. enterica* cells were applied to lettuce plants at various states of *X. vitians* infection. Specifically, *S. enterica* was applied to lettuce plants at 1-5 days post-infiltration (dpi) of *X. vitians* (**Figure 4A**). In addition, *S. enterica* was applied to healthy plants that had been infiltrated with H_2O 4 days prior to arrival as a control—these healthy leaves had an apparently dry apoplast at time of *S. enterica* arrival. *S. enterica* colonization was measured on UV-irradiated and non-irradiated lettuce samples at 2 days post-*S. enterica* arrival to determine how *X. vitians* infection progress impacts *S. enterica* survival and *S. enterica's* ability to colonize UV-protected niches.

S. enterica colonization of non-irradiated lettuce samples revealed that arrival of *S. enterica* at 1 dpi (Treatment X1) resulted in lower *S. enterica* populations than those on healthy (Treatment H) lettuce leaves (**Figure 4B**). For Treatments H and X1, *S. enterica* populations recovered from both non-irradiated and UV-irradiated leaves were significantly lower than the *S. enterica* inoculum level (**Figure 4B**). However, when *S. enterica* arrived between 2-4 dpi (Treatments X2-X4), *S. enterica* populations persisted at levels similar to the inoculum level, demonstrating that arrival of *S. enterica* on water soaked, *X. vitians*-infected leaves can prevent *S. enterica* population decline. Additionally, we observed that when comparing *S. enterica* populations on adjacent lettuce leaves, the relatively older leaves had significantly lower *S. enterica* populations ($P < 0.001$, Welch T-test).

The infection state of the lettuce apoplast impacted *S. enterica*'s ability to colonize the apoplast. When *S. enterica* was applied to healthy H_2O-infiltrated leaves (Treatment H) or *X. vitians*-infected leaves prior to the onset of water soaking (Treatment X1), the absence of an

aqueous apoplast in both treatments corresponded with the lowest *S. enterica* colonization of UV-protected niches at 2 days post-*S. enterica* arrival (**Figure 4b**). Contrastingly, *S. enterica* colonization of UV-protected niches was maximized following arrival on *X.* vitians-infected lettuce between 2-4 dpi, at which point water soaking, evident by enhanced translucency of the infiltrated area (**Supplementary Figure 3**), has occurred.

Confocal laser scanning microscopy (CLSM) was utilized to visualize *S. enterica* localization *in planta*. GFP-labelled *S. enterica* cells were applied at a relatively high level (~3 x 10^5 CFU/droplet) to the surface of leaves infiltrated with H_2O 4 days prior to arrival or *X. vitians* cells 1 or 4 days prior to arrival (analogous to **Figure 4A** Treatments H, X1, and X4, respectively). Leaves were then imaged at 2 days post-*S. enterica* arrival to determine whether *S. enterica* had successfully colonized the apoplast for any of these three treatments. For H_2O-infiltrated leaves, *S. enterica* colonization of the apoplast was not detected (e.g. **Figure 5,** panel D and J), although *S. enterica* cells were found to be localized within stomatal openings at the surface in 56% (N = 9) of fields imaged (e.g. **Figure 5,** panel A). By contrast, *S. enterica* colonization within substomatal areas of the apoplast was observed for *X. vitians*-infected leaves. Congruous to *in planta S. enterica* colonization results that showed *S. enterica* colonization of the UV-protected niches was higher following arrival at 4 dpi compared to 1 dpi (**Figure 4b**), CLSM revealed greater *S. enterica* colonization of the apoplast during late infection compared to early infection. *S. enterica* arrival at 4 dpi resulted in relatively frequent colonization of substomatal spaces within the apoplast, with 73% (N = 15) of image fields showing evidence of *S. enterica* ingress (e. g. **Figure 5**, panel F and L). In comparison, arrival of *S. enterica* on *X. vitians*-infected leaves as early at 1 dpi resulted in rare apoplast colonization (15% of fields imaged; N = 13), despite 100% of fields showing *S. enterica* interactions with surface stomatal

openings. **Figure 5,** panel B representatively illustrates this, with three stomatal openings clearly containing *S. enterica* cells at the surface, but only one of those three stomata containing *S. enterica* within its substomatal space (**Figure 5**, panels E and K). Our *S. enterica* population data (**Figure 4B**) and CLSM results support that *S. enterica* arrival to leaves with established *X. vitians* infection results in enhanced colonization of the UV-protected apoplast.

Infection progress coincided with *S. enterica's* ability to grow. We found that *X. vitians* populations and host electrolyte leakage had reached a maximum level during late infection (5-7 dpi, **Supplementary Figures 4A-B**). Arrival of *S. enterica* between 2-5 dpi resulted in the highest proportions of lettuce samples for which *S. enterica* colonization exceeded the inoculum, indicating high incidence rates of *S. enterica* replication (**Table 2**). For Treatment X4 in particular, arrival of *S. enterica* at 4 dpi resulted in 72% of non-irradiated samples and 61% of UV-irradiated samples being colonized with higher *S. enterica* populations than the inoculum (**Table 2**). However, *S. enterica* arrival during late infection at 5 dpi resulted in UV-protected *S. enterica* populations that were significantly lower than the inoculum (**Figure 4B**). Altogether, these results provide evidence that there is a window of time during *X. vitians* infection when *S. enterica* persistence and potential to colonize UV-protected niches is optimized—this window opens at water soaking onset and closes during necrosis onset later in disease progression.

***S. enterica* grows in a sucrose-rich environment in the presence of *X. vitians*.** Sucrose is one of the most prevalent carbohydrate sources available in plant tissues (36), yet sucrose is a poor carbon source for *S. enterica* and not utilizable by most *S. enterica* strains (37). Having observed that *S. enterica* successfully replicates *in planta* in the presence of an established *X. vitians* infection (**Figures 1- 2, Table 2**) and that *S. enterica* (shown in green) and *X. vitians* cells (shown in yellow) localize closely to each other *in planta* (**Figure 5**), we hypothesized that *X.*

vitians catabolism of plant compounds directly aids in *S. enterica* replication via 1) cell-dependent contact or 2) compounds secreted by *X. vitians.* To test this hypothesis, we developed an *in vitro* experiment in which *S. enterica* cells were introduced to turbid *X. vitians* cultures that had grown in M9 minimal media containing only sucrose as a carbon source (M9 sucrose) for 24 or 48 hours. Cell-free supernatant (CFS) was produced from the *X. vitians* cultures and also introduced to *S. enterica* to compare the effect of intact *X. vitians* cells versus media containing secreted compounds. In addition, *S. enterica* cells were added to M9 sucrose or M9 glucose media alone as negative and positive controls.

