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Detection of *Ralstonia solanacearum* in natural substrates using phage amplification integrated with real-time PCR assay

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ABSTRACT

A sensitive, selective, and rapid protocol for detecting *Ralstonia solanacearum* from soil and plant tissues was developed based on the integration of the rapid self-replicating ability of bacteriophages with quantitative PCR (q-PCR). Six bacteriophages were isolated and selected for their ability to specifically infect and lyse *R. solanacearum*. Sixty-three strains of *R. solanacearum* and 72 isolates of other bacterial species were tested for their susceptibility to the bacteriophages. Based on the large host range and observed replication speed and reproductive burst sizes in ginger infecting *R. solanacearum* strain GW-1, phage M_DS1 was selected for the development of the phage-based indirect assay. With primers based on the phage genome, the protocol was used to detect *R. solanacearum* from a number of substrates. In pure *R. solanacearum* cultures, the protocol consistently detected approximately 3.3 CFU/ml after an hour's incubation with 5.3×10^2 PFU/ml M_DS1. We used the protocol to confirm the presence of the pathogen in infected potted ginger plants, detecting levels near 10^2 CFU/g in 0.1 g of leaf tissue and levels near 10^3 CFU/ml in drainage water from the pots. In soils emended with the bacteria, we observed detection limits down to approximately 10^2 CFU/g.

1. Introduction

Bacterial wilt caused by *Ralstonia solanacearum* is deemed to be one of the most important plant diseases in tropical agriculture (Hayward, 1991). The disease affects more than 50 plant families including crops and wild species and includes strains adapted to infecting potato in temperate climates (Olsson, 1976; Hayward, 1991). The first line of defense against the spread of *R. solanacearum* is the restricted movement of infected plants. Identification of infected plants is made primarily by visual inspection. However, latent infections in plant tissues and propagating stocks can escape visual detection. Therefore, to effectively control the spread of the bacterium, more sensitive detection systems have been developed (Elphinstone et al., 1996).

The use of PCR to amplify selected regions of the bacterial genome unique to *R. solanacearum* (Poussier et al., 2002; Caruso et al., 2003; Dittapongpitch and Surat, 2003; Schonfeld et al., 2003; Van der Wolf et al., 2004) has been proposed for improving the speed and sensitivity of pathogen detection. However, PCR is not routinely used for detection of *R. solanacearum* in field samples because compounds in soil and plant tissues inhibit the process (Steffan et al., 1988; Picard et al., 1992; Tsai and Olson, 1992; Young et al., 1993; McGregor et al., 1996; Wilson, 1997). Dilution of samples to reduce the effects of inhibitory compounds can circumvent this limitation, but has the disadvantage of decreasing assay sensitivity and increasing the probability of false negative results (Picard et al., 1992). The target bacterium can be enriched by culturing samples on a semi-selective medium prior to PCR detection but *R. solanacearum* grows slowly and it can take up to 60 h to achieve a sensitivity of about 10^2 CFU/g of infected soil (Elphinstone et al., 1998; Pradhanang et al., 2000; Poussier et al., 2002).

The aim of this work is to improve sensitivity and speed of the detection protocol by using *R. solanacearum* specific bacteriophages to achieve rapid target amplification prior to molecular detection. Unique *R. solanacearum* specific bacteriophage gene sequences were employed in the development of primers for PCR.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The *R. solanacearum* and other bacterial isolates used to determine the host specificity of the isolated phages are shown in Table 1. *R. solanacearum* strains comprising all of the major different races and biovars were included in the analysis. The ginger infecting strain GW-1 was used for detailed analysis of phage/*R. solanacearum* interactions. Other bacterial species used in determining the host specificity of the

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Table 1
Strains of *Ralstonia solanacearum* and other species used in this study

Race	No. of strains	Host (no. of strains)	Origin (no. of strains)
1	23	Pepper (1), tomato (8), peanut (6), tobacco (4), olive (1), squash (1), snapbean (1), *Melanopodium perfliatum* (1)	South East Asia (9), The Caribbean (3), Australia (1), North America (5), South America (2), Pacific Islands (3)
2	10	Banana (5), heliconia (4), *Musa* sp. (1)	The Caribbean (1), Pacific Islands (5) South East Asia (1)
3	8	Potato (6), geranium (2)	Middle East (1) South East Asia (2), Middle East (1), South America (1), Africa (2), The Caribbean (1)
4	20	Ginger (20)	Australia (2), South East Asia (2), Pacific Islands (16)
5	2	Mulberry (2)	China (2)

