A quantitative PCR assay for detection of *Xanthomonas fragariae*

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Introduction

Angular leaf spot is an important disease of cultivated strawberry. The European Plant Protection Organization (EPPO) lists *X. fragariae* as an A2 quarantine pathogen and in certain European markets, U.S. nurseries must certify plants pathogen free if they wish to export to their customers. PCR is the desired tool for certification because of its sensitivity, specificity, and ease of use and Pooler et al. (Appl. Env. Microbiol., 62:3121-27) developed three primer sets for specific detection of *X. fragariae*: 241 (557 bp), 245 (305 bp), 295 (634 bp). However, real-time or quantitative-PCR is rapidly becoming the preferred platform because: 1) Q-PCR can be completed very rapidly since no manipulations are required post-amplification, 2) Identification of the amplification product by probe detection adds another level of specificity and is highly accurate compared w/ size analysis on gels, and 3) Analysis of the progress of the reaction allows accurate quantification of the target sequence over a wide dynamic range.

Objectives: 1) Develop Q-PCR primers for specific detection and quantification of X. fragariae; and 2) Evaluate the sensitivity of the primers for detection and quantification of X. fragariae in strawberry crown tissue.

Materials and Methods (and some results)

Bacterial Strains and DNA Extractions: *X. fragariae* strain XF3 (D. Ritchie, NC) was used in all experimental studies. The bacteria were grown for 4-6 days at room temperature in sucrose-peptone broth on an orbital table shaker. Bacterial DNA was extracted and purified using Qiagen's DNeasy Tissue Kit (Qiagen Inc., Valencia, CA.).

Primer Development: Three sets of Q-PCR primers were developed from DNA sequences corresponding with the products amplified by the oligonucleotide primers 241, 245 and 295 designed by Pooler et al (1996). The PCR products amplified by the three primer sets were prepared as described by Pooler et al. (1996) and cloned in to plasmid DNA (strain TOP10) with the TOPO TA Cloning Kit according to manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). The plasmid DNA was extracted using the Wizard Plus Minipreps DNA Purification System (Promega Corp., Madison, WI) and the DNA was sequenced at the University of Maryland's Sequencing Facility. Specific primers and TaqMan probes were developed from these sequences. No matches were found in a BLAST search of GenBank.

SET Q241	**q241F**	5'-GCC CGA CGC GAG TTG AAT C-3'
	q241R	5'-GGT CGA AAG TTA CCG CCA-3'
	PROBE	5'FAM-AGC ATC TCC AAG CGA ATG C-3'BHQ (Black Hole Quencher)
SET Q245	**q245F**	5'-TTC TGT CGG CAG AAA TGT CCA-3'
	q245R	5'-GCG TGC CAG AAC TAG CAG-3'
	PROBE	5'FAM-TTC GTT ACG CTG AGT ACT GG-3'BHQ
SET Q295	**q295F**	5'-CGT TCC TGG CCG ATT AAT AG-3'
	q295R	5'-GTA ATT TTC AGT TTG GTG ATA TTA-3'
	PROBE	5'-FAM-AAG CGA CAA GAA TGA AAC CG-3' BHQ

Reaction Optimization: A set of reactions were run to determine optimal reaction concentrations for the forward and reverse primers, TaqMan probe, and $MgCl_2$ in a 25-µL reaction for each primer/probe set. Reactions were run in a Cepheid SmartCycler (Cepheid Inc., Sunnyvale, CA) under the following conditions: DNA was denatured at 94°C for 20 seconds followed by an amplification profile of 94°C for 1 sec (optics off) and 58°C for 40 sec (optics on) for 40 cycles. Optimum concentrations, based on the Ct value, magnitude of fluorescence, and the shape of the reaction curve were determined to be: PCR buffer (20 mM Tris-HCL [pH 8.4], 50 mM KCl), 6 mM $MgCl_2$, 1 mM dNTP, 0.36 µM each of forward and reverse primer, 0.20 µM of probe (0.17 µM for 295), and 0.04 U/µL of Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA).

Primer Sensitivity: *Extracted DNA, whole cells, and plant/bacteria mixtures.* An aliquot from a 4-day-old liquid broth culture was adjusted to an optical density (OD_{620}) of 1.0. DNA was extracted from 1 ml aliquots of the standardized culture using Qiagen's DNeasy Tissue Kit (appropriate for cultured cells) and Plant Mini Kit (used in practice for extractions from potentially infected plants) and the quantity was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologis, Wilmington, DE). Ten-fold serial dilutions were prepared from: 1) the DNA extracts and 2) Bacteria (whole cells) from the standardized culture. The whole cell dilution series was plated on sucrose peptone agar to obtain an estimate of the cell density in the standardized culture ("plate counts"). Ten microliters of each of the whole cell dilutions was added to a plant tissue extract prepared by macerating ~20 mg of vascular tissue excised from the crown of a cold-stored strawberry plant, thus each tube contained the same quantity of plant tissue but decreasing amounts of bacteria. DNA was extracted from each mixture with Qiagen's Plant Mini Kit. Standard PCR and Q-PCR were performed on all dilution series as described above.

