Epiphytic Survival of *Pantoea ananatis* on *Richardia scabra* L. in Georgia

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Abstract

Pantoea ananatis, the causal organism of center rot of onion (*Allium cepa* L.), can survive on different weeds but, in a previous survey, it was most commonly found on Florida pusley (*Richardia scabra* L.). The epiphytic survival of *P. ananatis* on *R. scabra* under different temperature and moisture regimes was investigated. Weed seedlings were spray inoculated with rifampicin-resistant strain PNA 97-1^{rif} at either 10³ or 10⁸ CFU/ml and incubated in a growth chamber at 15.5 or 21.1°C at 65% relative humidity for 96 h postinoculation (hpi), which represented the mean environmental conditions during mid-March to mid-May in Vidalia, GA when onion production and *R. scabra* presence overlap. For plants inoculated with *P. ananatis* at 10³ CFU/ml, the bacterium survived for 96 hpi when incubated at 21.1°C, with mean populations of 1.7 × 10² CFU/g of leaf tissue. In contrast, no viable bacteria

were detected after 72 hpi at 15.5°C. For plants inoculated with *P. ananatis* at 10⁸ CFU/ml, the bacterium survived for 96 hpi at 21.1°C (3.8 × 10⁵ CFU/g) whereas, during the sample time period, viable bacterial populations were not detected at 15.5°C. Survival of *P. ananatis* on *R. scabra* was also monitored during alternating 12 h wet and 12 h dry periods, or continuous wet or dry periods for 96 hpi at 15.5 or 21.1°C. Compared with initial or continuous dry periods, *P. ananatis* survived significantly better with a 12 h wet/12 h dry cycle or a continuous 96 hpi wet period at both 15.5 and 21.1°C. Unlike at 15.5°C, *P. ananatis* populations (7.4 × 10² CFU/g) survived for 96 hpi at 21.1°C under a cycle of 12 h dry and 12 h wet. These results demonstrate that *P. ananatis* can survive on *R. scabra* leaves under conditions of 21.1°C and prolonged leaf wetness and may potentially serve as a source of inoculum to onion.

Center rot of onion (*Allium cepa* L.) is caused by a gram-negative bacterium, *Pantoea ananatis* (Serrano) Mergaert. The pathogen was first associated with center rot in Georgia in 1997 on 'Vidalia' onion. Since then, it has been spreading to different onion-growing regions in the United States. The symptoms of center rot start with water-soaking lesions on foliage which run along the length of the leaf. The lesions may gradually turn necrotic and produce a blighted appearance with disease progression. In severe cases, infected plants may wilt and exhibit a bleached appearance of the foliage (Dutta et al. 2014; Gitaitis and Gay 1997). *P. ananatis* can also invade the bulb tissue and cause bulb discoloration. The bacterial invasion can predispose the bulb to postharvest rot caused by the secondary microbes in storage. Under favorable conditions, yield losses to this disease may be as high as 100% (Gitaitis and Gay 1997).

Multiple sources of *P. ananatis* inoculum have been associated with center rot epidemics (Dutta et al. 2014; Gitaitis et al. 2002, 2003; Walcott et al. 2002). Infested onion seed can introduce inoculum to onion field. *P. ananatis* is both seedborne and seed transmitted in onion and, under favorable conditions, the bacterium can cause considerable economic losses (Goszczynska et al. 2006; Walcott et al. 2002). *P. ananatis* can also be transmitted to onion by two known insect vectors, *Frankliniella fusca* (tobacco thrips) and *Thrips tabaci* (onion thrips) (Dutta et al. 2014, 2016; Gitaitis et al. 2003; Wells et al. 2002). The mode of bacterial transmission was reported to be passive through contaminated thrips feces (Dutta et al. 2014, 2016). *P. ananatis* has also been reported to survive as an epiphyte on weeds in the farmscapes of Georgia in the United States (Gitaitis et al. 2002). At least 25 asymptomatic weed species, including *Digitaria sanguinalis*, Florida beggarweed (*Desmodium tortuosum*), Florida pusley (*Richardia scabra*), sicklepod (*Cassia obtusifolia*), Texas Millet (*Panicum texanum*), tall verbena (*Verbena bonariensis*), and yellow nutsedge (*Cyperus esculentus*), and crop plants such as

Bermuda grass (*Cynodon dactylon*), cowpea (*Vigna unguiculata*), and soybean (*Glycine max*), were reported to harbor epiphytic populations of *Pantoea ananatis* (Gitaitis et al. 2002). Among these weeds, *P. ananatis* was consistently recovered from *R. scabra*, which is a common weed in and around onion fields in the southeastern United States. Although the epidemiological role of these weeds in center rot epidemics is not clear, it is speculated that they may potentially contribute to the source of *P. ananatis* inoculum in onion field.

