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TECHNICAL NOTE

A rapid method for the detection and quantification of the vector-borne bacterium '*Candidatus* Liberibacter solanacearum' in the tomato potato psyllid, *Bactericera cockerelli*

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Introduction

The tomato potato psyllid (TPP), *Bactericera cockerelli* (Šulc) (Hemiptera: Psyllidae), is a serious pest of solanaceous crops and is widespread in areas of the USA, Mexico, Central America, and New Zealand (NZ). The species vectors the unculturable phloem-limited bacterial pathogen '*Candidatus* Liberibacter solanacearum' (Lso) which is accepted as the causal agent of economically important diseases in several crops including potato, tomato, capsicum, and tamarillo (Liefting et al., 2008, 2009; Munyaneza et al., 2008; Abad et al., 2009; Crosslin & Bester, 2009; Lin et al., 2009). Recently, Lso has been associated with disease in carrots affected by the psyllid *Trioza apicalis* Foerster in Finland, Sweden, Norway, and in carrots affected by psyllids in the genus *Bactericera* in mainland Spain and the Canary Islands (Alfaro-Fernández et al., 2012a,b; Munyaneza et al., 2010, 2012a,b).

Effective management of vector-borne plant disease depends on rapid, specific, and sensitive detection of the pathogen in both the insect vector and the plant host. The Lso bacterium cannot be cultured and therefore PCR-based methods are required for Lso diagnostics. Here, we compare two methods, using CTAB and ZyGEM reagents, respectively, for the preparation of DNA for the detection and quantitation of Lso in the TPP host using qPCR. The ZyGEM method is a rapid and high-yielding method for the preparation of PCR-ready DNA from TPP in a single step using *prep*GEM™ reagents (ZyGEM, Hamilton, NZ) (e.g., see Ball & Armstrong, 2008). The method involves proteolytic digestion of the insect sample followed by heat-inactivation of the proteinase. No sample transfer or clean-up steps are required. This protocol can be completed in just over 1 h, compared with ca. 6 h required for extraction via CTAB, and is easily adaptable for high-throughput processing.

The effectiveness of the two methods was assessed by comparing the yield of TPP and Lso target DNA as measured by qPCR. In addition, we describe a qPCR assay targeting the TPP Internal Transcribed Spacer region 2 (ITS2) for use as an internal control in conjunction with an Lso-specific qPCR assay for the quantitation and normalization of Lso titer in infective TPP.

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Materials and methods

Tomato potato psyllid and DNA extraction

Twenty-four adult TPP were collected from an Lso-positive colony (collected in 2009 from tomato plants in Auckland, NZ (36°50′S, 174°44′E), maintained on tomato plants in a controlled room at 25 °C, 40% r.h., and L16:D8 photoperiod. Twelve TPP were processed for DNA extraction using a CTAB extraction (modified from Zeh et al., 1993); the remainder were processed using ZyGEM reagents. Tomato potato psyllids were processed as whole individuals. For CTAB extractions, TPP were ground in a 1.7-ml centrifuge tube with a micropestle, then 100 µl 2× CTAB buffer (2% CTAB, 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1.4 M NaCl) was added and the sample was incubated for 2 h at 65 °C. Chloroform:IAA (24:1; 100 µl) was added and samples agitated for 2–5 min prior to centrifugation at 6 000 ***g*** for 15 min. Supernatant (80 µl) was removed to a fresh 1.7-ml centrifuge tube containing 80 µl isopropanol, inverted to mix, and subsequently incubated at −20 °C for 2 h. Samples were centrifuged at 16 000 ***g*** for 15 min, the supernatant was discarded and the pellet washed with 70% ethanol.

The pellet was air-dried and resuspended in 50 µl EB (10 mM Tris-HCL pH 8.5). For ZyGEM extractions, TPP were ground in a 1.7-ml centrifuge tube with a micropestle, then 50 µl of a reagent mix containing 1 µl *prep*GEM enzyme, 5 µl Buffer Green, and 44 µl water was added (*prep*GEM Bacteria Kit, ZyGEM). Samples were incubated at 75 °C for 60 min before being transferred to a second heat block preheated to 95 °C and incubated for 10 min. Following incubation, the sample was allowed to cool to room temperature, then was centrifuged at 16 000 ***g*** for 2 min to pellet debris. Supernatant aliquots were used for qPCR reactions. CTAB-purified DNA was quantified using a Nanodrop 100 spectrophotometer (NanoDrop, Wilmington, DE, USA); DNA prepared by the ZyGEM protocol is not suitable for spectrophotometric quantification.