Control strains

Species (no. of species)	Host	Origin
Enterobacter sp. (2)	Ginger (2)	Pacific Islands (2)
Pseudomonas sp. (2)	Anthurium (2)	Pacific Islands (2)
Ralstonia eutropha (1)		
Xanthmonas sp. (2)	Citrus (1), Anthurium (1)	Pacific Islands (2)

Environmental strains

Species (no. of species)	Location[a]
Acinetobacter sp. (3)	Wn (3)
Arthrobacter sp. (4)	Ha (1), Ka (1), La (1), SLH (1)
Bacillus sp. (16)	Ha (1), Ka (5), La (2), SLH (2), Wa (1), Wn (5)
Bacterium rA3 (1)	Wa (1)
Bacterium str (1)	Ha (1)
Citrobacter sp. (1)	Wn (1)
Corynebacterium sp. (1)	Ha (1)
Erwinia sp. (1)	Mi (1)
Escherichia sp. (1)	Ka (1)
Flavobacterium sp. (2)	Wn (2)
Glacial ice bacterium (1)	Ha (1)
Microbacterium sp. (3)	Ha (1), Ka (1), La (1)
Paenibacillus sp. (2)	Ka (2)
Pantoea (1)	Ka (1)
Paracoccus sp. (5)	Ha (1), La (2), SLH (1), Wn (1)
Pseudomonas sp. (11)	Ha (1), Ka (2), La (3), SLH (3), Ma (2)
Rhodococcus sp. (1)	Ka (1)
Stenotrophomonas sp. (2)	Ka (1), Wn (1)
Uncultured bacterium (5)	Ka (1), La (2), SLH (1), Wn (1)
Vibrio sp. (1)	Ka (1)
Xanthomonas sp. (3)	La (1), SLH (1), Wn (1)

[a] Ha is Hale iwa; Ka is Kahuku; La is Laie; Ma is Makaha Valley; SLH is St. Louis Heights; Wa is Waimanalo, and; Wn is Wai anae.

phages included those that were isolated from the same soil samples from which the phages were isolated and others that were obtained from existing collections. Bacteria species that were isolated with the phages were identified on the basis of their 16S rDNA sequence homologies with strains in GenBank.

2.2. Sampling for bacteriophages

Soils from farms on the Hawaiian island of Oahu where solanaceous crops are cultivated were sampled for phages that can infect *R. solanacearum*. For phage isolation, 1 g of soil was suspended in 5 ml of SM (per liter: 5.8 g NaCl, 2 g $MgSO_4$•$7H_2O$, Tris–Cl (1 M, pH 7.5) 0.002% w/v gelatin) buffer, filtered through a 0.45 μm membrane, and added to a culture of *R. solanacearum* cells in 20 ml CPG (Kelman, 1954) medium suspension (per liter, 10 g of tryptone, 5 g of glucose, 1 g of Casamino Acids, 1 g of yeast extract, and 15 g of agar) and cultured overnight in an incubated orbital shaker (New Brunswick Scientific C-24, Edison, NJ) at 250 rpm at 28 °C. Chloroform was then added to a concentration of 0.5% and the culture shaken vigorously for 10 min. The culture was centrifuged and the supernatant was assayed for the presence of phages infecting various strains of *R. solanacearum* by using a plaque-forming assay (Adams, 1959). Phages were initially typed on the basis of plaque size and shape and then by DNA banding after nuclease digestion and gel electrophoresis, as well as by imaging with a Transmission Electron Microscope.

2.3. Host range and sensitivity analysis

Bacteriophages isolated from various farms were tested for their ability to infect and lyse *R. solanacearum*. Sixty-three strains of *R. solanacearum* and 72 isolates of other bacterial species were tested for their susceptibility to the bacteriophages by the plaque-forming assay. Susceptibility was scored by observation of plaque formation after infecting host culture with an initial OD_{600} of 0.3 with 1×10^6 PFU/ml phage solution. Phages that exhibited a broad host range within *R. solanacearum* but did not infect the other bacterial species were selected for further analysis.