R. scabra is distributed throughout the onion-growing region of Georgia. This weed is prevalent during the months of March to October in southeastern Georgia and overlaps with onion production only for 3 months (March, April, and May). Interestingly, center rot epidemic reports are more frequent during these months. In preliminary experiments, we demonstrated that transmission of *P. ananatis* can occur from thrips that fed on contaminated *R. scabra* (naturally occurring epiphytic populations) under greenhouse conditions. These observations suggest that contaminated thrips and weeds may play an important role in center rot epidemics. However, factors that involve pathogen survival on weeds, pathogen acquisition from weeds by thrips, and further transmission to onion remain unclear. In this article, we demonstrate *P. ananatis* survival on *R. scabra* under conditions that represented the mean environmental conditions during mid-March to mid-May in Vidalia, GA when onion production and *R. scabra* presence overlap.

Materials and Methods

Bacterial strains and inoculum preparation. A rifampicin-resistant strain of *P. ananatis* (PNA 97-1^{rif}), generated specifically for this study, was used. This strain was developed from a parent strain of PNA 97-1 (Gitaitis and Gay 1997). The strain PNA 97-1^{rif} was pathogenic on onion and displayed growth in rifampicin-amended nutrient broth similar to that of its parent strain (PNA 97-1) in nutrient broth.

The inoculum was prepared by inoculating 3 ml of nutrient broth amended with rifampicin at 100 µg/ml, with single colonies of *P. ananatis* taken from 48-h-old antibiotic-amended nutrient agar (NA) plates. The inoculated culture was incubated overnight at 28°C on a rotary shaker (Innova; New Brunswick Scientific, Edison, NJ) at 150 rpm. After 24 h of incubation, 3 ml of bacterial suspension was centrifuged at 6,000 × g (Allegra™ 25R; Beckman Coulter, Fullerton, CA) for 5 min, supernatant was discarded, and the resulting pellet was resuspended in 0.1 M phosphate-buffered saline

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*The e-Xtra logo stands for "electronic extra" and indicates that one supplementary table is published online.

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solution (PBS). Further, bacterial concentration was adjusted to an optical density of 0.3 at 600 nm (approximately 1×10^8 CFU/ml) using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY). Ten-fold serial dilutions in 0.9 ml of 0.1 M PBS was done to reach a final concentration of approximately 1×10^3 CFU/ml. For our study, two inoculum levels were used: 1×10^8 and 1×10^3 CFU/ml.

Collection of *R. scabra*. In 2015, *R. scabra* samples were collected from the perimeter of the onion fields that had significant levels of center rot, or from areas that were left fallow in the current season but had been traditionally used for growing onion in the recent past. The identity of the weeds from which the leaf samples were taken were marked by flags and unique identification numbers for the weed and the sample site were assigned. Both sites were from the Tift County, GA. Leaf samples from weeds ($n = 25/\text{location}$) were collected from the edge of fields. Samples were collected in plastic bags, stored in an ice cooler, and brought back to the laboratory for processing. In the laboratory, samples were temporarily stored at 4°C until they were processed.

Processing of weed samples. Leaf samples (2 g) were placed in 20 ml of 0.1 M PBS and shaken overnight at approximately 150 rpm on a rotary shaker at 25°C. After incubation, 1.0 ml of the aliquot was removed and transferred to a 2-ml microcentrifuge tube. Samples were further centrifuged for 5 min at $5,000 \times g$ (Allegra 25R) and the supernatant was decanted, leaving a pellet that was then resuspended in 0.1 M PBS. Microbial genomic DNA extraction was conducted on the pellet using an UltraClean Microbial DNA Kit (MO BIO, Carlsbad, CA) according to the manufacturer's protocol. DNA samples (5 µl of 1 ng/µl) were amplified in 25 µl of reaction mix with polymerase chain reaction (PCR) mastermix (Eppendorf Real Master Mix Probe; Eppendorf, Hamburg, Germany or Bio-Rad iQ Supermix; Bio-Rad Laboratories, Hercules, CA), 25 µM of each primer PanITS1 (5'-GTCTGATAGAAAGATAAAGAC-3') and EC5 (5'-CGGTGGATGCCCTGGCA-3') (Walcott et al. 2002), and 10 µM TaqMan probe 6-FAM TAGCGGTTAGGACTCCGCCCTTTCA-BHQ. DNA amplification was carried out in a Cepheid Smart Cycler (Sunnyvale, CA) using the following thermal profile: denaturation at 95°C for 180 s and 35 cycles each of denaturation at 95°C for 15 s and annealing at 60°C for 40 s. Samples with cycle threshold values less than 35 were considered positive for *P. ananatis* (Dutta et al. 2014; Walcott et al. 2002).