We found that *S. enterica* cells incubated in co-culture with *X. vitians* cells or CFS replicated to population levels that exceeded the initial cell concentrations after 28 hours of incubation (**Figure 6**). By contrast, *S. enterica* incubation in M9 sucrose in monoculture (negative control; light blue bar) resulted in no growth after 28 hours. Specifically, introduction of *S. enterica* to *X. vitians* cells that had been growing in M9 sucrose for 24 hours (yellow bar) resulted in *S. enterica* cell concentrations that were greater than the negative control and greater than either CFS treatment (emerald and dark green bars), showing evidence for bacterial cross-feeding supporting *S. enterica* growth. Meanwhile, introducing *S. enterica* to a 48-hour *X. vitians* culture (orange bar) resulted in higher variability in *S. enterica* cell concentration than the 24-hour culture (yellow bar), and resulted in similar *S. enterica* concentrations to both the negative control and CFS treatments. Notably, the 24-hour *X. vitians* culture exhibited additional *X. vitians* growth during the 28-hour period (**Supplementary Figure 5**), whereas the 48-hour culture did not, suggesting that replicating *X. vitians* cells confer the greatest nutritional benefit to *S. enterica.*

DISCUSSION

In this study, we investigated the relationship between an enteric pathogen and a plant pathogen colonizing a lettuce host and examined how environmental conditions may act on the enteric pathogen's ability to benefit from plant disease. *S. enterica* was found to thrive on lettuce plants affected by bacterial spot disease (**Figures 1B, 2B,** and **4B**). The potential for *S. enterica* to survive and replicate on lettuce plants was linked to *X. vitians* infection progress (**Figure 4B**) and exposure to high humidity **(Figure 2B**), with our results showing that the combination of water soaking caused by disease and high humidity supported the highest *S. enterica* populations *in planta***.** Lastly, by measuring *S. enterica* growth in a sucrose-based minimal media, we identified bacterial cross-feeding as a nutrient source for *S. enterica* in association with *X. vitians* **(Figure 6**)**.**

Water—as a habitat, vehicle for bacteria movement, and vital source of moisture needed for growth—influences *S. enterica's* lifestyle in agricultural environments. Irrigation water is a common reservoir for *S. enterica* (14, 38, 39) and has been previously implicated in enteric pathogen outbreaks associated with raw produce (40, 41). The prevalence of *S. enterica* in bodies of water, including irrigation ponds, has been shown to correlate with rainfall (42–44) and summer climates (42), demonstrating how precipitation and seasonality influence *S. enterica* abundance. *S. enterica's* presence in irrigation water offers the potential for *S. enterica* to migrate to leaves and fruit. Sprinkler and microsprinkler irrigation are commonly used for agricultural fields in the United States (45, 46), including California and Arizona where most leafy greens are cultivated (47). However, this form of irrigation has inherent risk. Splash dispersal has been implicated as a vehicle for microbial dispersal on plant foliage (48, 49).

Regarding food safety, previous work has supported subsurface irrigation as a safer method for preventing colonization of plants with enteric pathogens (50, 51). Our previous study, which modelled a contamination route via splash dispersal, found that *S. enterica* applied to tomato leaves via surface droplet could become internalized into the leaf apoplast (24). A wet apoplast, either abiotically water-logged or water-soaked from phytobacterial disease, could enable the physical absorption of surface water and passive ingress of *S. enterica* in a non-flagellar motility-dependent manner (24).

This study builds on our previous work using the splash dispersal model by introducing lettuce as a novel host that supports *S. enterica* growth in the presence of *Xanthomonas* and establishing how humidity can affect the fate of *S. enterica* in concert with disease. Humidity is recognized as a driver of plant-bacterial interactions (52). High humidity has been shown to correlate with increases in phytobacterial disease (3, 53), including bacterial spot caused by *X. vitians* (54). In addition, enteric pathogen populations such as *S. enterica* have been shown to exhibit slower population decline on healthy plants exposed to high humidity (55–58). To determine how humidity impacts *S. enterica's* ability to benefit from *X. vitians* disease, we tested the impact of a 24-hour and 72-hour exposure to >95% RH conditions (**Figure 2A**). Our data showed that *S. enterica* populations were higher on *X. vitians*-infected plants exposed to high humidity, yet unaffected by humidity on healthy plants **(Figure 2B).** Short-term exposure to >95% RH prevented *S. enterica* population decline on *X. vitians*-infected lettuce, but multi-day exposure to >95% RH (Treatment III) enabled *S. enterica* replication on *X.* vitians-infected lettuce (**Figure 2B**). *S. enterica* ingress still occurred on *X. vitians*-infected leaves without high humidity exposure (**Figure 3B**), demonstrating that migration to the apoplast did not explain *S. enterica's* ability to replicate under high humidity conditions. Meanwhile, *X. vitians* replicated

successfully *in planta* at 50-70% RH (data not shown), suggesting infection liberates apoplast nutrients even under moderate humidity conditions. Therefore, these results support the idea that extended high humidity shifts the nutrient composition of a *X. vitians*-infected apoplast in favor of nutrients *S. enterica* can successfully metabolize. Whether high humidity conditions must be sustained continuously or if the accumulation of multiple high humidity events is sufficient to promote *S. enterica* growth on *Xanthomonas*-infected leaves should be determined through future work.

Stomata serve as critical entry points for bacteria in the phyllosphere (59). *Xanthomonas* infection has been previously shown to promote ingress of surface bacteria into the apoplast of tomato leaves via stomata (24). In this study, we observed that *S. enterica* became internalized within *X. vitians*-infected lettuce leaves, based on evidence of *S. enterica* protection from UV irradiation (**Figures 1B, 3B. 4B**) and colonization of *S. enterica* within substomatal chambers of the apoplast (**Figure 5**). At the micro-scale, CLSM revealed that *S. enterica* ingress varied among stomata. *S. enterica* colonization of stomatal openings, likely influenced by *S. enterica* chemotaxis (60), did not always result in colonization of the lower substomatal space (**Figure 5**). Syringe infiltration of *X. vitians* into the apoplast is assumed to create a consistent dispersal of cells within the infiltration zone (61). However, the mottled appearance of the infection area suggests that infection onset is not consistent throughout the infection zone (**Supplementary Figure** 3). Clonal *Pseudomonas syringae* cells have been shown to exhibit heterogeneity in T3SS expression within the apoplast (62). We speculate that differences in gene expression among *X. vitians* microcolonies in combination with heterogeneity of plant tissue may differentially impact *S. enterica's* interactions with stomata.