2.4. Quantification of phage amplification efficiencies

The number of phage progeny produced per infected cell during an infection cycle was determined by initially inoculating a known quantity of host cells with a given number of phage particles and incubating to the mid-way point of the predetermined eclipse period, which was defined as the period required for phage progeny to emerge from the infected host. Residual phage particles from the original inoculum were then inactivated with 10 mM of the virucidal agent ferric ammonium sulfate (FAS) (Park et al., 2003) and the phage progeny released from infected cells were counted using the plaque-forming assay immediately after the completion of the eclipse period but before the beginning of the next infection cycle.

2.5. DNA manipulation

Standard DNA techniques were employed to clone and sequence phage DNA (Sambrook and Russell, 2001).

2.6. Real time PCR amplification

Real time amplification (q-PCR) of phage DNA fragments was carried out on a commercial instrument (MJ-Mini, Bio-Rad, Hercules, CA, USA) using the iQ™ SYBR Green Supermix (Bio-Rad) with forward (5′CGCCATCACGTTATCAACACG 3′) and reverse (5′CTGACCGTTTAACG-TCACTGACAGC 3′) primers designed from the partially sequenced genome of the isolated phage M_DS1. PCR was performed by initially denaturing at 95 °C for 5 min and then cycling 34 times with a 30 s denaturation step at 95 °C, a 30 s annealing step at 55 °C, and a 1 min elongation/extension step at 72 °C. The product of this PCR is expected to be 180 base pair in size. Positive amplification was scored when the SYBR Green fluorescence exceeded a threshold of 0.015 arbitrary units at any cycle in the PCR process.

2.7. Indirect detection of R. solanacearum

To validate the combined phage amplification/q-PCR capability to indirectly detect low concentrations of *R. solanacearum*, an initial optimization experiment was set up consisting of phage M_DS1 added to *R. solanacearum* (GW-1) in CPG culture at varying multiplicities of infection (MOI). After vortexing each culture tube, 100 µl of supernatant representing phage culture at time zero (T_0) was removed and frozen at −20 °C. The remaining cultures were then incubated and shaken at 28 °C at 250 rpm for 1 h after which another 100 µl supernatant representing phage concentration after 1 h was withdrawn (T_1). Quantitative-PCRs (q-PCR) were performed using the withdrawn supernatants as templates. Indirect detection of *R. solanacearum* was scored when the threshold cycle for the time zero reaction exceeded that for the 1 h sample using a one-tailed *t*-test at the 0.05 significance level, and the detection limit was taken as the minimum amount of culturable bacteria resulting in positive detection using the same statistical comparison.

To evaluate the selectivity of the phage amplification/q-PCR protocol, q-PCR was performed as described above on a variety of sample mixtures in CPG with or without phage M_DS1, other *R. solanacearum* specific (S6-S1, ISO-2, M_DL, S3_S, and S5_5) and non-specific (nJV-4, nJV-9, nS3, nS5, nDM-1, and nKK) phages isolated in the course of this study, *R. solanacearum* strain GW-1, and/or a consortia of ten other bacteria (*Bacillus megaterium*, *Arthrobacter globiformis*, *Flavobacterium* sp., *Pseudomonas* sp., *Pseudomonas stutzeri*, *Pantoea stewartii*, *Microbacterium natoriense*, *Citrobacter selakii*, *Paenibacillus* sp., and *Acinetobacter baumannii*). Mixtures with phage M_DS1 contained quantities approximately in the range optimized in the protocol above for low detection limit (~5×10^2 PFU/ml), and similar quantities of other phages were used in mixtures containing them. Mixtures with *R. solanacearum* contained approximately 10^2 CFU/ml of the bacteria, and consortia of other bacteria were collected from media inoculated with them all and grown to OD_{600} of 0.3. Prior to PCR, samples were incubated in an orbital shaker for 1 h at 28 °C to allow the opportunity for phage proliferation. Positive detection was scored when a visible DNA product of the expected size was observed by ethidium bromide staining on an agarose gel after electrophoresis, and when SYBR Green fluorescence exceeded a threshold value of 0.015 arbitrary units during the course of the amplification.

