Survival of *Ralstonia solanacearum* Race 4 in Drainage Water and Soil and Detection with Immunodiagnostic and DNA-based Assays

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SUMMARY. *Ralstonia solanacearum* (Rs) race 4 strains cause bacterial wilt of edible ginger (*Zingiber officinale*). The survival of the pathogen was studied in plant-free soil and potting medium in the presence of plants inoculated by different methods (non-wounded, rhizome-wounded, and stem-wounded) and irrigated on different schedules (alternate and daily). Detection thresholds for Rs were determined for an enzyme-linked immunoabsorbent assay (ELISA), immunostrip assay, and polymerase chain reaction (PCR) using drainage water from soil and potting medium containing known concentrations of Rs. In the absence of a plant or in the presence of non-wounded plants, Rs populations declined rapidly in drainage water from potting medium during the first 9 days and were undetectable after 81 days. When plants were stem- or rhizome-wounded, Rs populations increased by two to three orders of magnitude from the initial population levels for the first 9 to 19 days and then gradually declined and became undetectable after 89 days. Results were similar in experiments with soil except for non-wounded ginger plants, where the initial decline in Rs populations was followed by an abrupt increase after day 11, reaching 7 log cfu/mL on day 21, then declining gradually to non-detectable levels after 137 days. The increase was attributed to natural infection of the plants followed by release of high populations of Rs into the irrigation water when plants wilted. When rhizome-inoculated plants were watered on alternate days, Rs was recovered from 97 to 129 days in soil and potting medium, but when the plants were watered daily, Rs was recovered in soil and potting medium up to 153 days after plant inoculation. ELISA using Ps1a monoclonal antibody detected the pathogen from >95% of the samples from soil and potting medium when viable populations were >5 log cfu/mL. The immunostrip assay (using the same antibody) detected the pathogen from 100% of the samples when viable populations were >3 log cfu/mL. PCR based on the flagellin gene *fliC* detected the pathogen from >95% of the samples from soil and from >74% of the samples from potting medium when viable populations were >4 log cfu/mL.

Edible ginger (Fig. 1A) is one of the most valuable and widely cultivated spice crops in the world (Ravindran and Babu, 2005). The global area under cultivation of

edible ginger in 2008 was 426,998 ha with a harvested yield of 34,104 kg·ha⁻¹ (Food and Agricultural Organization of the United Nations, 2010). One of the major production constraints of edible ginger is bacterial wilt disease (Fig. 1B) caused by *Ralstonia solanacearum*. Major crop losses due to the disease have been reported from almost all edible-ginger growing areas

(Hayward, 1994). The bacterium is known to cause severe wilting in many crops, including banana (*Musa* spp.), geranium (*Geranium* spp.), heliconia (*Heliconia* spp.), mulberry (*Morus alba*), peanut (*Arachis hypogaea*), plantain (*Musa paradisiaca*), potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), and tomato (*Solanum lycopersicum*) in the tropics, subtropics, and temperate regions (European Plant Protection Organization, 2004; Hayward, 1994; Kelman, 1953).

R. solanacearum is a soil-borne organism and is easily transmitted through field soil, runoff water, and infected planting material, and survives on weeds and latently infected potato, geranium, and edible ginger, as well as many other hosts (Janse et al., 2004; Pradhanang et al., 2000a; Shintaku et al., 2006). Previous studies have shown that high populations of Rs were released into drainage water when infected ginger plants were grown in potting medium (Paret et al., 2008a). However, no studies have reported survival of the ginger strains of Rs in field soil following different types of inoculation and under different irrigation schedules.