S. enterica was also influenced by the temporal dynamics of *X. vitians* disease progress. Our study investigated the fate of *S. enterica* following arrival at various times during *X. vitians* infection progress and essentially used *S. enterica* as a biological reporter for changes to a *X. vitians*-infected apoplast. Although *X. vitians* infection was found to be generally beneficial to *S. enterica*, (**Figures 1-2, 5**), the early infection court was a hostile environment for *S. enterica* (**Figure 4**). Arrival of *S. enterica* during early infection at 1 dpi led to a reduction in *S. enterica* populations, sometimes to non-detectable levels two days later (**Figure 4,** Treatment X1). We hypothesize that *S. enterica's* response in the host experiencing an early *X. vitians* infection is attributed to the host defense response. Infiltration of bacteria into the apoplast can elicit MAMP (microbe-associated molecular pattern)-triggered immune (MTI) response (63). Recognition of phytobacterial invaders may trigger phytohormone signaling and apoplast accumulation of reactive oxygen and nitrogen species (64, 65), which has been demonstrated to limit *S. enterica* growth *in planta* (66). *S. enterica* flagellin is a known elicitor of MTI (Garcia and Hirt 2014), and *S. enterica* may benefit from suppression of MTI by phytobacteria based on its ability to thrive in the presence of disease (24). However, as demonstrated in our study, the timing of co-colonization is important. Previously, it was shown that *S. enterica* achieved greater colonization of tomato foliage when seeds were co-treated with *Xanthomonas euvesicatoria* (formerly known as *X. campestris* pv. vesicatoria) via inoculated soil (25). However, at the early tomato seedling stage, *S. enterica* populations were lower in the presence of *X. euvesicatoria*. Additionally, Potnis et al. (67) demonstrated that *S. enterica* persistence on tomato plants is enhanced by *Xanthomonas perforans* infection but diminished by an avirulent *X. perforans* strain that can neither cause disease, nor suppress MTI. Hence, *S. enterica* responds to both aggravation and suppression of host defenses by phytobacteria.

Phytobacterial infection likely also supports *S. enterica* survival in the phyllosphere by providing nutrients for growth. *S. enterica* metabolism is incredibly flexible, enabling it to utilize many different types of carbon and nitrogen sources in an animal host, even at scant concentrations (68). However, *S. enterica* growth is disadvantaged on plants. *S. enterica* cannot liberate nutrients from plant cells on its own because it lacks cell-wall degrading enzymes and the ability to manipulate the host environment via type-III effectors (69–71). Additionally, as previously mentioned, *S. enterica* is rarely able to utilize sucrose, one of the most abundant carbon sources on plants (72, 73). On healthy plants, *S. enterica* can persist in the phyllosphere for months (16), but exposure to abiotic and biotic stresses, including low nutrient availability, generally results in *S. enterica* population decline (15, 23, 74). Contrastingly, in the presence of *X. vitians* infection, *S. enterica* replicates *in planta* (**Figures 1-2, Table 2**), indicating that infection increases nutrient availability for *S. enterica*. However, as *X. vitians* infection leads to tissue necrosis (**Supplementary Figure 3**) and presumably a decrease in available nutrients, *S. enterica* populations in a *X. vitians*-infected apoplast decline, as evident by *S. enterica* populations dropping below the original inoculum level following arrival during at 5 dpi (**Figure 4b**, Treatment X5). Late in infection, phytobacterial pathogens migrate away from necrotic tissue, presumably to locate available nutrients or to disperse to another host. Future studies will examine the dispersal of *S. enterica* beyond initially infected leaf tissue to determine if the human and plant pathogens co-migrate as they seek nutrient sources.

Nutrients metabolized by *S. enterica* in the presence of disease are likely host derived. *Xanthomonas* replication in the apoplast during infection is driven by leakage of plant cell constituents (8, 23), which *S. enterica* may be able to easily scavenge in the presence of *X. vitians* infection. However, we also hypothesize that bacterial cross-feeding (75) between *S.*

enterica and *X. vitians* contributes to *S. enterica's* ability to grow *in planta.* Previously, cross-feeding has been demonstrated to influence nutritional niches, thus affecting phytobacterial community composition (76). Our CLSM observation that *S. enterica* cells were observed near *X. vitians* cells *in planta* (**Figure 5**) led us to test whether these cells interact directly via nutrient flow from *X. vitians* to *S. enterica.* The results of our *in vitro* experiment demonstrated that although sucrose is a poor nutrient source for *S. enterica*, *S. enterica* could grow in sucrose-based minimal media if replicating *X. vitians* cells were present (**Figure 6**). Cell-free supernatant (CFS) derived from *X. vitians* cultures did not support *S. enterica* growth as well as cultures containing *X. vitians* cells. *Xanthomonas* strains are known for producing sticky extracellular polysaccharides, including xanthan gum (77), which may have contributed to the exclusion of nutrients during the filtering of *X. vitians* culture supernatant. The identity of the nutrients utilized by *S. enterica* via cross-feeding are unknown, but could include amino acids, carbohydrates, or organic acids (72). Future studies may elucidate the carbon sources metabolized by *S. enterica in planta* in the presence of disease.

Previous work investigating relationships between enteric pathogen persistence, phytopathogen infection, and environment has generally studied 1-2 of these elements in isolation. In reality, interplay between enteric pathogens, plant pathogens, and climate factors are all likely to influence the microbial communities in the phyllosphere. In this study, we found that *S. enterica* survival on lettuce is enhanced by bacterial spot disease caused by *X. vitians* and that *S. enterica* proliferation was conditional on a variety of factors **(Figure 7).** Overall, it was the combination of high humidity exposure and the timing of *S. enterica* arrival with respect to bacterial spot disease progress that maximized *S. enterica* survival and access to nutrients in an otherwise inhospitable host. As climate patterns continue to evolve, microbial dynamics in the

phyllosphere will evolve too, underlining the importance of studying host-microbial interactions under various environmental conditions.

