Development of Ethidium Monoazide (EMA) Real-Time Polymerase Chain Reaction (EMA-qPCR) Technique for Detection and Assessment of Live *'Candidatus* Liberibacter asiaticus' Cells in *Citrus* spp. Tissues

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Manuscript Received: September 2016 Accepted: 11 May 2017

Abstract

'*Candidatus* Liberibacter asiaticus' (Las) causal agent of Citrus huanglongbing (HLB) is a phloem-limited, gram-negative bacterium associated with three species of α-Proteobacterium includes; '*Candidatus* Liberibacter asiaticus' (Las), '*Candidatus* Liberibacter americanus' (Lam) and '*Candidatus* Liberibacter africanus' (Laf). HLB is one of the most serious disease of citrus worldwide. Infected trees express various symptoms of blotchy mottle and yellowing, shoot stunting and slowly decline. The citrus HLB pathogen has been unculturable in artificial media. Detection of HLB bacterium is based mainly by conventional polymerase chain reaction (PCR) and quantitative real-time PCR (qPCR) with species-specific primers. However, the disadvantage of qPCR on undistinguishable between live and dead cells is a limiting factor to detect active HLB population *in planta*. In this study, the EMA-qPCR technique was developed to quantify live Las cells with the aid of Ethidium Monoazide (EMA) to overcome the limitation of conventional and quantitative PCR techniques. Comparison on efficacy of EMA and Propidium Monoazide (PMA) for suitable time of citrus tissues treatment indicated a longer incubation for 20 minutes by soaking the tissue either in EMA or PMA solution was better than a shorter incubation for 5 minutes. However, EMA was significantly higher detection of Las live cells than PMA due to more penetration into the tissue. Therefore, a suitable method of citrus tissue preparation by soaking, pulverizing and pulverizing with grinding in liquid nitrogen in 100 µg/mL EMA solution for 20 minutes was determined for live Las cells detection by EMA-qPCR technique. Soaking method was the most suitable among the others which was more significantly efficient, simple and convenience. A minimum live Las cells for HLB symptom expression detection by this technique in asymptomatic and symptomatic leaves found that all asymptomatic leave of *Citrus* spp. has low live cells than symptomatic leaves ranging from 3.38-22.09% and 24.70-37.71%, respectively which was significantly different at $P < 0.05$. The efficacy of EMA-qPCR technique to quantify live Las cells in mature and immature seeds was 65.56% and 12.73%, respectively which was significantly different ($P < 0.05$). This work evidently shown the developed EMA-qPCR technique was sensitive and useful for early detection of live Las cells in immature seed as the first significant step for producing HLB-free citrus scion and root stock. This technique was also reliable supported early detection of live Las cells for monitoring HLB epidemic in citrus grove before an effective application of HLB management measures.

Additionally, this technique can be useful as a basis for development on detection of other uncultured live bacteria and phytoplasma pathogens.

Keywords: Citrus huanglongbing, 'Candidatus Liberibacter asiaticus', Ethidium Monoazide, live cell, EMA-qPCR, Citrus spp.

Introduction

Citrus Huanglongbing (HLB) also called citrus greening is one of the most devastating diseases of citrus, causing severe losses and significantly affecting the world citrus industry (Halbert and Manjunath, 2004). The causal agent of HLB disease is identified as a phloem-restricted bacterium belonging to a new genus in the alpha-subdivision of the α-Proteobacteria. Koch's postulates have never been completed for this pathogen (Jagoueix et al., 1994). Three species of HLB have been identified which differ in their vector specificity and environmental conditions; '*Candidatus* Liberibacter asiaticus'(Las) is a heat-tolerant species and is transmitted by both *Diaphorina citri* Kuwayama and *Trioza erytreae* Del Guercio; '*Candidatus* Liberibacter americanus'(Lam) is a heat-tolerant species vectored by *D. citri* Kuwayama; and '*Candidatus* Liberibacter africanus'(Laf) is a heat-sensitive species vectored by both *T. erytreae* Del Guercio and *D. citri* Kuwayama (Bové, 2006). Infected citrus trees show various symptoms, generally on their leaves, shoot and fruits. The leaves symptoms include blotchy mottle and yellowing. Infected shoots are stunted and slowly died as the symptoms appear in other zone of the tree. Fruit from affected branches can be lopsided, color deviation, seed abort and discolor (Gottwald et al., 2007).