Enzyme-linked immunoabsorbent assay (ELISA), immunostrip assays (Immunostrips®; Agdia, Elkhart, IN), polymerase chain reaction (PCR), and culturing are four methods widely used for detection and identification of Rs. ELISA and immunostrips use an Rs-specific monoclonal antibody (Mab) that reacts with an exopolysaccharide (EPS) antigen (Alvarez, 2004; Alvarez et al., 1993; McGarvey et al., 1998). The PCR assay uses specific primers to amplify *fliC* fragments of Rs that are present in the ginger strains of Rs (Schonfeld et al., 2003). Detection limits for Rs with ELISA and PCR were reported as >5 log and >2 log cfu/mL, respectively (Alvarez, 2004; Horita et al., 2004). Immunostrips

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Units

To convert U.S. to SI, multiply by	U.S. unit	SI unit	To convert SI to U.S., multiply by
0.4047	acre(s)	ha	2.4711
29,574	fl oz	μL	3.3814 × 10 ⁻⁵
29.5735	fl oz	mL	0.0338
1.1209	lb/acre	kg·ha ⁻¹	0.8922
1	micron	μm	1
28.3495	oz	g	0.0353
0.001	ppm	g·L ⁻¹	1000
0.001	ppm	mL·L ⁻¹	1000
(°F - 32) ÷ 1.8	°F	°C	(1.8 × °C) + 32

Fig. 1. (A) Healthy 5-month-old edible ginger plants in the field. (B) Yellowing and inward curling of leaves in edible ginger plants infected with the bacterial wilt pathogen, *Ralstonia solanacearum* race 4. The symptoms are caused by plugging of the vascular tissues leading to blockage of water movement to upward parts of the stem and leaves.

were more sensitive than ELISA, with a detection limit of $>4 \log \text{ cfu/mL}$ for pure cultures in water (Paret et al., 2008b). However, the strips have not been evaluated for detection of the bacterium in soil from ginger fields or in drainage water from soil and potting medium. Problems associated with direct analysis of environmental samples are many, and include low sensitivity, as in ELISA (Alvarez, 2004), possible inhibitors in PCR, and overgrowth of enterics during culturing (Pradhanang et al., 2000b). Inability to estimate soil-borne populations of Rs in ginger fields due to overgrowth of enterics like *Enterobacter* species in culture plates is a major constraint. Thus, soil treatments for bacterial wilt management are not initiated on time, leading to major crop losses.

The objectives of this work were to determine the survival of the Rs race 4 in soil without plants or with host plants inoculated by different methods and watered with different irrigation schedules, to determine population levels in drainage water from pots containing plants, and to evaluate immunodiagnostic and DNA-based techniques for direct detection of Rs from soil and water samples in comparison with culturing. The studies were carried out under controlled conditions in the greenhouse to determine the potential for disease spread through drainage water.

Materials and methods

BACTERIAL STRAIN. A well-characterized, representative strain of Rs race 4 (A4515) isolated from edible

ginger in Hawaii was used for the experiment (Yu et al., 2003). This strain belongs to phylotype I (R. Kubota, unpublished data) based on the recently proposed classification scheme for Rs (Fegan and Prior, 2005). The Rs strain was streaked onto a modified tetrazolium chloride (TZC) medium (Norman and Alvarez, 1989) and was incubated for 48 to 72 h at 28 °C. Typical mucoid white colonies of Rs were transferred to sterile distilled water (dH_2O), vortexed, and adjusted spectrophotometrically to $A_{600\text{nm}} = 0.1$. Based on viable plate counts on TZC medium, this was equivalent to 10^8 ($8 \log$) cfu/mL (Paret et al., 2008a).

SURVIVAL OF *RALSTONIA SOLANACEARUM* IN FIELD SOIL AND POTTING MEDIUM. Two types of growth media were used for the experiments: soil (Hilo silty clay loam; $\text{pH} = 4.4$) was collected from a field not planted with ginger or any host crop of Rs at the Waikaea Research Station on the island of Hawaii, and a commercial potting medium ($\text{pH} = 5.9$, Sunshine Mix 4 Aggregate Plus; Sun Gro Horticulture Canada, Vancouver, BC, Canada) used by the ginger industry (Paret et al., 2008a). Bacterial wilt-free edible ginger rhizomes produced at the research station (Hepperly et al., 2004) were planted in pots containing one of the two substrates and irrigated with 200 mL of water daily for 5 months before the start of the experiments.