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M.H.D and J.D.B. developed the experiments. M.H.D., D.N., and S.C.Z. performed the experiments. M.H.D. and J.D.B. wrote the manuscript, and all authors reviewed the manuscript. M.H.D. created the original illustrations.

TABLES

TABLE 1. Bacterial strains used in this study.

Strain name and description	Reference or source
Xanthomonas vitians L7	J. Jones
Xanthomonas vitians L7; Cm^R transformed with pNO-mCherry	This study
Escherichia coli; Cm^R; pNO-mCherry	Z. Gitai
Salmonella enterica serovar Typhimurium 14028s; Nal^R	(79)
Salmonella enterica serovar Typhimurium 14028s; Kan^R; transformed with pKT-Kan; GFP	(80)

^{R} = resistance to chloramphenicol (Cm), nalidixic acid (Nal), or kanamycin (Kan)

TABLE 2: Incidence[a] of *S. enterica in planta* growth depending on *X. vitians* infection progress at time of *S. enterica* arrival.

UV Treatment Group[c]	Infiltration Treatment Group[b]					
	H	**X1**	**X2**	**X3**	**X4**	**X5**
Non-irradiated	7% A[d]	17% AB	56% BC	56% BC	72% C	50% BC
UV-irradiated	0% a	0% a	33% ab	50% b	61% b	28% ab

[a]Percentage of samples for which *in planta S. enterica* colonization exceeds inoculum.

[b,c]Treatment groups refer directly to experiment described in **Figure 2**.

[d]Letters indicate significance between treatment groups among non-irradiated leaves (uppercase) and among UV-treated leaves (lowercase) based on Fisher's exact tests ($P < 0.05$).

FIGURE LEGENDS

Figure 1: *X. vitians* infection facilitates *S. enterica* replication and colonization of UV-protected niches *in planta*. **(A)** Schematic of experimental design. Lettuce plants were divided into four treatment groups. *S. enterica* (*Se*) cells were applied to leaves previously infiltrated with H_2O or *X. vitians* (*Xvit*) at 4 dpi, and plants were sampled at 1- or 3-days post-*Se* arrival (dpa). **(B)** *Se* colonization of H_2O-infiltrated (blue boxplots) and *X. vitians*-infiltrated (orange boxplots) plants. Darker boxplot colors for 3 dpa data signify exposure to high humidity (>90% RH) prior to sampling. Data represents sum of *Se* populations measured from surface swab and homogenized lettuce for each leaf. Leaves were irradiated with UV immediately prior to sampling (unfilled triangle points) or left non-irradiated (filled circle points). Letters above the boxplots indicate significance between treatment groups among leaves sampled at 1 dpa (uppercase) and among leaves sampled at 3 dpa (lowercase) based on Welch-ANOVA and *post hoc* Games-Howell means comparison tests ($P < 0.05$). Daggers (†) indicate significant increases in *Se* colonization between 1 dpa and 3 dpa within the same infiltration treatment and UV treatment groups based on two-sample T-tests ($P < 0.05$). Solid line indicates average *Se* inoculum applied to each sampled area, and dashed line indicates limit of detection (LOD). Values below LOD are defined in Materials and Methods. $N = 30$ leaves/treatment group; data is pooled from five independent experimental replicates.

Figure 2: High humidity (>90% RH) exposure and *X. vitians* infection additively enhance *S. enterica* colonization of lettuce. **(A)** Schematic of experimental design. Lettuce plants were divided into six treatment groups. *S. enterica* (*Se*) cells were applied to leaves previously

infiltrated with H_2O or *X. vitians* (*Xvit*) at 3 days post-infiltration. Plants were sampled following 0, 24, or 72 hours of exposure to high humidity (>90% RH) at 4 days post-*Se* arrival (Humidity Treatments I, II, and III, respectively). **(B)** *Se* colonization of H_2O-infiltrated (blue boxplots) and *X. vitians*-infiltrated (orange boxplots) plants at 2 days post-arrival (dpa) in response to varying humidity treatments. Letters above the boxplots indicate significance between treatment groups based on Welch-ANOVA and *post hoc* Games-Howell means comparison tests ($P < 0.05$). Asterisks indicate that *Se in planta* colonization is significantly less than (*<) or greater than (*>) inoculum based on one-sample Wilcoxon tests ($P < 0.05$). Solid line indicates average *Se* inoculum applied to each sampled area, and dashed line indicates limit of detection (LOD). < LOD values are defined in Materials and Methods. $N = 24$ leaves/treatment group; data is pooled from three independent experimental replicates.

Figure 3: High humidity (>90% RH) exposure is not required for *S. enterica* to colonize UV-protected niches of *X. vitians*-infected lettuce. **(A)** Schematic of experimental design. Lettuce plants were divided into two treatment groups. *S. enterica* (*Se*) cells were applied to leaves previously infiltrated with *X. vitians* (*Xvit*) at 3 days post-infiltration. Plants were sampled following 0 or 24 hours of exposure to high humidity (>90% RH) prior to sampling (Humidity Treatments i and ii, respectively). **(B)** *S. enterica* colonization of *X. vitians*-infiltrated plants at 2 days post-arrival (dpa) in response to varying humidity treatments. Leaf halves were irradiated with UV immediately prior to sampling (unfilled triangle points) or left non-irradiated (filled circle points). Letters above the boxplots indicate significance between treatment groups based on Welch-ANOVA and *post hoc* Games-Howell means comparison tests ($P < 0.05$). Asterisks (*) indicate that *S. enterica in planta* colonization is significantly less than inoculum based on

one-sample Wilcoxon tests ($P < 0.05$). Solid line indicates average *S. enterica* inoculum applied to each sampled area, and dashed line indicates limit of detection (LOD). $N = 18$ leaves/treatment group; data is pooled from three independent experimental replicates.