The samples that came negative for *P. ananatis* by real-time PCR assay were recorded. Later, sample sites were revisited and the entire plants (for which leaf samples were negative for *P. ananatis*) were uprooted with intact root, brought back to the greenhouse at the University of Georgia, Tifton, and planted in 10-liter plastic pots containing commercial potting mix (Scotts Miracle-Gro Company, Marysville, OH).

Effect of temperature on epiphytic survival of *P. ananatis* on *R. scabra*. *R. scabra* seedlings or plants ($n = 15$ each for treatment and control) negative for harboring *P. ananatis* as epiphyte were established in 10-liter plastic pots containing commercial potting mix. Pots were spaced evenly on benches in the growth chamber (Controlled Environments Inc., Pembina, ND). Plants were fertilized with Osmocote smart-release plant food (14-14-14) (Scotts Miracle-Gro Company). Once plants were established, foliage was spray inoculated with a *P. ananatis* suspension containing 10^3 or 10^8 CFU/ml. The inoculum was sprayed using a hand-trigger sprayer (model 916CN; Contico International, St. Louis) until runoff. Later, plants were incubated under conditions of either 15.5 or 21.1°C and 65% relative humidity (RH) for 96 h and a photoperiod cycle of 12 h of light and 12 h of darkness. The conditions tested in this study represented 12-year (2004 to 2015) mean average temperature and RH for the months of March and May in Vidalia, GA (Supplementary Table S1). The WatchDog Data Logger (model 150; Spectrum Technologies, Aurora, IL) was used to monitor temperature and RH. At each sampling period (0, 6, 24, 48, 72, 96 h post-inoculation [hpi]), three inoculated leaves were arbitrarily sampled, weighed, and processed for *P. ananatis* enumeration as follows. For enumeration, leaf samples were individually placed in 5 ml of 0.1 M PBS and shaken

overnight at approximately 150 rpm on a rotary shaker at 25°C. After incubation, leaf samples were removed and the suspension was transferred to a 15-ml polypropylene tube. Samples were further centrifuged for 5 min at $5,000 \times g$ (Allegra 25R) and the supernatant was decanted, leaving a pellet that was then resuspended in 1 ml of 0.1 M PBS. The suspension was vortexed vigorously for 30 s, followed by 10-fold serial dilutions in 1 ml of PBS. Subsequently, 100-µl aliquots were spread plated onto NA plates amended with rifampicin at 100 µg/ml and incubated for 48 h at 28°C. After incubation, bacterial colonies were enumerated and recorded from each sampling point. *R. scabra* plants treated with PBS and processed as described above served as a negative control. Two independent experiments were conducted and treatments were arranged in a split-plot design. Area under the growth progress curve (AUGPC) for each replicate per treatment was also calculated as follows: $\sum_{i=1}^n [(Y_{i+1} + Y_i)]/2 [X_{i+1} - X_i]$, where Y_i = bacterial population at the i th observation, X_i = time in hours at the i th observation, and n = the total number of observations. Analysis of variance was conducted on the AUGPC values to determine significance of treatment effects on *P. ananatis* populations, and Fischer's least significant difference (LSD) test ($P < 0.05$) was used for mean separation.