Quantitative PCR

Primer3plus (http://primer3plus.com/) was used to design the primer pair TPP ITS2F (AAAGCGACGTGTGGAAG AACC) and TPP ITS2R (GGTTGTGTGTGTCCGGGG AAG) against a representative sequence for the TPP Internal Transcribed Spacer 2 sequence (GQ249868), to provide an internal control qPCR for Lso screening of TPP. The primers were checked against an alignment of 92 TPP ITS2 sequences obtained from sequencing of NZ isolates of TPP (IAW Scott, unpubl.), as well as 29 sequences deposited in National Center for Biotechnology Information (NCBI), to ensure primers did not encompass regions containing known SNPs.

Tomato potato psyllid ITS2 SYBR Green qPCR was performed in 10 µl reactions containing 1× iTaq SYBR Green Mastermix with ROX (Biorad, Hercules, CA, USA), 1 µl template, 300 nM of primer TPP ITS2F (AAAGCGACGT GTGGAAGAACC), and 300 nM of primer TPP ITS2R (GGTTGTGTGTGTCCGGGGAAG). The amplification parameters were: 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s, with data capture during the 72 °C step, followed by a melt curve analysis (65–90 °C at 0.3 °C s^{-1}). All samples were analyzed in triplicate, and average values were calculated for each sample.

Single-step seminested Lso SYBR Green qPCR was performed in 10-µl reactions containing 1× iTaq SYBR Green Mastermix with ROX (Biorad), 1 µl template, 50 nM primer LsoF (GTCGAGCGCTTATTTTTAATAG GA), 300 nM of primer Lso16SF (ATACCGTATACGCC CTGAGAAG), and 300 nM of primer Lso16SRI (TCGTA GCCTTGGTAGGCATT). The amplification parameters were: 95 °C for 3 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s, with data capture during the 72 °C step, followed by a melt curve analysis (65–90 °C at 0.3 °C s^{-1}).

qPCR data and statistical analysis

The absolute quantification standard curve method was used to quantify target gene quantity in TPP samples. A plasmid, pZCn, containing one copy each of the target sequences for Lso 16S rRNA, '*Candidatus* Phytoplasma australiense' 16S rRNA, Potato Elongation factor 1 alpha (EF1a), and TPP ITS2 (constructed from reference sequences EU834130, FJ943262, AB061263, and GQ249868, respectively) was synthesized by GenScript (Piscataway, NJ, USA). The plasmid was linearized with the restriction enzyme *Sca*I. DNA was purified using a Promega Wizard SV PCR clean up Kit (Promega, Madison, WI, USA), then quantified using a Nanodrop spectrophotometer (NanoDrop). Plasmid serial dilutions were prepared in water to specific plasmid copy numbers according to the formula: number of copies = $(6.02 \times 10^{23}$ per mw) × mass (mw = molecular weight of linearized pZCn plasmid; mass = mass of linearized pZCn in g). All samples were analyzed in triplicate and averages calculated for cycle threshold (Ct) and quantity (copy number) values. Triplicate averages were used to calculate the average Ct and quantity values for both TPP ITS2 and Lso targets for each extraction protocol. Data for each sample were normalized to Lso ratio according to the formula: (mean of Lso quantity)/(mean of TPP ITS2 quantity).

Non-parametric and parametric statistical tests were used to detect whether the CTAB and ZyGEM DNA extraction protocols differed in their efficiency. The non-parametric, distribution-free, Kolmogorov-Smirnov (K-S) test (implemented at http://www.physics.csbsju.edu/stats/KS-test.html) was used initially to establish whether Ct and quantity data were normally distributed allowing the use of parametric statistical tests. If normally distributed, two-sample F-tests of variance were performed to establish whether the ZyGEM and CTAB data were distributed equally as a preliminary step to testing for mean effects using two-sample t- and z-tests. These tests were implemented within Microsoft Excel (Office 2007). If not normally distributed, K-S tests were used to determine whether the two datasets differed significantly.

