DEVELOPMENT OF A CULTURE-INDEPENDENT REAL-TIME PCR ASSAY FOR DETECTION OF *AGROBACTERIUM TUMEFACIENS* IN SOIL

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ABSTRACT

Crown gall disease caused by the bacterium *Agrobacterium tumefaciens* can cause significant economic loss in both commercial walnut orchards and in nursery operations in California. This results from the fact that, Paradox, one of the most popular walnut rootstocks in California, is extremely susceptible to *A. tumefaciens* infection and Crown Gall formation. By combining direct soil-DNA extraction with PCR amplification of target DNA followed by resolution of the PCR products on an agarose gel; as few as 200 colony forming units (CFU) of *A. tumefaciens* cells g^{-1} soil were detected using $virD_2$ primers. Real-time PCR analysis of the same soil lowered the detection limit to 20 CFU g^{-1} soil. The real time PCR cycling parameters reported here facilitate *A. tumefaciens* detection and quantification within 3 hr after soil DNA extraction. Dilution plating of spiked soil samples on the *Agrobacterium* selective tellurite-1A based medium detected as few as 10^2 CFU g^{-1} of soil, 20% of the time. The probability of detection rose to 90% when *Agrobacterium* populations rose to 10^3 CFU g^{-1} soil. The development of these detection tools will facilitate a more detailed examination of *A. tumefaciens* ecology and pathology, under field conditions.

OBJECTIVES

1. Develop a robust, culture independent PCR-based system for the detection of Ti plasmid containing, i.e. virulent, strains of *A. tumefaciens*.

PROCEDURES

Bacterial isolates and strains:
A. tumefaciens strains examined in this study were isolated from soil samples using the semi-selective medium, 1A amended with tellurite (60 µg/mL) (Table 1, Mougel et al.2001). Colonies with typical *Agrobacterium* morphology were isolated onto trypticase soy broth agar (TSBA) containing cycloheximide 200µg/mL. Selected colonies were identified by 16S rDNA analysis using rD1 and fP1 primers (Weisburg et al. 1991). Isolates were also tested by culturing on lactose agar to detect the production of 3-ketolactose (Schaad et al. 2001).

***A. tumefaciens* detection limit in soil by dilution plating:** *A. tumefaciens* detection limits in soil was determined by spiking 1mg of sterile soil with *A. tumefaciens* EC1 ranging from 10 to 106 CFU g^{-1} soil. Each of the 10 replications at each initial spiked concentration was added to 9mL of sterile water and vortexed at high speed for 1 min. The slurry was then serially diluted, plated onto the tellurite amended 1A medium developed by Mougel et al. (2001) and incubated at 26°C. Colonies were counted 48 hr post plating.

DNA extraction:
Cells from -80^{o}C glycerol stocks were streaked on trypticase soy broth agar (TSBA) and incubated at 28^{o}C for 2-3days. A single colony from each isolate or strain was inoculated into 2 mL of trypticase soy broth (TSB) and grown overnight at 28^{o}C.

DNA was extracted from each overnight culture using the MasterPure DNA purification kit (Epicentre, Madison, WI) and suspended in 50 µL of sterile Milli-Q H_2O or 0.4x TE pH 8.0.

PCR conditions:
$VirD_2$ PCR conditions: We used primers virD2A and virD2E described by Haas et al. (1995) for detection of pathogenic *A. tumefaciens* strains. PCR cycling parameters were changed substantially from reports published by Haas et al. (1995). PCR amplification reactions included 5 min initial incubation at 94°C followed by 35 cycles at 94, 66 and 72°C for 30 sec at each temperature and a final incubation at 72°C for 5 minutes. Amplified fragments were resolved in a 2% agarose gel. PCR primers were synthesized by Operon Biotechnologies, Inc (Huntsville, AL). PCR reactions were performed in 25 µl reaction mix containing 1X PCR buffer (Invitrogen, Carlsbad, CA), 1.5 mM MgCl2, 10 mM Tris-HCl (pH 8.4), 25 mM KCl, 1.5 mM $MgCl_2$, a 100 µM concentration of each deoxynucleoside triphosphate, 10 pmol of each primer.

16S rDNA PCR conditions: Purified DNA (1 µL) from each bacterial isolate was used as template in 25 µL reactions containing 529 µM forward primer fD1, 591 µM reverse primer rD1, 1.5 mM $MgCl_2$, 200 µM each dNTP, 1x PCR buffer (Eppendorf, New York, NY) 1.25 U Taq polymerase (Eppendorf). After denaturation for 2 min at 94^{o}C, the target DNA fragment was amplified in 29 cycles each consisting of denaturation for 30 sec at 94^{o}C followed by annealing for 1 min at 50^{o}C and extension for 1 min 30 sec at 72^{o}C. In the final step the reaction was incubated for 3 min at 72^{o}C. Fragments were resolved on a 2% w/v agarose gel stained with ethidium bromide.