The citrus HLB pathogen has been unculturable on artificial media. Several methods using DNA probes, enzyme-linked immunosorbent assay, electron microscopy and biological assay have been used to detect the HLB bacterium (Bové et al., 1974; Catling et al., 1978; Garnier et al., 1991; Villechanoux et al., 1992). Recently, conventional polymerase chain reaction (PCR) and quantitative real-time PCR (qPCR) with species-specific primers were developed based on 16S rDNA and β-operon based mainly to detect of '*Candidatus* Liberibacter spp.' (Jagoueix et al., 1996; Li et al., 2006; Li et al., 2007). Detection of the HLB bacterium based on PCR methodology has achieved popularity when compared with other methods from the past due to its simplicity, sensitivity and reliability. However, PCR methodology possibly reveals false negatives and false positives due to low concentration and uneven distribution in both host plants and vector psyllids. Subsequently, Li et al. (2006) has developed quantitative TaqMan PCR using 16S rDNA-based TaqMan primer–probe sets specific to detection *Candidatus* Liberibacter species which has been successful to detection, identification and quantification to confirm of HLB. However, the weakness of qPCR does not distinguish between live and dead cells, unable estimate live cells in hosts. Continuously detection error results were revealed from false negatives and false positives due to bacterial genomic DNA can remain stable up to 3 weeks after cell death (Josephson et al., 1993). Therefore, qPCR assays are likely to considerably overestimate the population of HLB pathogen in the hosts. Considering the situation of huanglongbing disease on citrus industry, there is a critical need to accurately quantify live cells of Las bacteria, which should improve disease management, disease-free citrus production and understanding infection of the infection mechanism of Las bacteria.

Ethidium Monoazide with quantitative real-time PCR (EMA-qPCR) has been reported to effectively distinguish between live and dead cells (Nocker et al., 2006; Rudi et al., 2005, Wang and Levin, 2006). Discrimination of live and dead cells is obtained by covalent binding of EMA to DNA of dead cells by photo-activation (Wagner et al., 2008). EMA penetrates only the dead cells with compromised membrane/cell systems. Subsequent photoinduced cross-linking inhibits the PCR amplification of DNA from dead cells (Soejima et al., 2007). The DNA from live cells is unstained while the DNA from the dead cells is covalently

bound to EMA. The unstained DNA from live cells is PCR amplified while the DNA from dead cells with bound EMA unable to amplify. Essentially, result from EMA-qPCR can use to estimate the proportion of live and dead cells. In the present study, we developed an efficient tool to solve the weakness of qPCR by using EMA-qPCR technique to qualify live cells from citrus tissues with an accurate assessment of the live HLB pathogen. This appropriate application of EMA-qPCR technique in HLB research to investigate disease epidemiology, production of disease-free citrus, biology and infection mechanism of Las is a crucial component for disease assessment and management of HLB in citrus industry worldwide.

Materials and Methods

Citrus Materials and Preparation

For vascular bundle analysis, citrus fruits were collected from citrus species smooth flat seville (*Citrus paradisi*, L.) and liane pummelo (*Citrus maxima,* (Burm.) Merr.) during the period of September 2013 to May 2014. The trees were planted in 1999-2000 in Picos farm at the USDA-ARS Horticultural Research Laboratory, Fort Pierce, FL, USA. Thus citrus trees became infected by insect transmission sometime after planting. Infected citrus trees showed typical foliar symptoms of HLB and prior investigation they demonstrated the presence of the causal agent, '*Candidatus* Liberibacter asiaticus' (Las) (data not presented). A subsample of seed was removed and tested for the presence of '*Candidatus* Liberibacter asiaticus' using real-time PCR (qPCR). Vascular bundles were excised from seed coats under a stereomicroscope approximately the amount of 5 vascular bundles per sample (0.01 g) (Figure 1a, b, c and d).

For petiole analysis, citrus leave samples were collected from three citrus cultivars consisted of red shaddock pummelo (*Citrus maxima,* (Burm.) Merr.), sour orange (*Citrus aurantium*, L.) and madam vinous sweet orange (*Citrus sinensis* (L.) Osbeck) which were previously PCR-confirmed and maintained in greenhouses and Picos farm of the USDA-ARS Horticultural Research Laboratory, Fort Pierce, FL, USA. The citrus trees were inoculated by grafting with infected budwood at least two years prior to start this experiment. Symptomatic leaf samples (fully expanded and hardened) were collected from Las-infected trees and asymptomatic leaf samples at a similar development stage were collected from healthy looking tree. Petioles were cut from the leaf with a sharp razor blade and pith was peeled from the bark (approximately 0.1 g per sample) (Figure 1e).