Effect of intermittent wet and dry cycle on epiphytic survival of *P. ananatis* on *R. scabra*. *R. scabra* plants confirmed as negative for *P. ananatis* as epiphyte were used for this experiment. Plants were established in a 10-liter pot and spray inoculated with *P. ananatis* at 10^3 CFU/ml until runoff, as described above. Later, plants were kept in growth chambers with an incubation temperature of either 15.5 or 21.1°C, each with the following wetting regimes: continuous wet or dry conditions for 96 hpi, 12 h wet alternated with 12 h dry periods for 96 hpi, or 12 h dry periods alternated with 12 h wet periods for 96 hpi or continuous wet or continuous dry for 96 hpi. For different wetting regimes, plants were kept in the temperature-controlled growth chambers with a humidifier (Trion 707U, Indian Trail, NC), which created high mean RH ($\geq 94\%$). For drying regimes, plants were moved to another temperature-controlled growth chamber with mean RH of 30%. Based on the duration of wetting and drying cycles, plants from each treatment were moved to temperature- and humidity-controlled growth chambers. Temperature and RH inside the growth chambers were also monitored using data loggers (WatchDog 150; Spectrum Technologies). The plants were maintained under a photoperiod of 12 h of light and 12 h of darkness. Three leaf samples per temperature and wetting regime conditions were arbitrarily sampled (0, 6, 24, 48, 72, and 96 hpi), weighed, and processed for *P. ananatis* enumeration, as described above. *R. scabra* plants treated with PBS and processed as described above served as a negative control. Fifteen plants per treatment were used in a single experiment and two independent experiments were conducted. The treatments were arranged in a split-plot design. Area under the disease progress curve (AUDPC) was calculated for each treatment and compared statistically by Fisher's LSD after confirming the treatment effect using analysis of variance (ANOVA) at the $P < 0.05$ level.

Confirmatory tests for *P. ananatis*. Bacterial colonies isolated on NA amended with rifampicin medium were confirmed as *P. ananatis* using specific real-time PCR assays, as described above.

Bacterial colonies were also tested for pathogenicity on onion. For the pathogenicity assay, three randomly selected colonies per plate per *R. scabra* plant were transferred to the nutrient broth and grown overnight at 28°C on a rotary shaker (Innova; New Brunswick Scientific) at 150 rpm. After 24 h of incubation, the bacterial suspension was centrifuged at $6,000 \times g$ (AllegraTM 25R; Beckman Coulter), supernatant was discarded, the resulting pellet was resuspended, and the *P. ananatis* concentration was adjusted to 0.3 at 600 nm (approximately 1×10^8 CFU/ml) using a spectrophotometer (Spectronic 20; Bausch and Lomb).

Onion seedlings ('Century') were planted in pots of 10 by 8 cm (diameter by height; Hummert International, Earth City, MO) in a commercial potting mix in the greenhouse and maintained at 25 to 28°C and 80 to 90% RH with a photoperiod of 12 h of light and 12 h of darkness. Mechanical inoculation was done by clipping

seedlings with a pair of sterile scissors at approximately 5 cm below the apex. Using a micropipette, four 10- μ l drops (diagonally opposite) of bacterial suspensions containing *P. ananatis* isolates at 1×10^8 CFU/ml (approximately 1×10^6 CFU/ml) from *R. scabra* were placed at the cut ends of the seedlings. Seedlings mechanically inoculated with PBS as described above were used as negative controls. Three replications per colony per plate per *R. scabra* plant were used in two independent trials. Seedlings were checked visually for symptom development (water-soaked, blighted leaves that gradually turned necrotic) at 3-day intervals for 15 days.

Statistical analysis. *P. ananatis* colony counts were $\log_{10}$ transformed as $\log_{10}$ CFU/g of fresh leaf weight. The $\log_{10}$ -transformed data of each treatment for both experiments were compared by ANOVA using PROC GLIMMIX (version 9.4; SAS Institute Inc., Cary, NC). The data for two experiments were pooled together after ascertaining that experimental effect was not significant ($P = 0.625$). The effect of main-plot treatment (temperature or wetness regime) was tested against the main-plot error, run-temperature, split-plot treatment, and sampling time, and interactions were tested against the residual error.

Results

Effect of temperature on epiphytic survival of *P. ananatis* on *R. scabra*. *R. scabra* plants were inoculated with 10^3 and 10^8 CFU/ml separately and incubated for 96 hpi under growth chamber conditions. For *R. scabra* plants inoculated with 10^3 CFU/ml, the mean *P. ananatis* populations recovered at 0 hpi from *R. scabra*