Pathogenicity testing:
Bacteria were tested for pathogenicity on tomato (cultivar Early Girl, 20-25 cm tall seedlings). Plants were grown under green house conditions at 22°-24° C and were inoculated by making stem incisions into which a bacterial suspension was introduced and wrapped with parafilm to keep the wound moist. Plants were checked for tumor formation 4-6 weeks post inoculation.

Real-time PCR conditions:
Quantitative PCR assay was performed in 25-µL volumes using optical PCR tubes and reagents supplied by Stratagene (La Jolla, CA). The PCR mixture included Brilliant SYBR® Green QPCR Master Mix (consisting PCR buffer, SYBR Green I dye, $MgCl_2$ 2.5mM, SureStart™ *Taq* DNA polymerase and standard dNTP mix), 0.5 µL of ROX reference dye (Invitrogen, Carlsbad, CA), 4 nM each of primers $virD_2A$ and $virD_2E$. The reactions were run for 40 cycles on an Mx3000P™ Real-Time PCR System (Stratagene, La Jolla, CA). PCR cycles consisted of 5 min of initial denaturation, followed by 40 cycles of 30 seconds each at 95°C, 66°C and 2 minutes at 72°C. Each experiment was repeated 3 times. Data analysis was carried out with Stratagene software as described by Stratagene.

Soil-DNA extraction:
Agrobacterium tumefaciens strain EC1 was grown for 16 hrs, cells were pelleted and washed once with sterile 180 mM potassium phosphate (pH 7.2). Cell density was adjusted spectrophotometrically (OD_{600}) to a concentration of 10^8 cells ml^{-1}. Cell suspensions were diluted serially and added to 500 mg sterile soil in 2 ml tubes. Soil moisture was adjusted to field capacity. Soil DNA was extracted as described by Wechter et al. (2003). Experiments to determine the limits of detection were repeated at least twice.

RESULTS AND DISCUSSION

Primer selection and specificity:
VirD2A and virD2E primer pair amplified the predicted 338-bp DNA fragment from all *A. tumefaciens* isolates that were able to induce tumor formation on tomato (Table 1). However, the virD2 primer pair also generated a 338 bp fragment from *A. tumefaciens* field isolates B2, and B8 that were shown to be avirulent in pathogenicity testing on tomato plants (Table 1). The occurrence of false PCR positives using virD2 primers on individual *Agrobacterium* isolates was reported previously (Haas et al., 1995). We are currently examining what influence this level of false positives will have on population estimates derived from a PCR based approach. In addition we are now working towards development of new PCR primers that will amplify genetic loci within the T-DNA of the Ti-plasmid which we hypothesize will yield a lower incidence of false positives.

Soil DNA extraction and PCR detection sensitivity:
Using the soil DNA extraction method (Wechter et al. 2003), we isolated >100 ng DNA g^{-1} soil from sterile soil samples spiked with *A. tumefaciens* EC1 (10^8 CFU g-1). PCR amplification of soil DNA extracted from samples spiked with serial dilutions of *A. tumefaciens* EC1 using virD2A and virD2E primers gave the predicted 338-bp DNA fragment. Using virD2 primers, an amplification product was detectable in soil samples containing as few as 200 cells g^{-1}soil. Plating soil samples on 1A media containing 60µg tellurite/mL facilitated detection of bacterial cells from soil samples containing 103 CFU g-1 soil with a 90% detection probability (Fig. 1). Detection limits for *A. tumefaciens* using real-time PCR analysis were lowered to 20 CFU g-1 soil (Fig. 2, Table 2).

Real-time PCR assay:
Real-time PCR assay was carried out on genomic DNA of *A. tumefaciens* EC1 grown in TSB broth and from soil DNA extracted from sterile soil samples spiked with serial dilutions of EC1 bacterial cell suspension. Using $virD_2$ primers, positive amplification results were obtained with *A. tumefaciens* EC1 DNA and the relationship was linear over 5 log cycles from 10 ng to 1 pg of DNA. No amplifications were observed in negative controls containing no template DNA. Soil DNA extracted from soil samples spiked with serial dilutions of EC1 strain was used as template in real-time PCR reactions (Fig. 2). When the threshold line was set to a fluorescence value of 0.07 for FAM, the threshold cycles numbers ranged from 10 to 26 using $virD_2$ primers. Combining the soil DNA extraction and real- time PCR methods, *A. tumefaciens* was detectable from soil samples containing 20 CFU g^{-1} soil using $virD_2$A and $virD_2$E primers (Fig. 2). The detection limit for real time PCR assay, 20 CFU/g soil is more sensitive than enumeration on selective media and conventional PCR methods. The assay is robust and reproducible and can be conducted within 3 to 4 hours. Detection limits were determined from at least four independent experiments.