For Expt. I, media in 2-L pots were inoculated by three different methods designed to simulate different situations that could occur during cultivation as follows: 1) 25 mL of an aqueous suspension containing $8 \log \text{ cfu/mL}$ of Rs was poured into pots with no plants, 2) 25 mL of Rs was poured into pots containing ginger plants, and 3) the rhizome was wounded with a sterile scalpel before pouring 25 mL of the Rs onto the base of the plant. Three replicate pots were used for each treatment. An additional inoculation method was used for pathogenicity testing in the greenhouse: 1 mL of Rs inoculated into each stem (three stems inoculated per plant) with a sterile syringe. Pots were positioned over a petri plate and were irrigated with 200 mL of water on alternate days for 153 d. Fifty milliliters of drainage water from the pots was collected subsequently on the petri plates and was vortexed.

A 1-mL subsample was then taken to initiate a 10-fold dilution series (1×10^{-1} to 1×10^{-7}) and 100 μ L of each dilution was plated onto a modified semiselective medium South Africa (SMSA) medium (Engelbrecht, 1994) to assess viable Rs populations. In addition to the assessment of bacterial populations in drainage water from soil and potting medium, the Rs populations in soil and potting medium were assessed directly by culturing once the populations in drainage water fell below 1 log cfu/mL. Pathogen survival was subsequently evaluated until growth no longer occurred on SMSA. A 1-g sample (soil or potting medium) was vortexed for 10 s in 10 mL of dH₂O and was allowed to settle. One milliliter of the supernatant was diluted and colonies were enumerated by the above methods. The presence of Rs in wilted plants was confirmed with Rs-specific Immunostrips as follows: a 1-g plant tissue was excised from the collar region of the infected plant, surface sterilized with 0.6% sodium hypochlorite for 2 min, rinsed with dH₂O three times, and ground with a mortar and pestle in 1.0 mL of dH₂O. One hundred microliters of the extraction buffer BEB1 buffer (Agdia) was mixed in a microcentrifuge tube with 100 μ L of the sample extract and tested with the Immunostrips. The experiment was repeated (Expt. II), and two pots were tested for each treatment.

In Expt. III, the plants were watered daily with 200 mL of water for 169 d and were tested with the methods to enumerate bacterial populations in drainage water, soil, and potting medium after inoculum was poured into pots containing rhizome-wounded ginger plants. Three pots were tested per treatment. The samples from both the experiments were analyzed until the pathogen populations became non-culturable on modified SMSA medium. Non-inoculated plants served as controls in both experiments. The daily and alternate day irrigation schedules created soil moistures in the range 75% to 100% and 50% to 75%, respectively, and are representative of two different scenarios that may occur in a field situation. The temperatures in the greenhouse were at a maximum of 32 ± 4 °C and a minimum of 18 ± 2 °C. The measurements were taken using Hygrochron® (Maxim Integrated Products, Sunnyvale, CA).

DETECTION OF *RALSTONIA SOLANACEARUM* FROM DRAINAGE WATER BY ELISA. The Ps1a antibody was used in ELISA to detect Rs in drainage water from soil and potting medium collected in Expt. III, and sensitivity of detection was evaluated for 145 d after inoculation. One hundred microliters of the drainage water was added into a 1.5-mL tube and was centrifuged at 16,000 g_n for 3 min. The supernatant was discarded and the pellet was resuspended in phosphate-buffered saline [PBS [8.0 g·L⁻¹ sodium chloride (NaCl), 1.15 g·L⁻¹ sodium phosphate (Na₂HPO₄), 0.2 g·L⁻¹ potassium phosphate (KH₂PO₄)] in dH₂O; pH 7.4] containing 0.5% (5 mL·L⁻¹) formalin and was incubated for 15 min. The samples were washed three times with 0.85% (8.5 g·L⁻¹) NaCl and resuspended in carbonate bicarbonate buffer [CBC [1.59 g·L⁻¹ sodium carbonate (Na₂CO₃), 2.93 g·L⁻¹ sodium bicarbonate (NaHCO₃)] in dH₂O; pH 9.6]. Samples (100 μ L) were pipetted into 96-well microtiter plates (three replications) and dried for 8 h in a circulating air incubator at 37 °C before initiating the ELISA assay (Kaneshiro et al., 2008). Samples with plate readings greater than twice the standard deviation above the average negative control were considered positive (Kaneshiro et al., 2008). A total of 366 drainage water samples (183 each from Rs-infested soil and potting medium) were analyzed with ELISA, Immunostrips, and PCR.