For validation of the protocol in soil and plant tissue samples, ten day old potted ginger plants were inoculated by pouring bacterial suspensions (*R. solanacearum* strain GW-1) to soil densities of approximately 10^{11} CFU/g after wounding the base of the stems with a one to two inch incision. Samples of plant tissue and drainage from the soil following daily irrigation were collected 14 days post-inoculation, before the onset of visible symptoms of bacterial wilt disease, for analysis with the phage amplification/q-PCR protocol. In the tissue experiments, 0.1 g (fresh weight) of root or leaf tissues of infected potted ginger was used. The tissues were ground in 1 ml of sterile distilled water and the concentration (CFU/g of tissue) of the *R. solanacearum* was determined by plate count on TZC medium (Kelman, 1954). Serially diluted phage solutions were then added to serial dilutions of tissue extract to give different MOIs. A 150 µl aliquot of the mixture was then added to 50 ml 3× CPG medium and a 100 µl aliquot was immediately withdrawn and stored at −20 °C to represent phage culture at time zero. The remaining culture was incubated and shaken at 28 °C at 250 rpm for 1 h to allow phage DNA proliferation. The soil experiments were carried out in two parts. In part one, irrigation water drained from infected potted ginger was tested using the protocol as described for the ginger tissue extracts. In the second set up, 1 g soil samples were emended with 0.1 ml of serially diluted (3.6×10^4, 3.6×10^3 and 3.6×10^2 CFU/ml) *R. solanacearum* (strain GW-1) culture. The inoculated soil samples were mixed with 2 ml sterile distilled water and the pathogen extracted and assayed as described above for plant tissues. For comparison, negative controls were taken for analysis from plant tissues of uninoculated potted ginger plants, and from uninfected soils.

3. Results

3.1. Phage isolation and characterization

Six *R. solanacearum* infecting phages with unique plaque morphology, nuclease digestion banding patterns, and physical appearance on Transmission Electron Microscope were isolated from cultivated lands on Oahu. Non-specific interactions of these phages were not observed with any of the 72 tested isolates of other bacterial species. Each of these phages was tested against 63 different strains of the *R. solanacearum* (Table 1) to identify host strains within the species. None of the bacteriophages infected all strains. In all cases though, the susceptibility rates were generally high, ranging from about 80% for S5_5 to about 97% for M_DS1 (Table 2). The two *R. solanacearum* strains not susceptible to phage M_DS1 included one Race 5 isolated from mulberry in China, and one Race 1 isolated from tomato in Hawaii. Significantly, all of the tested Race 3 strains of *R. solanacearum*, which are classified as a select agent for potential agro-terrorism, were susceptible to phage M_DS1.

In addition to the six phages isolated which were selective to bacteria within the *R. solanacearum* species complex, an additional six phages were isolated which were able to infect *R. solanacearum* strain GW-1 but which also formed plaques on cultures of other bacteria outside of the species. Aside from using these in selectivity studies described above for the phage proliferation/q-PCR protocol, no further characterization of these non-selective phages was performed.

Table 2
Characteristics of *R. solanacearum* specific phages isolated from cultivated lands on the island of Oahu

Characteristic	Phage isolate					
	M_DS1	S6-S1	ISO-2	M_DL	S3_S	S5_5
Origin[a]	SLH	La	Wa	SLH	La	La
Host range[b]	97% (2)	92% (5)	89% (7)	89% (7)	87% (8)	79% (13)
Adsorption time[c]	15–30 min	45 min	45 min	90 min	90 min	<15 min
Eclipse period[d]	15–30 min	<15 min	15–30 min	45 min	45 min	45 min
Reproductive burst[e]	160±21	13±1	220±13	22±2	8±2	120±32

[a] SLH is St. Louis Heights; La is Laie; Wa is Waimanalo.
[b] Percentage of the 63 tested *R. solanacearum* strains susceptible to the phage (number of non-susceptible strains).
[c] Time required for phage to infect the host bacteria.
[d] Time required for phage progeny to emerge after infection.
[e] Number of phage progeny released per infected host cell.