Figure 4: The fate of *S. enterica* on lettuce leaves is influenced by *X. vitians* infection progress at *S. enterica* arrival. **(A)** Schematic of experimental design. Lettuce plants were divided into six treatment groups. *S. enterica* (*Se*) cells were applied to leaves previously infiltrated with H_2O or *X. vitians* (*Xvit*) at various days post-infiltration (dpi). Plants were sampled following 24 hours of exposure to high humidity (>90% RH). **(B)** *Se* colonization of H_2O-infiltrated (blue boxplots, Treatment H) and *X. vitians*-infiltrated (orange boxplots, Treatments X#) plants at 2 days *post-Se* arrival (dpa). Leaf halves were irradiated with UV immediately prior to sampling (unfilled triangle points) or left non-irradiated (filled circle points). Letters above the boxplots indicate significance between treatment groups among non-irradiated leaves (uppercase) and among UV-treated leaves (lowercase) based on Welch-ANOVA and *post hoc* Games-Howell means comparison tests ($P < 0.05$). Asterisks (*) indicate that *Se in planta* colonization is significantly less than inoculum based on one-sample Wilcoxon tests ($P < 0.05$). Solid line indicates average *Se* inoculum applied to each sampled area, and dashed line indicates limit of detection (LOD). N = 15-18 leaves/treatment group; data is pooled from three independent experimental replicates.

Figure 5: *S. enterica* colonizes the apoplast of *X.* vitians-infected lettuce leaves. CLSM images depict GFP-labelled *S. enterica* colonization of the lettuce surface **(A-C, G-I)** and apoplast (D-F, J-L) for three sites **(columns A-C)** representative of three treatments and imaged at the given z-

depths (μm). *S. enterica* cells were applied to the surface of leaves previously infiltrated with H_2O (column A) or mCherry-labelled *X. vitians* cells **(columns B-C)**. Horizontal labels describe infiltration treatment and days-post infiltration (dpi) at time of *S. enterica* arrival. **A-F**: merged channels (GFP, green; mCherry, yellow; DIC, bright-field). **G-L**: GFP channel only for A-F images, respectively. Stomata (S) and substomatal *S. enterica* cells (sS) are highlighted with white arrows and white triangles. Scale bar = 20 μm.

Figure 6: *X. vitians* metabolism of sucrose produces nutrients *S. enterica* can utilize for growth. *S. enterica* cells were added to fresh M9 sucrose (M9 suc, light blue points) or M9 glucose (M9 glc, dark blue points), mixed with *X. vitians* (*Xvit*) cell cultures grown for 24 (yellow points) or 48 hours (orange points) in M9 sucrose, or mixed with *Xvit* cell-free supernatant (CFS, emerald green & dark green points) at time (t) = 0 hr. Dotted line represents the initial *S. enterica* cell concentration at t = 0 hr. Each horizontal colored bar represents the average *S. enterica* cell concentrations of four independent experiments at t = 28 hr. Letters above the boxplots indicate significance between treatment groups based on Welch-ANOVA and *post hoc* Games-Howell means comparison tests ($P < 0.05$). Daggers (†) indicate a significant increase in *S. enterica* cell concentration compared to t = 0 hr based on two-sample T-tests ($P < 0.05$).

Figure 7: The fate of *Salmonella enterica* in the phyllosphere is influenced by *Xanthomonas* infection progress and humidity. **(A)** Early *Xanthomonas* infection. *X. vitians* (yellow bacteria) begin to modify the host environment, but the infection court is not yet habitable for *S. enterica.* **(B)** Established *Xanthomonas* infection. *S. enterica* (green bacteria) successfully colonize the apoplast of *Xanthomonas*-infected leaves via ingress through stomata. **(C)** *Xanthomonas*

infection plus high humidity. High humidity (>90% RH) promotes replication of *S. enterica* within a *Xanthomonas*-infected apoplast, though is not required for *S. enterica* ingress.

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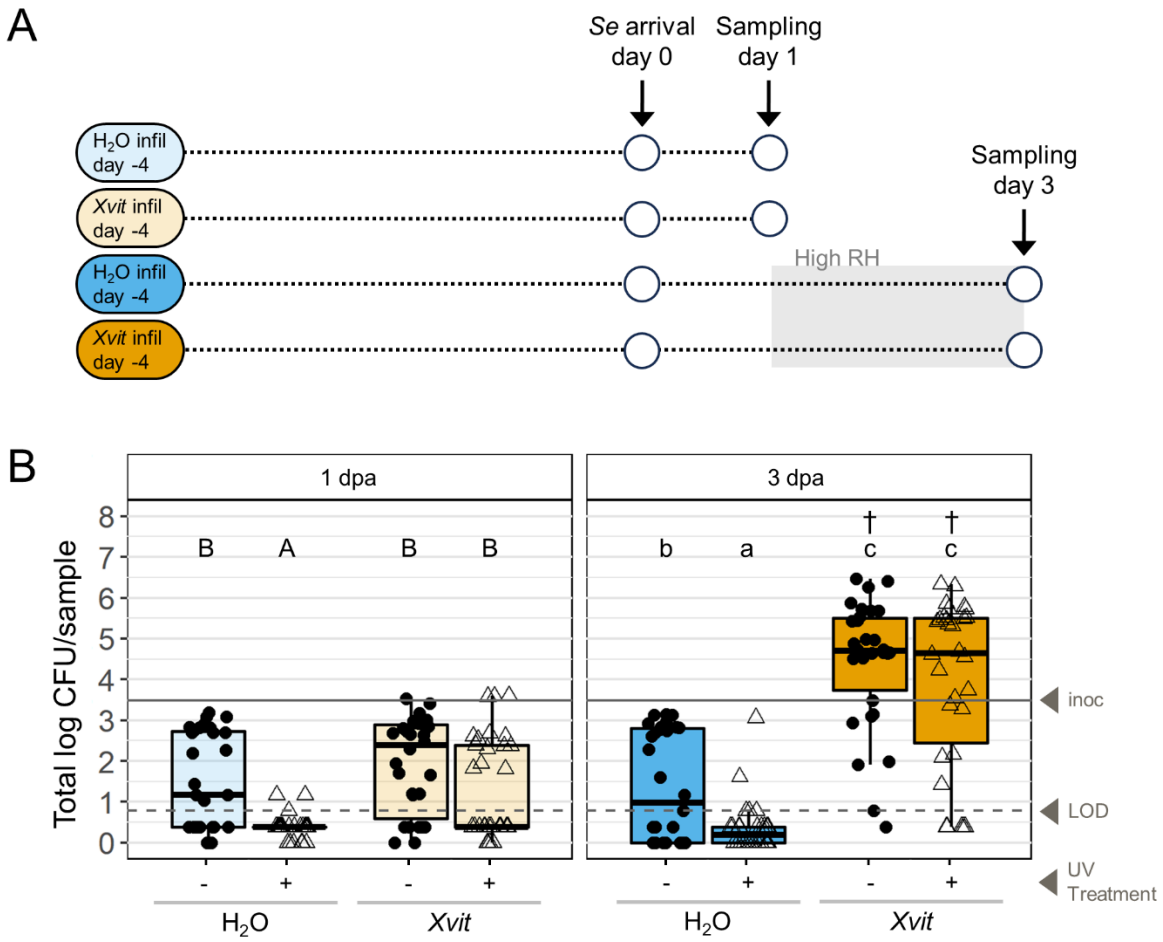
A
Se arrival
day 0
Sampling
day 1
Sampling
day 3
H2O infil
day -4
Xvit infil
day -4
H2O infil
day -4
Xvit infil
day -4
High RH
B
1 dpa
3 dpa
Total log CFU/sample
B
A
B
B
b
a
c
c
inoc
LOD
UV
Treatment
H2O
Xvit
H2O
Xvit

Figure 1.