Results

Tomato potato psyllid ITS2 qPCR

SYBR Green qPCR with LsoF-Lso16SF-Lso16SRI was performed using a serial dilution of pZCn DNA to establish the efficiency of the quantitative PCR and the theoretical limits of detection. A standard curve was obtained using the dilution series when log concentration was plotted against Ct within the range tested (5–5 000 000 copies of target DNA), with an amplification efficiency of 99.0%, $R^2 = 0.998$, and Y-intercept of 32.04 (Figure 1). The assay

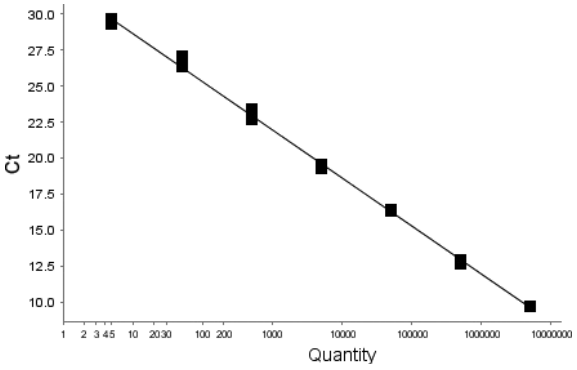

Figure 1 Standard curve for absolute quantification (qPCR) of tomato potato psyllid ITS2 target gene copy number. Templates were a 10-fold serial dilution of plasmid pZCn (5-5 000 000 copies of target DNA).

produced amplification against samples calculated to contain five copies of target DNA in all replicates tested, indicating that the limit of detection is below five copies of target DNA per reaction. A single amplification product with a melting point of ca. 83.4 °C was generated for all 24 samples from the two extraction methods, as well as from multiple CTAB-extracted samples from TPP collected from several different regions of NZ (individual data not shown because qPCR melt curves produced by Stepone-plus SYBR Green qPCR do not have sufficient resolution to distinguish between closely related target sequences, e.g., SNPs among individuals in a population).

Comparison of ZyGEM and CTAB protocols

Amplification was observed in all samples from each extraction procedure for both TPP ITS2 and Lso qPCR. For the TPP ITS2 assay, the average Ct and quantity were 12.48 and 1.1×10^6, respectively, for the 12 ZyGEM samples, and 11.68 and 1.7×10^6, respectively, for the 12 CTAB samples (Figure 2). For the Lso assay, the average Ct and quantity were 17.44 and 9.5×10^4, respectively, for the 12 ZyGEM samples, and 16.31 and 1.6×10^5, respectively, for the 12 CTAB samples. The average Lso ratio for ZyGEM and CTAB samples was 0.115 and 0.164, respectively.

Parametric and non-parametric statistical tests were carried out to determine whether the ZyGEM and CTAB methods differed in their efficiency. Triplicate average Ct and quantity scores for each sample for both TPP ITS2 and Lso were compared for ZyGEM and CTAB samples. K-S tests on Ct data indicated that these were normally distributed (data not shown); however, K-S tests on quantity and Lso ratio data indicated that these data were not normally distributed and did not meet the assumptions of parametric statistical tests. Two-sample F-tests indicated that the variance of ZyGEM and CTAB Ct data was equivalent for both TPP ITS2 and Lso assays (Table 1). Two-sample t- and z-tests of the equivalence of Ct means indicated that there was no significant difference between the values obtained from CTAB or ZyGEM-extracted samples for both TPP ITS2 and Lso assays (Table 1). K-S tests indicated that the distributions of data obtained from ZyGEM and CTAB methods were equivalent for quantity data from both assays, and for Lso ratio data (Table 1). Taken together, these data indicate that there was no significant difference in DNA yield between ZyGEM and CTAB methods.

Discussion

The results of this study demonstrate that a TPP DNA preparation protocol using *prep*GEM reagents provided template DNA for PCR with a DNA recovery that was statistically equal to that obtained by a CTAB extraction protocol. The ZyGEM protocol involves enzymatic proteolysis of a crushed insect sample and does not include