DETECTION OF *RALSTONIA SOLANACEARUM* FROM DRAINAGE WATER WITH IMMUNOSTRIPS. The sensitivity of Immunostrips was evaluated on drainage water samples for 145 d. A 1-mL subsample from the drainage water was vortexed for 10 s and 100 μ L of the sample was added to a 1.5-mL microcentrifuge tube containing 100 μ L of BEB1 buffer and was tested using the manufacturer's instructions.

DETECTION OF *RALSTONIA SOLANACEARUM* FROM DRAINAGE WATER BY PCR-*fliC*. A PCR detection method that uses *fliC* primers targeting the gene coding for the flagella subunit of Rs (Schonfeld et al., 2003) was used for analyzing drainage water samples. Samples were filtered to enhance trapping of the cells and were washed to minimize the inhibitors (Kubota et al., 2008). One milliliter of the drainage water

was trapped on a 0.2- μ m filter unit (Ultra-free MC GV column; Millipore, Billerica, MA) following centrifugation at 1000 g_n for 2 min. The filter was then washed with 1.5 mL of 1× TE buffer [10 mM Tris, pH 7.5; 1 mM ethylene diamine tetra acetic acid (EDTA), pH 8.0] and the unit was centrifuged at 1000 g_n for 2 min. The column was then crushed with a pair of pliers and the membrane was removed with sterile forceps and vortexed in 50 μ L of 1× TE buffer. The sample was incubated at 95 °C for 5 min followed by centrifugation at 2400 g_n for 1 min. Five microliters of the sample was used for PCR-*fliC* reaction.

PCR reactions were performed in 20- μ L reaction mixtures with 1 μ L of 1 μ M *fliC*-forward primer, 1 μ L of 1 μ M *fliC*-backward primer, 4 μ L of 5× Go Tag reaction buffer (Promega, Madison, WI), 0.16 μ L of 25 mM deoxynucleoside triphosphate (dNTP), 0.14 μ L of Taq DNA polymerase (5U), 8.7 μ L of double distilled water (ddH₂O), and 5 μ L of template DNA. The PCR reaction was carried out as per the protocol described by Schonfeld et al. (2003). The amplified products were electrophoresed at 80 V for 40 min through a 2% agarose gel (1× Tris-acetate-EDTA) and were stained with ethidium bromide. Appropriate size markers (Hyper ladder II; Bioline USA, Randolph, MA) were used for final confirmation of the amplified *fliC* DNA sequence.

STATISTICAL ANALYSIS. The greenhouse experiments were set up in a randomized complete block design. Days for wilting and survival of the pathogen in soil and potting medium were statistically analyzed using analysis of variance, and the means were compared using Student-Newman-Keuls test (SNK, $P \leq 0.05$). The analysis was performed with SAS (version 9.2; SAS Institute, Cary, NC).

Results and discussion

SURVIVAL OF *RALSTONIA SOLANACEARUM* IN SOIL AND POTTING MEDIUM. All wounded plants in all the treatments wilted within 12 d in alternate days of the irrigation schedule (Expts. I and II, Table 1) and in the daily irrigation schedule (Expt. III, data not shown). Non-wounded plants in soil took significantly longer (19.5–23 d) to wilt than wounded plants (Expts. I and II). The presence

Table 1. Disease development in edible ginger, following inoculation of *Ralstonia solanacearum* (Rs) race 4 strain A4515 into the stem or rhizome of 5-month-old plants and maintained under alternate days irrigation schedule.

Growth medium	Inoculation methods	Time to wilting (d)	
		Expt. I ^z	Expt. II ^z
Soil	Ginger: non-wounded ^y	23.0 ^u	19.5 ^u
	Ginger: rhizome wounded ^x	7.3	9.0
	Ginger: stem wounded ^w	8.7	8.5
Potting medium	Ginger: non-wounded	NW ^v	NW
	Ginger: rhizome wounded	7.7	8.5
	Ginger: stem wounded	7.7	9.5

^zExpt. I had three pots per treatment; Expt. II had two pots per treatment.