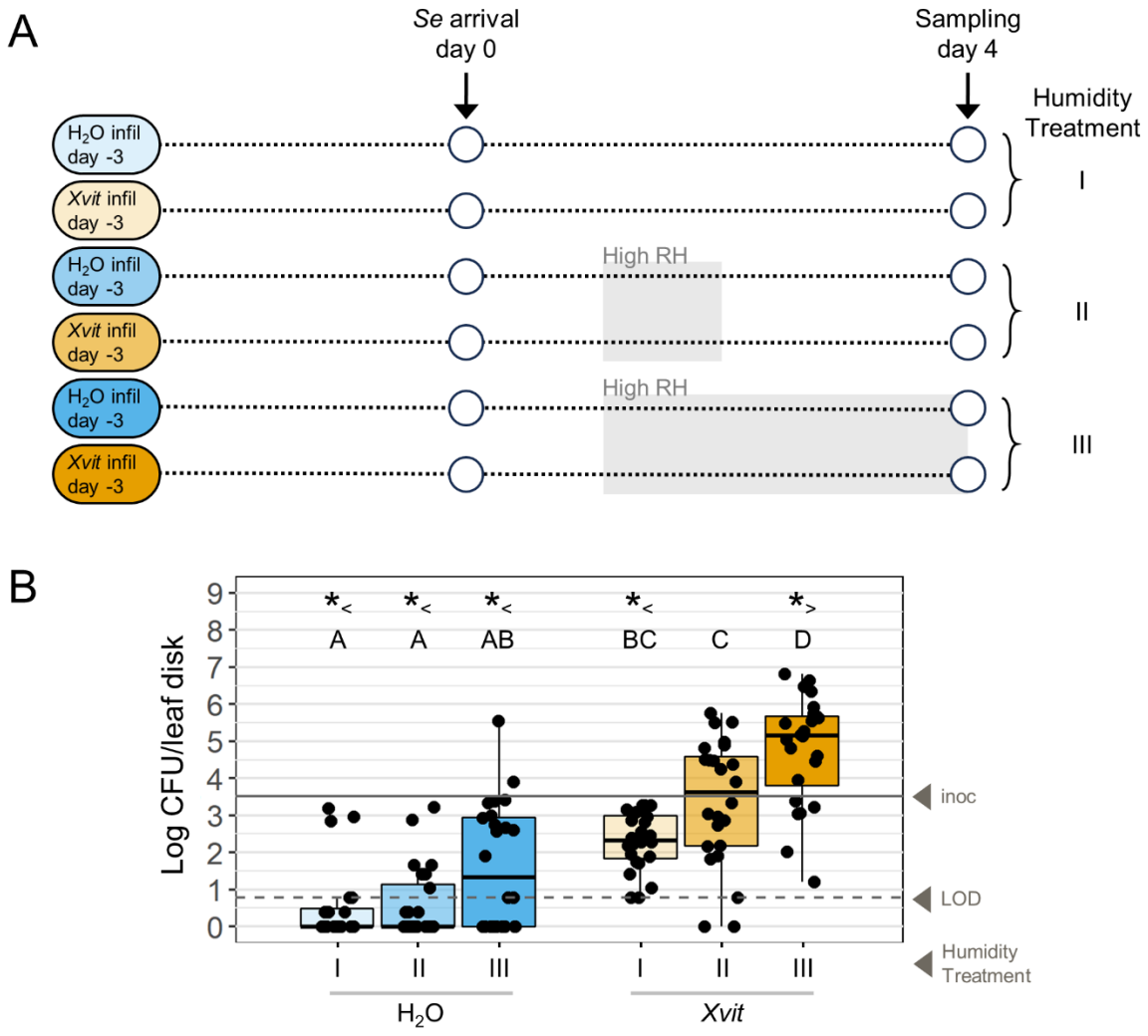
A
Se arrival
day 0
Sampling
day 4
Humidity
Treatment
H2O infil
day -3
Xvit infil
day -3
High RH
I
II
III
B
Log CFU/leaf disk
A
A
AB
BC
C
D
inoc
LOD
Humidity
Treatment
H2O
Xvit

Figure 2.

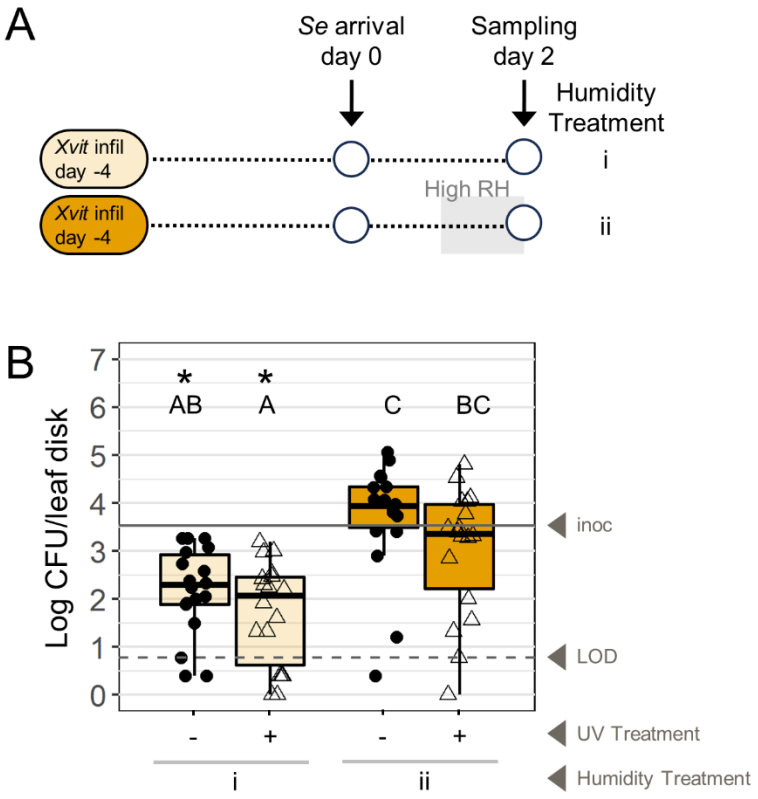
A
Se arrival
day 0
Sampling
day 2
Humidity
Treatment
Xvit infil
day -4
Xvit infil
day -4
High RH
i
ii
B
Log CFU/leaf disk
7
6
5
4
3
2
1
0
*
AB
*
A
C
BC
inoc
LOD
-
+
-
+
UV Treatment
i
ii
Humidity Treatment