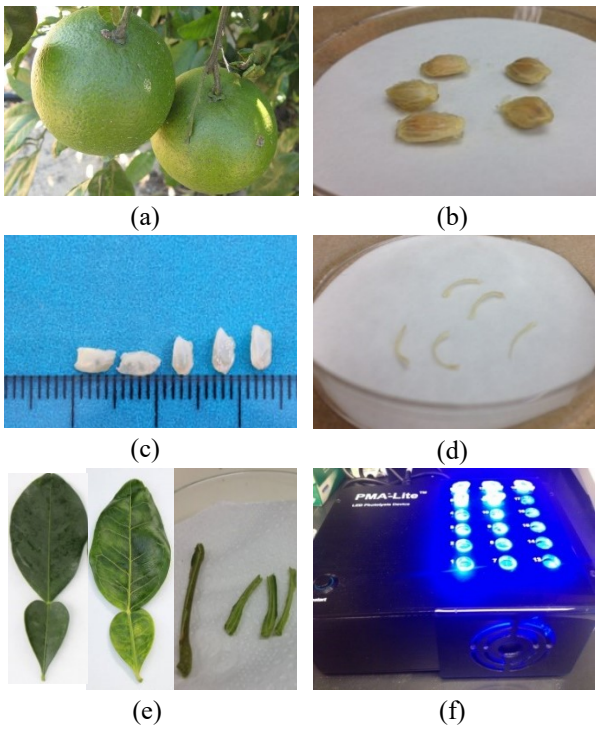

Figure 1 Citrus plant tissues from various sources and LED-Box used to quantify live Las cells by EMA-qPCR technique. **(**a) Smooth flat seville (*Citrus aurantium* L.) fruits from Picos Farm at the USDA-ARS Horticultural Research Laboratory, Fort Pierce, FL, USA., (b) Mature seed of smooth flat seville (*Citrus aurantium* L.), (c) Immature seed of liane pummelo (*Citrus maxima* (Burm.) Merr.), (d) Vascular bundle separated from seed, (e) Petioles and pith peeled from citrus and (f) plant sample tubes with or without EMA under photoinduced cross-linking by Biotium LED-Box (PMA-Lite™ LED, Biotium, Inc., Hayward, CA).

Chemical Treatment

Ethidium Monoazide (EMA; Molecular Probes, Invitrogen, Carlsbad, CA) and Propidium Monoazide (PMA; Biotium, Inc., Hayward, CA) were separately dissolved in sterile distilled water in the dark to a final stock solution of 50 ng/mL and 25 ng/mL, respectively then stored at -20˚C until used. For analysis of cross-linking ability approximate a portion of 0.01 g of vascular bundle or 0.1 g of petiole was placed in a 1.5 mL microtube and either EMA or PMA solution was added to a final concentration of 100 µg/mL. The sample tubes were incubated in the dark for 5 and 20 minutes with occasional inversion. Photoinduced cross-linking of the EMA and PMA with DNA in plant tissues was treated by a Biotium LED-Box (PMA-Lite™ LED, Biotium, Inc., Hayward, CA) for 20 minutes (Figure 1f). After light exposure, the sample tubes were centrifuged at 13,200 rpm for 5 minutes, the supernatant was removed. The plant tissue pellet was washed twice with 0.5 mL of isolation buffer (1M Sodium phosphate buffer pH7.2, 1 M Dithiothreitol, 5% of Polyvinyl-pyrrolidone, 20% Sucrose and 0.5 M EDTA) to remove residual of EMA or PMA. The plant tissue pellet was kept for further DNA extraction.