Results

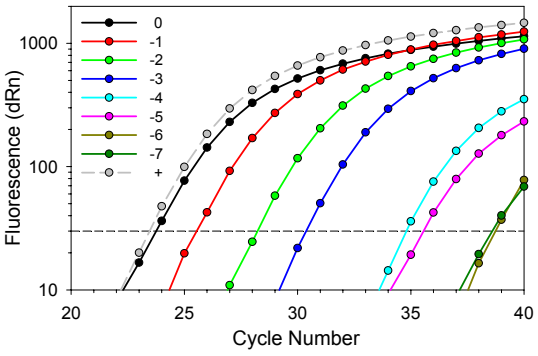
Table 1. Estimated number of bacterial cells added to PCR reaction tubes for serial dilutions of extracted DNA, whole cells, and the plant-bacteria mixture. For DNA extracted with Qiagen's Tissue Kit or Plant Kit, DNA quantities were calculated with a spectrophotometer and readings were converted to cell numbers by assuming a 5 Mb/genome for *X. fragariae*. Whole cell absorbance readings were converted to cell numbers based on the relationship between absorbance and cell density as reported by Hazel, Civerolo, and Bean (Plant Disease 64:178-181). Plate 'counts' are estimates based on the serial dilution.

	Extracted DNA & Whole Cell				*Plant-Bacteria Mixture*			
Dilution	**Tiss. Kit**	**Plant Kit**	**H,C,&B**	**Counts**	**Tiss. Kit**	**Plant Kit**	**H,C,&B**	**Counts**
-1	7.3×10^6	3.3×10^6	3.2×10^6	6.5×10^5	7300	3300	3200	650
-2	7.3×10^5	3.3×10^5	3.2×10^5	6.5×10^4	730	330	320	65
-3	7.3×10^4	3.3×10^4	3.2×10^4	6500	739	33	32	6.5
-4	7300	3300	3200	650	7.3	3.3	3.2	<1
-5	730	330	320	65	<1	<1	<1	<1
-6	73	33	32	6.5	<1	<1	<1	<1
-7	7.3	3.3	3.2	<1	<1	<1	<1	<1

Table 2. Binary results showing the sensitivity of 3 standard PCR primer sets ('S') and the 3 new real-time PCR primer sets ('Q') for detecting *X. fragariae* in dilution series of extracted DNA, whole cell bacteria and plant-bacteria extract. A '+' indicates the presence of a band at that dilution for standard PCR or that the Ct value exceeded the threshold for real-time PCR. A '*' indicates that the Ct value crossed the threshold, but one of the negative controls had a higher Ct value. Compare with Table 1 to determine sensitivity

	241 Primers						*245 Primers*						*295 Primers*					
	DNA Extract		**Whole Cell**		**Plant & Bact.**		**DNA Extract**		**Whole Cell**		**Plant & Bact.**		**DNA Extract**		**Whole Cell**		**Plant & Bact.**	
Dilution	**S**	**Q**	**S**	**Q**	**S**	**Q**	**S**	**Q**	**S**	**Q**	**S**	**Q**	**S**	**Q**	**S**	**Q**	**S**	**Q**
-1	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	−	+
-2	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	−	+
-3	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	−	+
-4	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	−	+
-5	−	+	−	+	−	*	+	+	−	+	−	+	+	+	+	+	−	*
-6	−	+	−	+	−	−	+	+	−	+	−	−	+	*	−	−	−	−
-7	−	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−

Figure 1. An example of the quantitative output using the q245 primers on whole cell bacteria. Note, the Ct values are not evenly distributed across the cycle number, presumably due to cell clumping.



Conclusions

The sensitivity of the Q-PCR protocol was greatest with extracted DNA, followed by whole cell DNA and then plant/bacteria extracts as expected. Based on the calculations in Table 1, Q-PCR was able to detect ~30 cells reliably with extracted DNA and often with whole cell DNA. Q-PCR is able to detect bacteria mixed with strawberry crown tissue extract, an application where standard PCR does not work due to PCR inhibitors in the tissue extract. Although evaluated with artificial inoculations, we have evidence that the Q-PCR procedure is capable of detecting natural infections with similar sensitivity. Although not shown here, all primers result in very efficient reactions (>90%). Moreover, we can show that the Ct values are linearly related to the quantity of DNA in the reaction. We are further testing the protocol on additional strains of the bacteria, and are testing its specificity with additional bacteria isolated from crown tissue. This and future work will define the sensitivity and specificity of the primers and we will use the information to develop a group or aggregate testing procedure for quantification of *X. fragariae* in strawberry nursery stock.