incubated at 15.5 and 21.1°C were 3.8×10^2 and 3.5×10^2 CFU/g, respectively (Fig. 1A). After 6 hpi, bacterial populations decreased to 1.95×10^1 CFU/g for plants incubated at 15.5°C whereas the mean *P. ananatis* population recovered from plants incubated at 21.1°C was 3.9×10^2 CFU/g. By 24 hpi, mean populations recovered from 15.5 and 21.1°C treatments were 1.3×10^1 and 4.3×10^3 CFU/g, respectively (Fig. 1A). The bacterial populations decreased rapidly for 15.5°C treatment after 48 hpi (1.05×10^1 CFU/g) and viable colonies of *P. ananatis* could not be recovered at 72 and 96 hpi. In contrast, although slight decreases in *P. ananatis* populations were observed at 48 hpi (3.8×10^2 CFU/g) and 72 hpi (5.4×10^2 CFU/g) for the 21.1°C treatment, viable bacterial colonies could still be recovered until 96 hpi (1.7×10^2 CFU/g) (Fig. 1A). The viable *P. ananatis* populations were not recovered from plants treated with 0.1 M PBS. The AUDPC values for plants incubated at 21.1°C (255.6) was significantly higher than the plants incubated at 15.5°C (80.1) ($P < 0.001$) (Fig. 1B).

For *R. scabra* plants inoculated with 10^8 CFU/ml, mean *P. ananatis* populations recovered at 6 hpi for 15.5 and 21.1°C treatments were 6.8×10^7 and 7.4×10^9 CFU/g, respectively. At 24 hpi, bacterial populations declined to 1.5×10^7 CFU/g for 15.5°C treatment whereas *P. ananatis* CFU remained around 1.5×10^9 CFU/g for 21.1°C treatment (Fig. 1C). By 48 hpi, mean populations remained at 2.7×10^9 CFU/g for 21.1°C whereas it decreased to 2-log order for plants incubated at 15.5°C (4.6×10^5 CFU/g) (Fig. 1C). The bacterial populations decreased rapidly for 15.5°C treatment after 72 hpi (1.05×10^1 CFU/g) and viable colonies of *P. ananatis* could not be recovered by 96 hpi. Although, decline in bacterial populations also

Fig. 1. Epiphytic survival of *Pantoea ananatis* 97-1^{rif} populations ($\log_{10}$ CFU/g of fresh leaf weight) and area under growth progress curve (AUGPC) on *Richardia scabra* incubated for 96 h postinoculation (hpi) under 15.5 and 21.1°C and 65% relative humidity. *R. scabra* foliage were spray inoculated with *P. ananatis* at either approximately 10^3 CFU/ml (A, population counts [CFU/g] and B, AUGPC) or approximately 10^8 CFU/ml (C, population counts [CFU/g] and D, AUGPC) and incubated in a growth chamber for 96 h. Three leaf samples per replicate per treatment were sampled at 0, 6, 24, 48, 72, and 96 hpi and populations of *P. ananatis* were enumerated on nutrient agar plates amended with 100 μ g/ml. Data points represent the mean bacterial populations at each sampling period in two independent experiments. Bars indicate the standard error of the mean.

occurred at 72 hpi (1.5×10^6 CFU/g) for 21.1°C treatment, high CFU of *P. ananatis* could still be recovered by 96 hpi (3.8×10^5 CFU/g) (Fig. 1C). The AUDPC values for plants incubated at 21.1°C (696.3) was significantly higher than for plants incubated at 15.5°C (453.2) ($P < 0.001$) (Fig. 1D).

Overall, the effect of temperature, sampling period, and inoculum concentrations were significant on *P. ananatis* survival ($P \leq 0.003$). In addition, significant interactions between temperature–inoculum concentrations, temperature–sampling period, and sampling period–inoculum concentrations on *P. ananatis* CFU were observed ($P \leq 0.001$) (Table 1).

Effect of intermittent wet and dry cycle on epiphytic survival of P. ananatis on R. scabra. The effect of wet and dry cycles on epiphytic

populations of *P. ananatis* was evaluated at 15.5 and 21.1°C on *R. scabra* for an initial inoculum level of 10^3 CFU/ml. At 15.5°C, mean bacterial populations at 0 hpi for 12 h wet/12 h dry and 12 h dry/12 h wet treatments were 8.7×10^2 and 9.05×10^2 CFU/g, respectively. During the same period, *P. ananatis* populations for continuous wet or continuous dry treatments were 8.7×10^2 and 1.05×10^3 CFU/g, respectively (Fig. 2A). By 24 hpi, bacterial populations for treatments with 12 h wet/12 h dry and 12 h dry/12 h wet treatments were 7.5×10^1 and 5.6×10^2 CFU/g, respectively (Fig. 2A). During the same time period, the treatments continuous wet (9.2×10^1 CFU/g) or continuous dry (1.8×10^1 CFU/g) had 1- and 2-log order reduction in *P. ananatis* populations, respectively (Fig. 2A). By 48 and

Table 1. Summary of analysis of variance of epiphytic populations of *Pantoea ananatis* on *Richardia scabra* foliage at 15.5 and 21.1°C^a