Table 3
Indirect detection of *R. solanacearum* in pure culture with q-PCR amplification of phage M_DS1 DNA

[Phage] PFU/ml	Host cell concentration CFU/ml						
		3.3×10^5	3.3×10^4	3.3×10^3	3.3×10^2	3.3×10^1	3.3
5.3×10^5	C_{T0}	1.36±0.17	1.7±0.46	1.6±0.23	1.66±0.25	1.94±0.46	1.97±0.84
	C_{T1}	1.15±0.02	1.15±0.02	1.19±0.02	1.16±0.04	1.17±0.11	1.19±0.03
		$p=0.921$	$p=0.322$	$p=0.871$	$p=0.337$	$p=0.476$	$p=0.691$
5.3×10^4	C_{T0}	4.25±1.06	4.46±0.81	4.43±0.36	4.91±1.35	5.01±0.92	4.37±1.08
	C_{T1}	1.31±0.01	1.64±0.64	1.38±0.05	2.23±0.77	4.31±0.75	4.7±0.92
		$p=0.022$	$p=0.039$	$p=0.009$	$p=0.022$	$p=0.087$	$p=0.711$
5.3×10^3	C_{T0}	16.26±4.71	15.72±2.69	15.15±0.78	13.37±1.09	15.3±0.39	15.68±0.78
	C_{T1}	1.43±0.11	1.65±0.2	1.53±0.03	6.7±1.06	10.65±0.6	14.3±1.7
		$p=0.012$	$p=0.006$	$p=0.002$	$p=0.013$	$p=0.038$	$p=0.101$
5.3×10^2	C_{T0}	24.72±1.75	24.04±0.93	26.14±4.91	26.62±0.67	25.8±1.97	27.44±2.64
	C_{T1}	5.47±0.16	4.61±0.92	4.58±0.64	6.31±0.32	16.44±1.08	20.48±2.36
		$p=0.007$	$p=0.003$	$p=0.005$	$p=0.009$	$p=0.011$	$p=0.038$

C_{T0} = time zero threshold cycle; C_{T1} = threshold cycle after 1 h incubation. Phage proliferation in metabolically active host is detected when C_{T1} is significantly lower than C_{T0} by *t*-test ($p<0.05$).

3.2. Phage generation times and burst size

The adsorption times required for phage infection of the host ranged from about 15 min for phage S5_5 to about 90 min for phages M_DL and S3_S (Table 2). Phage M_DS1 had an infection period of between 15 and 30 min and an eclipse period of similar duration (Table 2). On average the eclipse periods for the other phages ranged from about 45 to 75 min. The reproductive burst sizes determined for each of the phages ranged from a high of 220 to a low of about 10 phage progeny per infected host cell (Table 2).

Phage M_DS1 was selected as the most suitable candidate for indirect detection of *R. solanacearum* based on its relatively short replication cycle, prolific burst of progeny, infectivity to a wide variety of host strains of interest, and the fact that its genome is composed of double stranded DNA, thereby precluding the need for reverse polymerase reactions and the susceptibility to RNA degradation.

3.3. Indirect detection of R. solanacearum

Real-time assays based on the DNA sequence of phage M_DS1 were tested and validated for the detection of *R. solanacearum* in a variety of conditions. Table 3 shows the results of the phage proliferation/q-PCR protocol for various initial concentrations of the phage and cultured *R. solanacearum* cells. A significant increase in phage concentration after incubation was attributed to the presence of a metabolically active host. Increased phage concentration correlates positively with increased phage genetic material and hence to lower C_T values. Significant decline of C_T values for post-incubation cultures compared to those of pre-incubation cultures was observed for a number of phage to host ratios. When a high initial phage concentration was used (e.g. 5.3×10^5 PFU/ml), the ability to detect small amount of host was reduced, but detection limits steadily improved with lower numbers of initial phage counts (Table 3). Using a low initial phage concentration of 5.3×10^2 PFU/ml resulted in the detection of *R. solanacearum* at all concentrations tested, including 3.3 CFU/ml.