Detection of *A. tumefaciens* from soil samples:
Soil DNA was extracted from soil samples in duplicate sets and analyzed by real-time PCR. A standard curve was generated from serial dilutions of DNA extracted from soil spiked with known number of cells of *A. tumefaciens*. The assay was linear over initial DNA concentration range of 1 pg to 10 ng (R^2= 0.98). Soil samples collected from a high crown gall incidence field site had a cell density of 4.2 x 10^4 to 1.2 x10^5 CFU g^{-1} soil as determined by dilution plating. Soil from the low disease incidence site had plate counts of 3.5 x 10^1 to 1.7 x 10^2 CFU g^{-1}. Initial template concentrations ranged from 206 pg to 1.45 ng in high disease incidence soil samples and from 3.5 pg to 8.7 pg in low disease incidence soil samples as determined by real time PCR assay. There was a positive correlation between cell densities obtained using dilution plating and real time PCR estimates for the virulent (i.e. *vir*D_2 positive) subpopulation of *Agrobacterium*. Twenty five bacterial strains were selected from each soil type (high and low disease incidence) and were identified as *A. tumefaciens* by 16S rDNA sequence analysis.

In conclusion, we have developed a real-time PCR system coupled with direct DNA extraction from soil that facilitates the detection and enumeration of Agrobacterium in soil in a culture independent manner. This technique can be performed in 3-4 hours which will expedite detection of *A. tumefaciens* in soil, allow for enumeration of bacterial cells, and facilitate the study of Ti-plasmid ecology. Finally, this method provides us with a useful tool to explore and develop crown gall disease control strategies in both orchard and nursery environments.

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Table 1. Correlation of virulence with the generation of the diagnostic PCR fragment using virD2 primers specific for virulent *Agrobacterium tumefaciens*. DNA was extracted from bacterial cells grown on trypticase soy broth agar (TSBA). 1.0 µL from a 50 µL DNA extract was used as template in a 25 µL PCR using, $virD_2$ primers, (to amplify a target from the Ti plasmid) or 16S primers (designed to amplify a region of the 16S intergenic space). DNA samples which produced a ~1.5 kb band with 16S primers and a 338 bp band with $virD_2$ primers, are indicated with a (+) and samples with no visible amplification products are indicated with a (-). Bacterial cells grown on TSBA were inoculated into longitudinal slits cut into the stems of healthy tomato plants. Gall scoring was performed 6 weeks post-inoculation. Wounds with galls are indicated with (+) and wounds without galls are noted with a (-).

A. tumefaciens **Total DNA**	**$virD_2$ primers**	**16SrDNA primers**	**Gall formation Tomato**
A208	+	+	+
A356	+	+	+
ID135	+	+	+
ID1248	+	+	+
A348	+	+	+
EC-1	+	+	+
Chico isolate A2	-	+	-
Chico isolate A3	-	+	-
Chico isolate C6	+	+	+
Chico isolate D1	+	+	+
Chico isolate D2	+	+	+
Chico isolate D9	+	+	+
Chico isolate E2	+	+	+
Chico isolate E4	+	+	+
B2	+	+	-
B8	+	+	-

Table 2. Comparison of techniques for *A. tumefaciens* detection. Here we compare the sensitivities of three different *Agrobacterium* detection techniques, direct plating of serial dilutions, conventional PCR and real-time PCR. A (+) indicates that the diagnostic DNA fragment was detected and (-) indicates that the fragment or bacterial colonies were not observed.

Cells added to Sample (cells g^{-1} soil)	Plating on 1A-tellurite medium (CFU g^{-1} soil)	Conventional PCR (visualized DNA fragment in agarose gel)	Real-Time PCR
20,000	1.4×10^4	+	+
2000	1.02×10^3	+	+
200	-	+	+
20	-	-	+