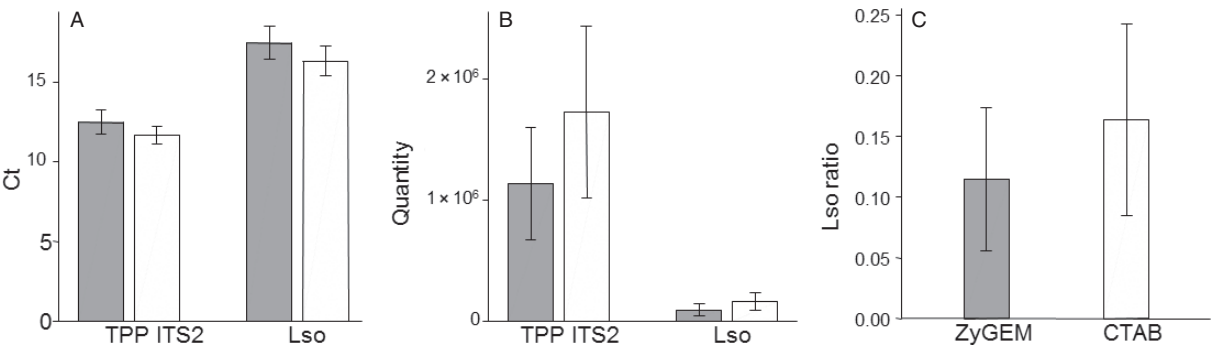

Figure 2 Summary of qPCR results. Mean (± 95% confidence interval) (A) Ct values for tomato potato psyllid (TPP) ITS2 and *Candidatus* Liberibacter solanacearum (Lso) assays, (B) quantity values for TPP ITS2 and Lso assays, and (C) Lso ratio for the 12 samples for each extraction method. Gray bars: ZyGEM method, white bars: CTAB method.

Table 1 Results of statistical tests comparing qPCR results obtained by ZyGEM and CTAB extraction methods for tomato potato psyllid (TPP) and *Candidatus* Liberibacter solanacearum (Lso)

	TPP ITS2	Lso	Ratio
Two-sample F-statistic[1]	1.855 (P = 0.16)	1.276 (P = 0.35)	na
Two-sample t-statistic[1]	−1.528 (P = 0.14)	−1.428 (P = 0.17)	na
Two-sample z-statistic[1]	−1.528 (P = 0.13)	−1.428 (P = 0.15)	na
K-S D-statistic[2]	0.299 (P = 0.11)	0.250 (P = 0.39)	0.250 (P = 0.79)

na, not applicable.
[1]Two-tailed tests performed on mean Ct data ($\alpha = 0.05$).
[2]Tests performed on mean quantity data (TPP ITS2 and Lso) or mean Lso ratio.

any purification steps. The *prep*GEM Bacteria Kit includes a lysozyme reagent; however, this was found not to affect the recovery of TPP ITS2 or Lso DNA. The resulting sample contains a pellet of solid debris with a supernatant that is suitable for PCR analysis. We observed that up to 5 μl of supernatant can be used in a single PCR reaction with no signs of inhibition. Samples are not intended for long-term storage, but may be suitable for rapid or high-volume diagnostic applications.

A SYBR Green qPCR assay targeting the TPP ITS2 gene was developed as an internal control gene for the normalization of Lso titer between TPP samples. To account for variation between samples in the amount of tissue sampled and DNA recovery, Lso titer was expressed as Lso ratio. The assay produced a linear standard curve over the range tested, and a single amplification product in all TPP samples tested, including TPP isolates collected from multiple regions of NZ.

The ZyGEM protocol offers several advantages over traditional CTAB DNA extraction for PCR screening applications. Sample preparation can be completed in approximately one-sixth of the time required for CTAB extraction. For both protocols, reagent cost is comparable and insignificant relative to the cost of labor. Sample preparation using ZYGEM involves no reagent addition or sample transfer after the initial step. The reduced sample handling minimizes the potential for contamination and cross-contamination of samples, which can lead to generation of false-positive results in diagnostic PCR (Ratcliff et al., 2007). Sample preparation can be completed in standard 0.2 ml PCR tubes or plates using a PCR thermocycler and is therefore adaptable to high-throughput processing.

Tomato potato psyllid samples prepared via the ZyGEM protocol are not suitable for DNA quantification by spectrophotometry, therefore DNA yield was measured by a qPCR assay targeting the TPP ITS2 sequence. Although an apparent 34% reduction in the average TPP ITS2 quantity was observed for ZyGEM samples compared with CTAB, no statistically significant differences in the TPP ITS2 Ct and quantity data were observed between the two protocols suggesting that the two protocols provide a very similar recovery of DNA preparation for PCR.

To assess the ability of both protocols to extract bacterial DNA from TPP samples, Lso titer in TPP was quantified using a qPCR assay targeting the Lso 16S rRNA. Lso titer in TPP varied among individuals. As with TPP ITS2, the apparent 42% reduction in the average Lso quantity observed for ZyGEM samples compared with CTAB was not statistically significant. Comparison of average Lso Ct and Lso ratio data demonstrated no statistically significant differences between the two methods, indicating that both methods are able to extract bacterial DNA with equal recovery.

Taken together, these results demonstrate that the ZyGEM method provides a rapid and simple protocol for the preparation of PCR-ready TPP samples for Lso diagnostics. We envisage that this method could be extended to other insect species for the rapid detection and quantitation of vector-borne microbes.

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