^y20 mL (0.6763 fl oz) of Rs (8 log cfu/mL) were poured into the growth medium (soil/potting medium) with no wound to ginger plants and the rhizome; 1 cfu/mL = 29.5735 cfu/fl oz.

^x20 mL of Rs (8 log cfu/mL) were poured into the growth medium (soil/potting medium) after wounding the rhizome by a sterile scalpel.

^w1 mL (0.0338 fl oz) of Rs (8 log cfu/mL) were inoculated into the stem of ginger plants with a sterile needle.

^vNW = no wilting (plants did not wilt until 153 and 129 d after inoculation for Expts. I and II, respectively).

^uColumn means are significantly different at ($P \leq 0.05$), based on Student-Newman-Keuls test.

of Rs in wilted plants was confirmed with Immunostrips, and the bacteria were cultured on modified SMSA medium. The plants in the potting medium with no wounds showed no bacterial wilt symptoms until the end of the experiments. Immunostrips tested negative and the pathogen was not cultured from these plant samples. The initial population of Rs in drainage water ranged from 3 to 5 log cfu/mL (Fig. 2, A–C) in all treatments except one (Fig. 2D, stem-wounded ginger plants in soil), where there was no recovery until 3 d, but the population increased to 5 log cfu/mL within 9 d. When plants were watered on alternate days (Expt. I), Rs was recovered up to 73 d from drainage water from all treatments (Fig. 2). There were no differences in recovery from pots with soil or potting medium in any treatments except two (Fig. 2B, non-wounded ginger plants in soil; Fig. 2D, discussed earlier). In the first case, the pathogen population increased abruptly after 11 d and continued to increase until 21 d when plants wilted. Populations then declined, gradually dropping to an undetectable level at 145 d. Rs was undetectable at only one other point (73 d). In the rhizome-wounded treatments, Rs was recovered from drainage water for 89 to 105 d (Fig. 2C), and for 89 d for ginger-stem wounded treatments (Fig. 2D). In each of the treatments, the highest population of Rs recovered in drainage water coincided with the wilt stage of the plant (Table 1, Fig. 2).

The days for survival of Rs in various treatments in Expt. II showed a similar trend as in Expt. I (Table 1,

Figs. 2 and 3). However, the survival was for a shorter duration in all the treatments except plants in the stem-wounded treatments in Expt. II. Rs was recovered in drainage water up to 121 d when plants were rhizome-wounded and irrigated daily (Expt. III), but only to 105 d in the alternate day irrigation schedule (Figs. 2–4). In soil also, Rs survived longer following a daily irrigation schedule (153 d; data not shown) than when plants were irrigated on alternate days (129 d, Table 2). No significant differences in Rs population levels were noted between treatments for any of the time points of analysis in Expt. I (105–153 d) and Expt. II (81–129 d), except for the Rs population in non-wounded treatment at 121 d in Expt. I, which showed significantly higher levels compared with other treatments. There were no significant differences in survival of Rs populations in soil and potting medium in Expt. III at any of the time points of analysis (113–176 d, data not shown).

The main sampling procedure used in the experiments was collection of drainage water from soil or potting medium followed by assessing Rs populations (cfu/mL). This was a non-destructive method of analysis and avoided injury to the tested plants. Wilting of the plants was thus due of the entry of Rs into the plant through the wounds created by the inoculation procedure in the experiment, and not due to the sampling procedure. However, because it also was important to know how long the pathogen survived in soil and potting medium (cfu/g), they were sampled

directly after Rs populations fell below log 1 cfu/mL in drainage water. The bacterium was recovered at later sampling times directly from soil/potting medium and not from the drainage water from these pots (Table 2, Figs. 2–3).