Survival, source	df ^b	Error ^c	F value	P > F
Experiment ^d	1	Experiment × temperature	6.67	0.762
Temperature ^e	1	Experiment × temperature	28.34	0.0034
Sampling period (Sampling) ^f	5	Residual error	267.28	0.0021
Inoculum concentrations (Inoculum)	1	Residual error	15.62	<0.001
Temperature × Sampling	5	Residual error	31.52	<0.001
Temperature × Inoculum	1	Residual error	45.52	<0.001
Inoculum × Sampling	5	Residual error	18.2	<0.001

^a Mean square error with residual error value was 13.8 ± 0.52 for survival at 15.5 and 21.1°C.

^b Degrees of freedom.

^c Error term used to test number of the experiment multiplied by temperature treatment.

^d Number of experiments.

^e Temperature in growth chambers.

^f Sampling period of leaves after inoculation.

Fig. 2. Epiphytic survival of *Pantoea ananatis* 97-116f populations (log₁₀ CFU/g), with population counts at **A**, 15.5 and **C**, 21.1°C, and area under growth progress curve (AUGPC) at **B**, 15.5 and **D**, 21.1°C, on *Richardia scabra* incubated for 96 h postinoculation (hpi) at 15.5 and 21.1°C under different moisture regimes. *R. scabra* plants were spray inoculated with *P. ananatis* at approximately 10^3 CFU/ml and incubated at four moisture regimes (12 h wet/12 h dry, 12 h dry/12 h wet, continuous wet, or continuous dry) for 96 hpi. Three leaf samples per replicate per treatment were sampled at 0, 6, 24, 48, 72, and 96 hpi and populations of *P. ananatis* were enumerated on nutrient agar plates amended with 100 µg/ml. Data points represent the mean bacterial populations at each sampling period in two independent experiments. Bars indicate the standard error of the mean.

72 hpi, *P. ananatis* CFU could be recovered for 12 h wet/12 h dry (48 hpi: 8.4×10^1 CFU/g; 72 hpi: 9.8×10^1 CFU/g) and 12 h dry/12 h wet treatments (48 hpi: 2.4×10^1 CFU/g; 72 hpi: 1.6×10^1 CFU/g) treatments. During the same sampling periods (48 and 72 hpi), mean bacterial populations declined for continuous wet (48 hpi: 1.3×10^1 CFU/g; 72 hpi: 1.86×10^1 CFU/g) and continuous dry (48 hpi: 1.2×10^1 CFU/g; 72 hpi: 0.8×10^1 CFU/g) treatments (Fig. 2A). At 96 hpi, mean *P. ananatis* populations for the 12 h wet/12 h dry treatment was 6.8×10^1 CFU/g, whereas viable CFU could not be recovered for 12 h dry/12 h wet treatment. During the same period, treatment with the continuous wet regime produced *P. ananatis* populations of 1.6×10^1 CFU/g, whereas bacterial CFU were not recovered for the continuous dry treatment. The AUGPC value for continuous dry (72.7) treatments was significantly lower than other treatments (Fig. 2B). The 12 h wet/12 h dry (156.2) and continuous wet (125.4) treatments had significantly higher AUGPC values than the 12 h dry/12 h wet (110.8) treatment (Fig. 2B).

At 21.1°C, mean bacterial populations at 0 hpi for 12 h wet/12 h dry or 12 h dry/12 h wet or continuous wet or continuous dry treatments were approximately 10^2 CFU/g. By 24 hpi, bacterial CFU declined for treatments with 12 h wet/12 h dry (6.8×10^2 CFU/g) and 12 h dry/12 h wet (3.5×10^2 CFU/g) regimes (Fig. 2C). The continuous wet (1.5×10^1 CFU/g) or continuous dry (1.3×10^1 CFU/g) treatments declined rapidly to the order of 2-log by 24 hpi (Fig. 2C). By 48 and 72 hpi, *P. ananatis* CFU could be recovered for 12 h wet/12 h dry (48 hpi: 2.6×10^2 CFU/g; 72 hpi: 6.8×10^1 CFU/g) and 12 h dry/12 h wet (48 hpi: 2.4×10^1 CFU/g; 72 hpi: 1.6×10^1 CFU/g) treatments. Mean bacterial populations for the continuous dry treatment declined rapidly (48 hpi: 1.2×10^1 CFU/g) and viable CFU could not be recovered at 72 and 96 hpi. In contrast, mean *P. ananatis* populations for the continuous wet treatment remained around 2.4×10^1 CFU/g (48 hpi) and 1.6×10^1 CFU/g (72 hpi) and, by 96 hpi, mean CFU recovered was 4.8×10^2 CFU/g. Also, by 96 hpi, mean *P. ananatis* populations for 12 h wet/12 h dry and 12 h dry/12 h wet treatments were 7.4×10^2 and 1.8×10^2 CFU/g, respectively.