Figure 3.

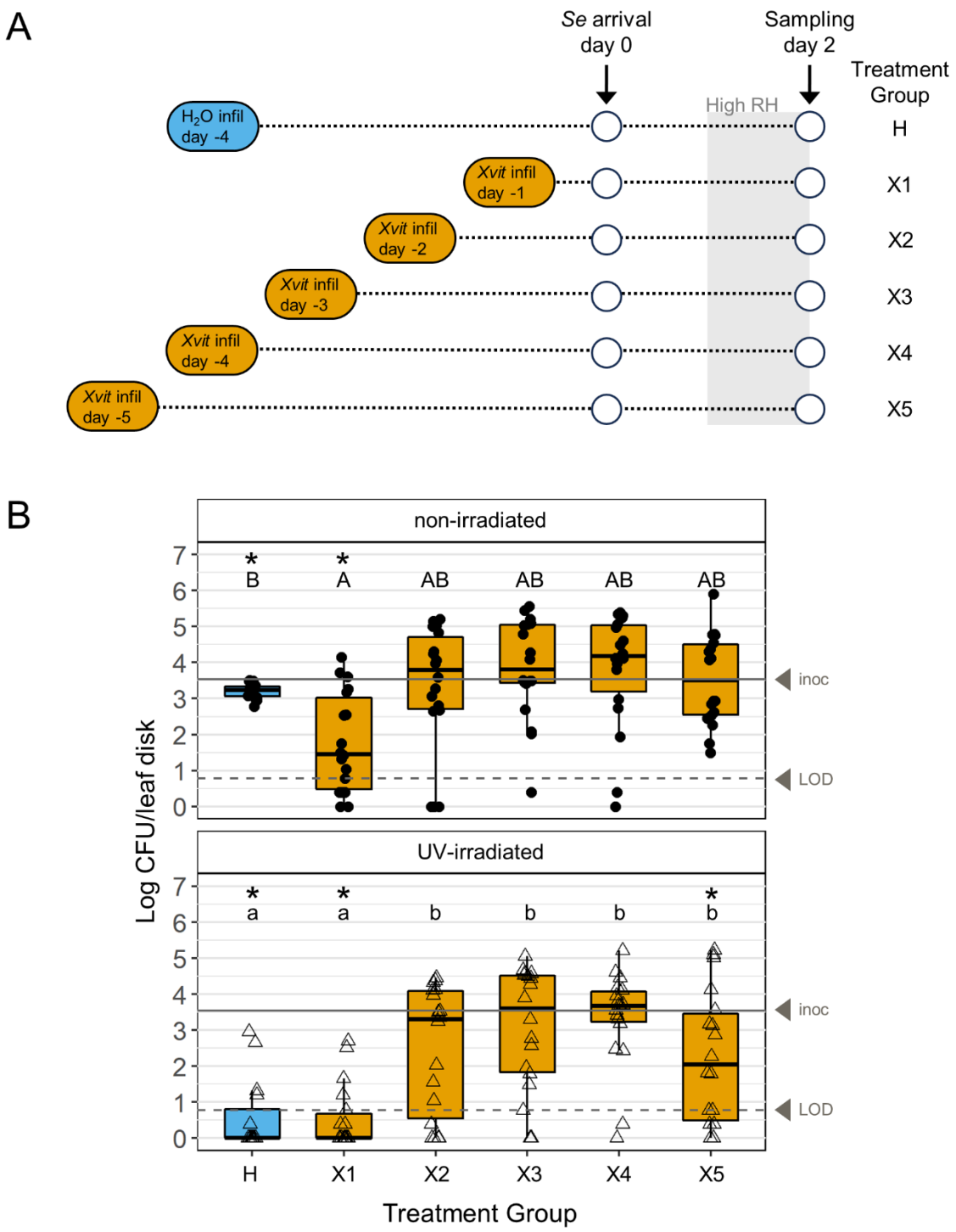
A
Se arrival
day 0
Sampling
day 2
Treatment
Group
High RH
H2O infil
day -4
Xvit infil
day -1
Xvit infil
day -2
Xvit infil
day -3
Xvit infil
day -4
Xvit infil
day -5
H
X1
X2
X3
X4
X5
B
non-irradiated
UV-irradiated
Log CFU/leaf disk
inoc
LOD
Treatment Group

Figure 4.