The edible ginger cropping period ranges from 8 to 10 months and there is a 2- to 4-month period between harvest and new plantings. Rs can be introduced into a fallowed field through irrigation water and may infect healthy unwounded edible ginger plants as well as ginger rhizomes injured by farm implements or nematodes. The study indicates that the Rs race 4 can survive for 3 to 5 months in soil under these different conditions, and can be released into drainage water, whether a plant is present or not. This study confirms that the pathogen is constantly released from infected plants, corroborating previous studies on survival and the spread of Rs race 3 strains infecting potato (Alvarez et al., 2008; Caruso et al., 2005). This study also shows that delayed appearance of wilting symptoms is associated with later release and longer survival of the pathogen in drainage water. This is of particular importance, as infection at a later stage of growth could be more damaging to the new edible ginger plantings in the subsequent season. The potting medium currently used to propagate ginger suppressed Rs population levels in the absence of a plant wound, and this indicates its potential value for managing diseases.

DETECTION OF *RALSTONIA SOLANACEARUM* IN DRAINAGE WATER BY ELISA. ELISA indicated positive results on all samples with Rs viable populations >7 log cfu/mL (Fig. 4, A and B). ELISA detected Rs in 97.4%, 97.1%, 79.3%, 52.4%, 16.7%, and 5% of the samples from soil, and 100%, 95.7%, 87.2%, 76.5%, 48%, and 22% of the samples from potting medium when populations were between 6 and 7 log, 5 and 6 log, 4 and 5 log, 3 and 4 log, 2 and 3 log, and 1 and 2 log cfu/mL, respectively. The assay did not show any positive results below viable Rs populations of 1 log cfu/mL. All of the samples in the control pots with no Rs inoculum tested negative. During the prewilt period of the edible ginger plants (0–10 d), Rs detection with ELISA was variable and the sensitivity ranged

Fig. 2. Population of *Ralstonia solanacearum* race 4 strain A4515 in drainage water from soil (□) and potting medium (■) with or without ginger, inoculated by various methods and irrigated on alternate days (Expt. I): (A) no ginger, (B) ginger: non-wounded, (C) ginger: rhizome wounded, and (D) ginger: stem wounded. Viable cells are reported as log cfu per milliliter (1 cfu/mL = 29.5735 cfu/fl oz). Each data point is the average of three samples and the errors bar represent SE.

Fig. 3. Population of *Ralstonia solanacearum* race 4 strain A4515 in drainage water from soil (□) and potting medium (■) with or without ginger, inoculated by various methods and irrigated on alternate days (Expt. II): (A) no ginger, (B) ginger: non-wounded, (C) ginger: rhizome wounded, and (D) ginger: stem wounded. Viable cells are reported as log cfu per milliliter (1 cfu/mL = 29.5735 cfu/fl oz). Each data point is the average of two samples and the errors bar represent SE.

Fig. 4. Evaluation of sensitivity of detection of *Ralstonia solanacearum* race 4 strain A4515 in drainage water from pots with infested soil and potting medium and irrigated daily (Expt. III). Plate counts on modified SMSA (semiselective medium South Africa) were compared with ELISA using Ps1a monoclonal antibody (A and B); Immunostrips® (Agdia, Elkhart, IN) using Ps1a monoclonal antibody (C and D); and polymerase chain reaction (PCR) using *fliC* primer (E and F). Black Circles (●) represent positive results, open circles (○) represent negative results, and grey circles (●) represent overlapping positive and negative samples. Viable cells are reported as log cfu per milliliter (1 cfu/mL = 29.5735 cfu/fl oz).

Fig. 4. (continued)

between 5 and 7 log cfu/mL (Fig. 4, A and B). The most consistent detection was during the post-wilting period (10–36 d). However, the pathogen was only detected a few times beyond 36 d. All of the samples from the control pots with no *Rs* inoculum tested negative. ELISA performed comparatively well on samples from potting medium compared with those from the soil.

ELISA is a traditional method for detecting *Rs* and other bacterial pathogens, but sensitivity usually is not more than 5 to 6 log cfu/mL (Alvarez, 2004). This is clearly validated in our study with >95% detection when *Rs* populations were >5 log cfu/mL in drainage water from soil and potting medium. However, the detection was inconsistent when *Rs* populations were <5 log cfu/mL. This was attributed to the numerous washing steps in the protocol, leading to loss of cells from microtiter plates, which compromised sensitivity.