The AUGPC value for the 24 h dry treatment (59.4) was significantly lower than other treatments. The treatments with 12 h wet/12 h dry (193.4) and continuous wet (186.6) had significantly higher AUGPC values than the 12 h dry/12 h wet treatment (149.2) (Fig. 2D). The viable *P. ananatis* populations were not recovered from plants treated with 0.1 M PBS and incubated at above temperature under wet and dry regimes. Overall, the effects of temperature, sampling period, and treatments were significant on *P. ananatis* survival ($P \leq 0.021$; Table 2). In addition, significant interactions between treatment–sampling period, treatment–temperature, and temperature–sampling period on *P. ananatis* CFU were observed ($P \leq 0.001$; Table 2).

Confirmatory tests for *P. ananatis*. Confirmation of presumptive *P. ananatis* colonies was conducted by a *P. ananatis*-specific real-time PCR assay, as described above. Of the colonies tested, 100% were amplified using this primer. In addition, 100% of the onion

seedlings displayed center rot symptoms under greenhouse conditions when inoculated with the *P. ananatis*-suspected colonies.

Discussion

The knowledge of factors affecting pathogen survival on a weed host is critical to understanding the epidemiology of bacterial dispersal and spread from weeds to host crops. The survival of *P. ananatis* on *R. scabra* (a well-known epiphytic host) was investigated under different temperature and moisture regimes. The conditions investigated in this research mimicked the mean temperature and RH during the months of March, April, and May in the Vidalia onion region of southeastern Georgia. During these months, overlapping of *R. scabra* and onion can be observed in this region. Such overlapping may potentially result in the risk of *P. ananatis* spread in onion crop via thrips or wind-driven rain.

According to earlier reports, epiphytic bacteria on leaf surfaces followed a log-normal distribution. Such distribution indicates that, in a subsample of infested or positive leaves, few samples may possess higher bacterial populations (10^6 to 10^8 CFU/g) whereas the majority of the leaf samples may harbor bacterial populations around 10^2 to 10^3 CFU/g (Hirano et al. 1982). Hence, in this study, we investigated *P. ananatis* survival at two levels of initial inoculum— 10^3 (low) and 10^8 (high) CFU—representing two spectra of epiphytic populations in nature. The low inoculum level was used to mimic mean epiphytic populations that generally occur under natural conditions (Beattie and Lindow 1995, 1999; Hirano et al. 1982). In contrast, the high inoculum level represented a worst-case scenario where high bacterial populations could be present in fewer *R. scabra* leaves. The results indicated that, when *R. scabra* leaves were inoculated with *P. ananatis* at 10^3 CFU/ml, the bacterium could survive to a population level of 1.05×10^1 CFU/g at 15.5°C for up to 48 h but could not be recovered at 72 and 96 hpi. In contrast, *P. ananatis* did survive for 96 hpi with a population level of 1.7×10^2 CFU/g at 21.1°C. In addition, AUDPC values were significantly higher for plants incubated at 21.1°C (255.6) than for plants incubated at 15.5°C (80.1). These observations suggest that, even at low initial *P. ananatis* populations, the bacterium could survive efficiently on *R. scabra* for a longer period of time (96 hpi) when incubated at 21.1 than at 15.5°C. Similar observations of reduced *P. ananatis* survival on *R. scabra* at 21.1 than at 15.5°C was also noted at a higher initial inoculum level (10^8 CFU/ml). Despite high initial inoculum, *P. ananatis* populations at 15.5°C reduced to 0 CFU/g by 96 hpi. In contrast, during the same time period (96 hpi), *P. ananatis* populations survived (3.8×10^5 CFU/g) at 21.1°C. The observations made in this study were in contrast to observations made on epiphytic survival of *Erwinia tracheiphila* (causal agent of bacterial wilt of cucumber) (Saalau Rojas and Gleason 2012). The researchers reported that *E. tracheiphila* survived longer (48 hpi) and to a higher population when cucumber seedlings were incubated at 10 and 20°C than at 30 and 35°C (Saalau Rojas and Gleason 2012). Also, in other studies, epiphytic bacterial survival was reduced with an increase in temperature (Beattie and Lindow 1994, 1999). However, in our study, *P. ananatis* survived comparatively better at 21.1 than at 15.5°C. It is possible