As expected the assay showed remarkable selectivity by accurately detecting the presence of *R. solanacearum* in mixed cultures containing *R. solanacearum* and associating bacteria but showing no proliferation of the selected phage DNA for negative samples containing other bacteria and phages (Fig. 1). Curiously, no phage DNA amplification was observed in mixtures containing phage M_DS1 but no *R. solanacearum*, suggesting that some amount of phage DNA proliferation occurred in the "C_{T0}" samples stored at −20 °C in Table 3, perhaps during the short intervals of time during freezing and thawing and prior to the initial PCR denaturation step. While the

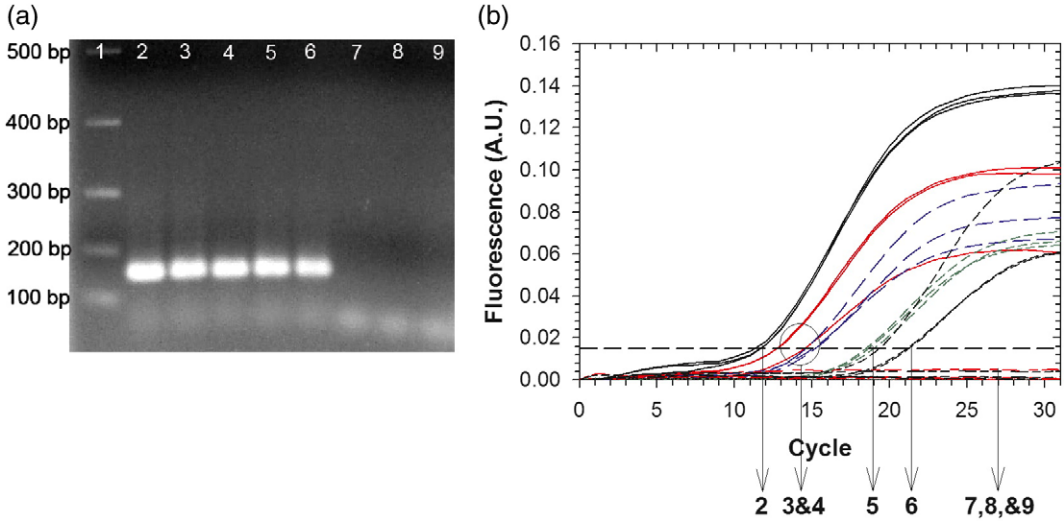

Fig. 1. PCR product banding patterns (a) and q-PCR fluorescence graphs (b) for phage M_DS1 specific primers applied to various consortia of bacteria and phages. Reaction mixes contain: 2) phage M_DS1 and *R. solanacearum*; 3) phage M_DS1, *R. solanacearum*, and non-host bacteria consortium; 4) phage M_DS1, other *R. solanacearum* infecting phages, and *R. solanacearum*; 5) phage M_DS1, non-*R. solanacearum* infecting phages, *R. solanacearum*, and non-host bacteria consortium; 6) phage M_DS1, other *R. solanacearum* infecting phages, *R. solanacearum*, and non-host bacteria consortium; 7) phage M_DS1, non-*R. solanacearum* infecting phages, and non-host bacteria consortium; 8) non-*R. solanacearum* infecting phages, *R. solanacearum*, and non-host bacteria consortium; 9) non-*R. solanacearum* infecting phages and non-host bacteria consortium. Lane 1 in gel (a) contains DNA size markers (Bioline HyperLadder IV, Taunton MA).

Table 4
Indirect detection of *R. solanacearum* from infected ginger tissues and environmental substrates

	[Initial host] CFU/ml	[Phage] PFU/ml	Mean C_T at		*p*-value
			T_0	T_1	
Root tissue	3.3×10^5	7.1×10^2	22.66±1.19	3.23±1.68	0.004
Leaf tissue	2.7×10^2	3.1×10^2	24.69±2.7	16.72±1.59	0.019
Drainage water	4.9×10^3	5.6×10^2	26.18±2.01	15.15±1.07	0.009
Soil emended	3.6×10^2	4.2×10^2	24.53±2.19	19.27±0.64	0.019

Detection was scored positive when mean C_T at T_0 was significantly higher than C_T at T_1. Data are mean±standard deviations from three replications.

presence of other phages and bacteria suppressed the efficiency of the detection method as illustrated by increased cycle threshold values (Fig. 1), detection occurred for each sample in which both phage M_DS1 and *R. solanacearum* were present and in none of the samples in which either was missing.