DETECTION OF *RALSTONIA SOLANACEARUM* IN DRAINAGE WATER WITH IMMUNOSTRIPS. This assay detected *Rs* from 100% of the samples

when the populations were >3 log cfu/mL (Fig. 4, C and D). No significant differences between soil and potting medium were observed for the Immunostrip detection assay, and 94.4%, 75%, and 45% of the samples from soil, and 100%, 88.9%, and 40.7% of the samples from potting medium tested positive when populations were between 2 and 3 log, 1 and 2 log, and 0 and 1 log cfu/mL, respectively. The detection was consistent during early wilting (0–10 d) and at severe wilting (10–15 d) stage. During the post-wilting (beyond 15 d), the assay consistently gave positive results up to 54 d. Positive results using Immunostrips were observed on samples collected as late as 129 d. None of the samples from the control pots (no *Rs* inoculum) tested positive. Immunostrips were more sensitive than ELISA in detecting *Rs* from drainage water (Fig. 4, A–D).

The role of EPSI as a virulence factor in *Rs*, and its ability to enhance symptom expression following root infection and vascular colonization of tomato plants, has been previously demonstrated (Araud-Razou et al.,

1998; Denny and Baek, 1991). Also, EPSI is produced abundantly by *Rs* in culture media and in planta (Orgambide et al., 1991). The most significant application of this information has been in the use of anti-EPSI-based monoclonal antibodies (Ps1 and Ps1a) for detection of *Rs* strains from pure cultures and field samples (Alvarez et al., 1993). Assays using this MAb are specific for *Rs* strains, highly sensitive (detection limit of 1 ng of purified EPSI by ELISA), and resistant to inhibitors in environmental samples (McGarvey et al., 1998).

A drawback of using EPSI-based MAb in ELISA is that the cells may be washed away easily during the procedure and the sensitivity tends to be low when viable *Rs* populations fall below 5 to 6 log cfu/mL, as shown in this study. Immunostrips created with MAb Ps1a rely on a different format, trapping the antigen onto a strip. The desirable outcome is a highly sensitive *Rs*-EPSI detection in drainage water from field samples. Detection of *Rs* at low levels (1–2 log cfu/mL) in drainage water in pot studies indicates that the Immunostrips

Table 2. Survival of *Ralstonia solanacearum* (Rs) race 4 strain A4515 in soil and potting medium with or without ginger and maintained under alternate days irrigation schedule. Samples were taken directly from the medium after the plants wilted. The shaded region indicates the last day the pathogen was recovered directly from soil or potting medium. Viable cells are reported as log cfu per gram (1 cfu/g = 28.3495 cfu/fl oz).

Growth medium and inoculation methods	Populations of <i>Ralstonia solanacearum</i> [log (cfu/g + 1)]									
	Time after inoculation (d)									
	81	89	97	105	113	121	129	137	145	153
Expt. I										
Soil										
No ginger ^z	— ^y	—	—	1.2	0.9	0.0	0.0	0.0	0.0	0.0
Ginger: non-wounded ^x	—	—	—	0.9	0.9	0.3	0.7	0.3	0.0	0.0
Ginger: rhizome wounded ^w	—	—	—	1.6	0.8	0.3	0.8	0.0	0.0	0.0
Ginger: stem wounded ^v	—	—	—	1.2	0.0	0.0	0.0	0.0	0.0	0.0
Potting medium										
No ginger	—	—	—	1.1	0.4	0.0	0.0	0.0	0.0	0.0
Ginger: non-wounded	—	—	—	0.4	1.7	1.2 ^u	0.3	0.0	0.0	0.0
Ginger: rhizome wounded	—	—	—	1.2	0.0	0.0	0.0	0.0	0.0	0.0
Ginger: stem wounded	—	—	—	0.7	1.5	0.3	0.0	0.0	0.0	0.0
Expt. II										
Soil										
No ginger	0.7	1.2	0.6	0.0	0.0	0.0	0.0	— ^y	—	—
Ginger: non-wounded	2.8	1.7	1.8	0.6	1.8	0.0	0.0	—	—	—
Ginger: rhizome wounded	2.9	2.9	1.2	0.0	0.0	0.0	0.0	—	—	—
Ginger: stem wounded	2.4	2.5	1.8	0.0	0.0	0.0	0.0	—	—	—
Potting medium										
No ginger	1.2	0.7	0.6	0.0	0.0	0.0	0.0	—	—	—
Ginger: non-wounded	2.1	0.7	0.6	0.0	0.7	0.0	0.0	—	—	—
Ginger: rhizome wounded	0.7	0.7	0.0	0.6	1.0	0.0	0.0	—	—	—
Ginger: stem wounded	2.3	0.8	0.0	0.0	0.0	0.0	0.0	—	—	—