Table 2. Summary of analysis of variance of epiphytic populations of *Pantoea ananatis* on *Richardia scabra* foliage under different wet and dry regimes^a

Survival, source	df ^b	Error ^c	F value	P > F
Experiment ^d	1	Experiment × treatment	3.82	0.284
Treatment ^e	3	Experiment × treatment	32.51	0.0214
Temperature	1	Experiment × temperature	42.8	0.015
Sampling period ^f	4	Residual error	253.52	0.0072
Treatment × sampling period	12	Residual error	24.31	<0.001
Treatment × temperature	3	Residual error	23.7	<0.001
Sampling period × temperature	4	Residual error	244.28	<0.001

^a Mean square error with residual error value was 1.62 ± 0.17 for survival under different wet and dry regimes.

^b Degrees of freedom.

^c Error term used to test number of the experiment multiplied by treatments.

^d Number of experiments.

^e Wet and dry regimes in growth chambers.

^f Sampling period of leaves after inoculation.

that the upper limit (21.1°C) of temperature tested could certainly fall within temperature optima for *P. ananatis* survival on biological surfaces, which could partly explain why the bacterium survived better at 21.1°C.

In order to mimic variabilities of alternating moisture regimes (rainfall or overhead irrigation followed by a dry spell or vice versa) under field conditions, *P. ananatis* survival was also evaluated at 12 h wet/12 h dry or 12 h dry/12 h wet or continuous wet or continuous dry conditions for 96 hpi. These moisture regimes were evaluated at both 15.5 and 21.1°C with an initial inoculum population of 10^3 CFU/ml. At 15.5°C, *P. ananatis* survived for 96 hpi with populations of 6.8×10^1 and 1.6×10^1 CFU/g in 12 h wet/12 h dry and continuous wet regimes, respectively, whereas, during the same time period, viable bacterial populations could not be recovered for 12 h dry/12 h wet or continuous dry regimes. In contrast, at 21.1°C, bacterial populations survived for 96 hpi under 12 h wet/12 h dry, 12 h dry/12 h wet, or continuous wet regimes but *P. ananatis* populations could not be recovered from a continuous dry regime for 96 hpi. These observations indicate that *P. ananatis* can survive on *R. scabra* leaves under conditions of 21.1°C and prolonged leaf wetness. Moreover, the survival was significantly better at 21.1 than at 15.5°C under different moisture regimes. Even under alternating dry or continuous dry conditions, *P. ananatis* survived for 72 hpi, indicating that the bacterium could survive stress for a shorter duration. The possible explanation for this phenomenon could be due to selection of a population that can tolerate stress (Beattie and Lindow 1999), or perhaps *P. ananatis* survived in microsites on the leaf surface, thus helping bacterial populations to evade environmental stress (Beattie and Lindow 1995).

Although the epidemiological significance of *R. scabra* is not well determined, our preliminary investigations indicate that it can potentially act as a link between thrips and onion. Our preliminary investigations under greenhouse conditions showed that *F. fusca* thrips could efficiently acquire epiphytic *P. ananatis* populations from *R. scabra* and were able to transmit the bacterium to onion seedlings, resulting in center rot (data not shown). These data indicate that *R. scabra* can act as a reservoir for epiphytic populations of *P. ananatis* and may serve as an important link in center rot epidemic. A better understanding of the environmental factors that may affect *P. ananatis* survival on *R. scabra* and subsequent spread to an onion crop may potentially improve center rot management.

Overall, for the first time, it was demonstrated that *P. ananatis* can survive as an epiphyte on *R. scabra* under different temperature and moisture regimes that mimicked conditions for the months of March,

April, and May in the Vidalia onion region of Georgia. The results suggest that *P. ananatis* can survive on *R. scabra* leaves under conditions of 21.1°C and prolonged leaf wetness and can potentially serve as a source of inoculum to onion. However, the role of weeds in the epidemiology of *P. ananatis* is still not well understood. Perhaps detailed investigations on the role of epiphytic survival of *P. ananatis* and subsequent spread to onion crops may provide necessary information for center rot management. Future studies with more *P. ananatis* strains under a wider range of environmental conditions combined with genetic factors affecting epiphytic survival of *P. ananatis* may provide important clues for bacterial survival.

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