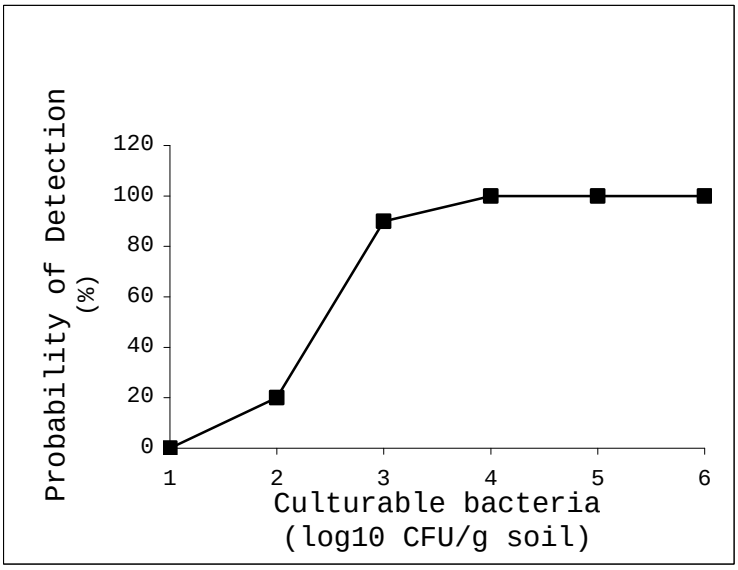

Figure 1. Probability of detecting *Agrobacterium* in soil samples using the tellurite amended 1A medium developed by Mougel et al. (2001). Soil samples spiked with known levels of *A. tumefaciens* EC1 ranging from 10 to 10^6cells were serially diluted and plated onto tellurite::1A and incubated at 26°C. The presence of *Agrobacterium* colonies were recorded 48hr post plating. Each point on the curve represents the % of the replications, at each concentration level, that exhibit Agrobacterium-like colonies. We examined 10 replications at each concentration level.

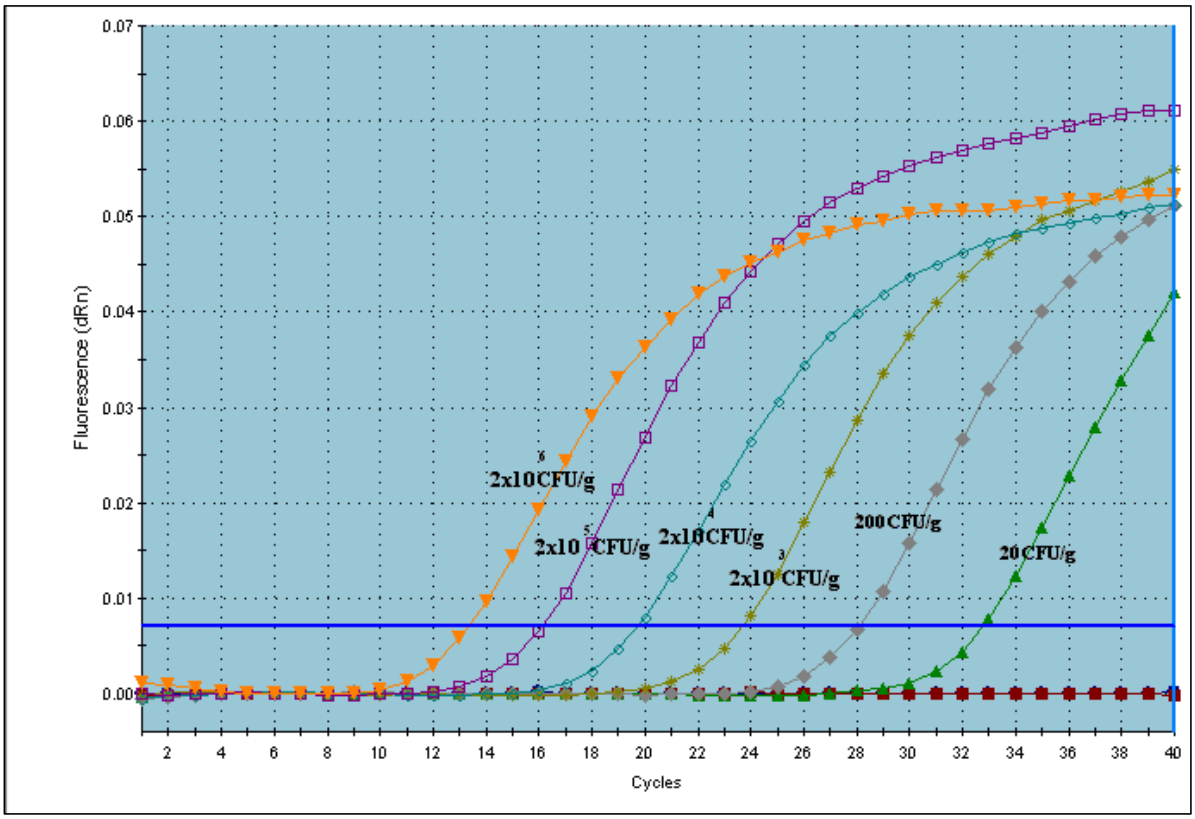

Figure 2. Limits of Agrobacterium detection using virD$_2$ primers in real-time PCR. *Agrobacterium* cell suspensions were diluted serially and added to 500 mg sterile soil in 2 ml tubes (*Agrobacterium* concentrations ranged from 10 cells/g soil up to 10^6 cells g^{-1} soil). Soil moisture was adjusted to field capacity. Soil DNA was extracted as described by Wechter et al. (2003) and used in a real-time PCR system with virD$_2$ DNA primers. Experiments to determine the limits of detection were repeated twice.