^z20 mL (0.6763 fl oz) of Rs (8 log cfu/mL) were poured into the growth medium (soil/potting medium) with no ginger plants.

^y— indicates that no samples were taken for analysis.

^x20 mL of Rs (8 log cfu/mL) were poured into the growth medium (soil/potting medium) with no wounds to the ginger plants and the rhizome.

^w20 mL of Rs (8 log cfu/mL) were poured into the growth medium (soil/potting medium) after wounding the rhizome of the ginger plants by a sterile scalpel.

^v1 mL (0.0338 fl oz) of Rs (8 log cfu/mL) were inoculated into the stem of ginger plants with a sterile needle.

^uColumn means in the same experiment are significantly different at ($P \leq 0.05$), based on Student-Newman-Keuls test.

may be suitable for testing field samples before and after planting and for regular monitoring of irrigation water sources and runoff from adjacent fields. Immunostrip results were unaffected by the type of growth medium used (soil or potting medium) in this study, indicating that this assay may be used for testing diverse environmental samples.

DETECTION OF *RALSTONIA SOLANACEARUM* IN DRAINAGE WATER BY PCR. PCR-*fliC* showed positive amplification for all samples from soil with viable Rs populations >7 log cfu/mL (Fig. 4, E and F). The assay detected Rs in 100%, 97.1%, 96.6%, 81%, 61.1%, 50%, and 15.9% of the samples from soil, and in 83.3%, 82.6%, 61.5%, 38.2%, 20%, 11.1%, and 14.8% of the samples from potting medium when populations were between 6 and 7 log, 5 and 6 log, 4 and 5 log, 3 and 4 log, 2 and 3 log, 1 and 2 log, and 0 and 1 log cfu/mL, respectively. PCR-*fliC* performed significantly better on soil

samples than those from potting medium. This may be due to the presence of numerous PCR inhibitors in potting medium that lead to inconsistent results, thereby compromising sensitivity, as seen in our studies. The assay detected Rs throughout the experimental period (until 105 d). However, numerous samples with high populations were not detected before, during, or after plants wilted. None of the samples in the control pots with no Rs inoculum tested positive.

The detection capability of PCR-*fliC* was above 80% when populations of Rs race 4 in drainage water from soil were above 3 log cfu/mL. An even more sensitive real-time PCR assay detected Rs race 4 at even lower levels of 1 to 2 log cfu/mL in ginger rhizomes when samples were enriched in a semiselective medium for 24 h (Thammakijawat et al., 2006). However, our studies were focused on the testing of numerous drainage water samples from soil and potting

medium for the presence of the bacterium, and hence, were different from previous studies.

Conclusions

Rs survived in field soil in the presence or absence of ginger debris for 120 to 150 d, indicating that the bacterium should survive the fallow period for ginger cultivation, increasing the chances of infection in the new crop. The potting medium used in this experiment reduced the survival of Rs race 4, and in the absence of a wound, the ginger plants were not infected. Immunostrips had the lowest detection limit among all assays tested and was the most rapid (1 min) and least cumbersome method for detecting the bacterium. Based on the results of this study, Immunostrips have been further selected for environmental monitoring of Rs in ginger fields as well as for evaluating the effects of biofumigants in Rs-infested soils and potting medium.

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