R. solanacearum was detected in different tissues of infected potted ginger (leaves and roots), effluent water from pots with infected ginger plants, and from soil emended with *R. solanacearum* following 100-fold dilution of the sample extracts. At this dilution level, detection occurred in all of the plant tissues and soil drainage water from infected potted plants tested, including levels of 3.3×10^5 CFU/g in root tissue using a starting phage count of 7.1×10^2 PFU/ml and 3.4×10^2 CFU/g in leaf tissue using a starting phage count of 3.1×10^2 PFU/ml, and levels as low as 4.9×10^3 in drainage water using initial phage concentrations of 5.6×10^2 of PFU/ml (Table 4). The detection limit for *R. solanacearum* in emended soils was 3.6×10^2 CFU/g using an initial phage concentration of 4.2×10^2 of PFU/g of soil (Table 4), as detection did not occur in soil emended with 36 CFU/g *R. solanacearum*. No detection was observed in any of the negative controls.

4. Discussion

The primary importance of this work was the coupling of the selectivity of the phage–host interaction and the amplification and detection of phage DNA by q-PCR to improve the sensitivity of *R. solanacearum* detection. Real-time assays based on the DNA sequence of the phage were tested and validated for the detection of *R. solanacearum* in a variety of settings. In order to be able to use the phage DNA as an indirect indicator for *R. solanacearum* detection, it was necessary to isolate a *R. solanacearum*-specific bacteriophage. Parameters that were of importance in selecting the phage of choice for the development of detection primers were the number of progeny that each infecting phage produced (burst size) and their generation times. These give an indication of the magnitude of nucleic acid amplification that can be expected and the times that would be required for the amplification process. In our study we selected a phage that had a burst size of about a 100 progeny per infected host and a generation time of less than 1 h. Thus we expected about a 100-fold increase in our phage DNA concentration after about an hour's incubation with the host, corresponding to a depression in the threshold cycle for q-PCR of at least 6 depending on the amplification efficiency. We observed a saturation effect at high initial phage concentrations, where an insufficient amount of host was available to replicate the phage to levels easily discriminated from the starting amount, so that much lower detection limits were possible using relatively small amounts of initial phage (Table 3).

A major setback to using PCR for detecting *R. solanacearum* from infected soils and plant tissues has been the presence of PCR inhibitors in these substrates (Steffan et al., 1988; Picard et al., 1992; Tsai and Olson, 1992; Young et al., 1993; Seal, 1995; McGregor et al., 1996; Wilson, 1997). Diluting the samples to decrease the concentration of these inhibitors increases the likelihood of false negative outcomes. In our protocol it was shown that indirectly targeting *R. solanacearum* selective phage DNA can result in positive identification of the bacterium from diluted samples as the phage used had a burst size that under ideal conditions can increase the target phage DNA 100-fold after only an hour's incubation with *R. solanacearum*.

The findings of our greenhouse experiments demonstrate that the new method is promising for the detection of *R. solanacearum* from real soil, irrigation water, and plant tissues. While PCR was inhibited by undiluted extracts from these materials, detection of phage DNA was possible after 100-fold dilution. Detection at levels down to a 4.9×10^3 CFU/ml *R. solanacearum* was observed in irrigation water with an initial addition of 5.6×10^2 PFU/ml phage load (Table 4). Similarly, *R. solanacearum* was detected in plant tissue extract at 2.7×10^2 CFU/g using an initial phage concentration of 3.1×10^2 PFU/ml (Table 4). These values compare favorably to detection limits reported in previous studies of PCR detection of *R. solanacearum*, which range from 10^2 to 10^5 CFU/ml in pure suspensions and are even higher ($\geq10^6$ CFU/g) in plant tissues (Elphinstone et al., 1996; Fegan et al., 1998). Significantly, we were able to detect latent infections in plant tissues containing only 2.7×10^2 CFU/g starting with only 0.1 g of tissue. Existing protocols for screening plants for *R. solanacearum* infection require whole plant destruction due to the relatively low sensitivity of the protocol (Swanson et al., 2005). The method reported here is promising as a sensitive and a relatively non-destructive means of screening many more plants than is presently feasible.

A couple of advantages were also realized by using the combined q-PCR/phage amplification assay to indirectly detect *R. solanacearum*. First there was no need for an elaborate DNA extraction procedure. The supernatant contained sufficient phage DNA to support PCR amplification. Another advantage of the method is that, unlike PCR used alone, this protocol selectively detects metabolically active cells, which are the most likely to result in spread of plant disease.

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