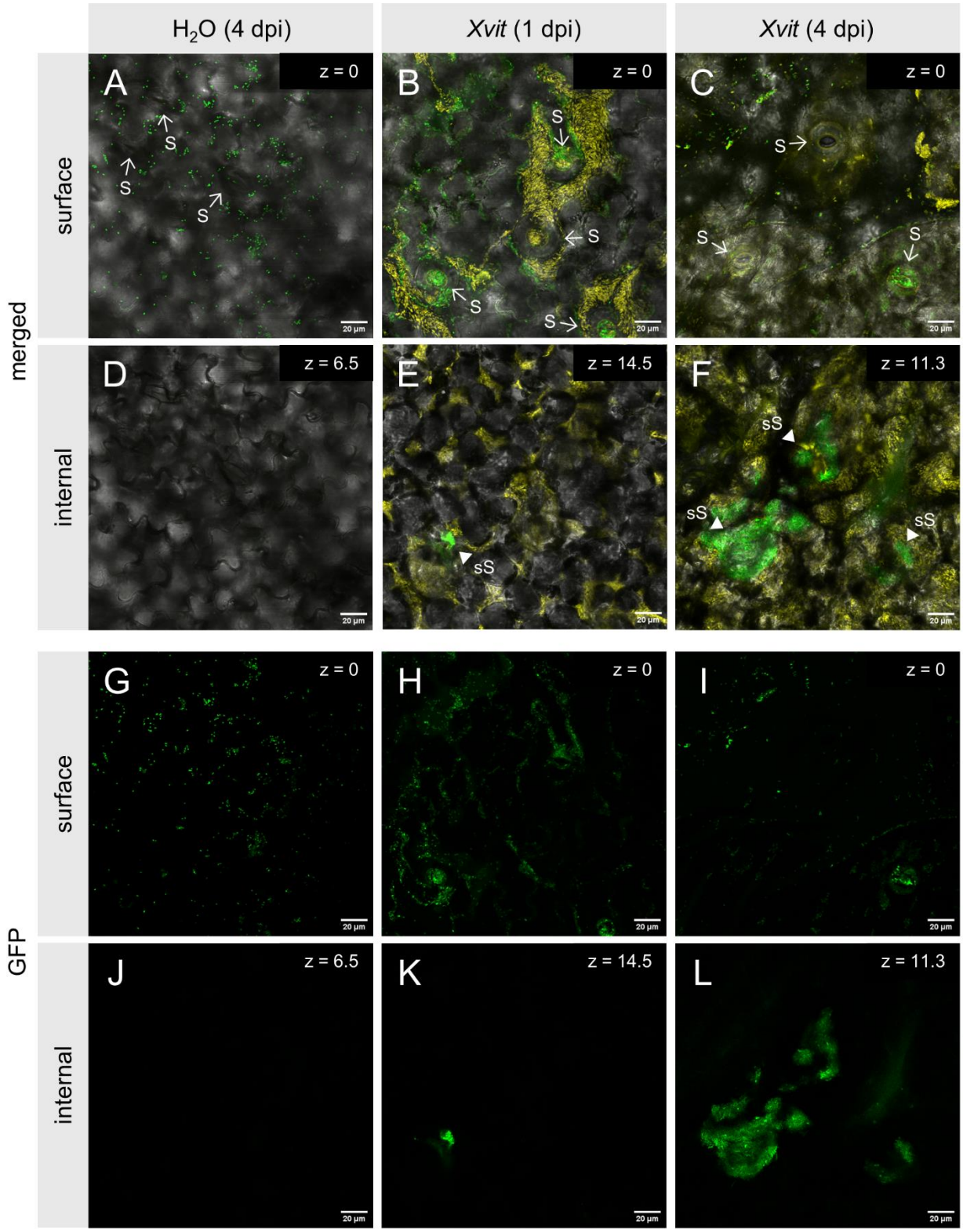
H2O (4 dpi)
Xvit (1 dpi)
Xvit (4 dpi)
merged
GFP
surface
internal
A
B
C
D
E
F
G
H
I
J
K
L
z = 0
z = 6.5
z = 14.5
z = 11.3
s
sS
20 µm

Figure 5.

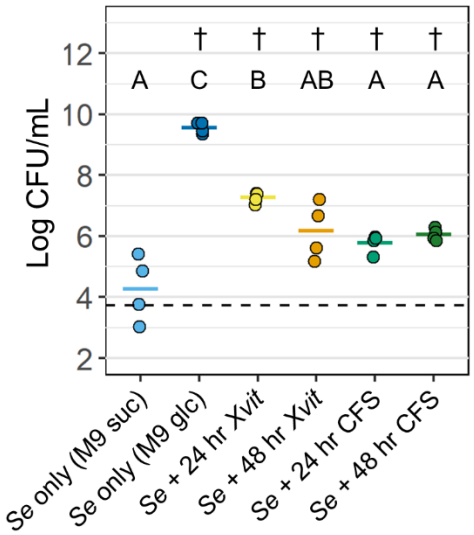
Log CFU/mL
12
10
8
6
4
2
A
C
B
AB
A
A
†
†
†
†
†
Se only (M9 suc)
Se only (M9 glc)
Se + 24 hr Xvit
Se + 48 hr Xvit
Se + 24 hr CFS
Se + 48 hr CFS

Figure 6.

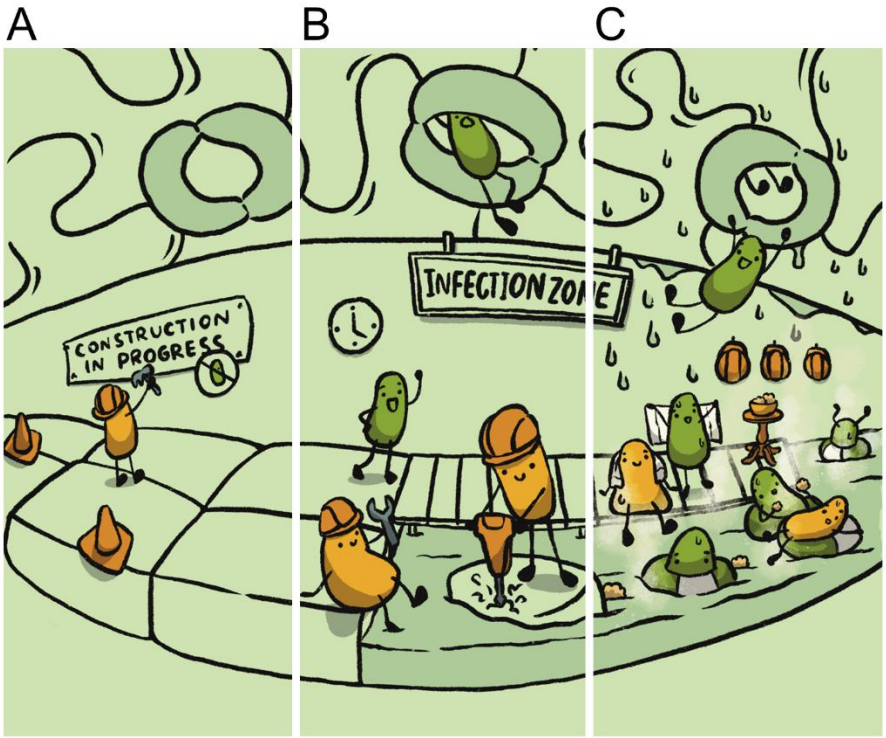

Figure 7.