Citrus Tissue Preparation for EMA-qPCR Technique

Three different methods of citrus tissues preparation for optimum detection of live Las cells by EMA-qPCR were compared between soaking, pulverizing and pulverizing with grinding in liquid nitrogen (N_2). Citrus pith peeled from petiole was exposed to EMA by three different methods consisting of (i) soaking; the sample was soaked in 500 µl of isolation buffer, (ii) pulverizing; the sample was soaked in 500 µl of isolation buffer and pulverized for 3 minutes with a Mini-Beadbeater (Biospec Products Inc., Bartlesville, OK), and (iii) pulverizing with grinding in liquid nitrogen (N_2); sample was soaked in 500 µl of isolation buffer and pulverized for 3 minutes with a Mini-Beadbeater (Biospec Products Inc., Bartlesville, OK) then frozen in liquid nitrogen (N_2), and before quickly grinding to fine powder. A 5 µl of EMA solution (100 ng/mL) was separately added into each of 3 sample tubes then incubated in the dark for 20 minutes with occasional inversion. Photoinduced cross-linking of the EMA with DNA by using a Biotium LED-Box (PMA-Lite™ LED, Biotium, Inc., Hayward, CA) for 20 minutes (Figure 1f).

Extraction of EMA-treated DNA and Quantitative Real-Time qPCR assays (EMA-qPCR)

The pellet of plant tissue from EMA or PMA treated was placed into a 2.0 mL screw-cap tube (USA Scientific, Ocala, FL) in the presence of the 400 µl buffer AP1 and 4 µl RNase A stock solution (100 mg/mL) and vortex vigorously. Sample tubes were pulverized for 3 minutes with a Mini-Beadbeater (Biospec Products Inc., Bartlesville, OK). Extraction of nucleic acids from pulverized tissues was done with a DNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol.

Verification the present of '*Candidatus* Liberibacter asiaticus' (Las) sequences in DNA extracts was performed by qPCR with primer and probe oligonucleotides specific at a portion of the '*Candidatus* Liberibacter asiaticus' 16S rRNA gene (Li et al., 2006) by HLBasf; 5'-TCGAGCGCGTATGCGAATACG-3', HLBasp; 5'-GCGTTATCCCGTAGAAAAAGGTAG-3' ,and HLBasr; 5'-AGACGGGTGAGTAACGCG-3' using an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). Duplicate of 20 µL reactions containing 25 ng of DNA, 0.25 µM each of the forward and reverse primers and 0.15 µM probe and 2xTaqMan Fast Universal PCR Master Mix (Applied Biosystems, Carlsbad, CA) were subjected to one cycle of denaturation at 95°C for 20 seconds followed by 40 cycles of denaturation for 3 seconds at 95°C and annealing/extension at 60°C for 30 seconds.

The presence of target template in a sample was confirmed if the cycle threshold (Ct) value did not exceed 36.0 (Hilf, 2011; Turechek et al., 2009). In all qPCR analyses, duplicated samples of nucleic acid extracted from foliar tissues of '*Candidatus* Liberibacter asiaticus' (Las) infected and non-infected trees maintained in insect-proof greenhouses were included as positive and negative

controls. The Ct values were converted to the estimated bacterial titers using the grand universal regression equation $Y = 13.82\text{-}0.2866X$, where X is the mean Ct value and Y is the log concentration of the target DNA copies (Li et al., 2006). Calculating of live cells was estimated from proportion number of Las population from sample of EMA treated (EMA+) with EMA non-treated sample (EMA-).

Statistical Analysis

The data were analyzed using Analysis of Variance (ANOVA), signification difference between treatments compared by Duncan's multiple range test (DMRT) method with statistical analysis system (SAS Version 9, SAS Institute Inc., USA) to separate the treatment means at the $P \leq 0.05$.

Results and Discussion

Results

Efficacy of Ethidium Monoazide (EMA) and Propidium Monoazide (PMA) for Detection of Live Cells of '*Candidatus* Liberibacter asiaticus' (Las) by Quantitative Real-Time PCR (qPCR)

Duration of plant tissue exposure duration of plant tissue to the cross-linking chemical is important for the effectiveness of live Las cell detection. Ethidium Monoazide (EMA) and Propidium Monoazide (PMA) were compared to select the suitable time of plant tissue incubation period for 5 and 20 minutes which were conducted by using vascular bundle of smooth flat seville cultivar. In treatment of control, EMA and PMA for 5 minutes incubation period, the Ct value and Las population (cell/g of plant tissue) of control (none-chemical treated), EMA and PMA treatments were 21.04 ± 0.50 and 6.10×10^6, 25.14 ± .21 and 0.41×10^6 and 25.17 ± 0.18 and 0.40×10^6, respectively. Control treatment gave significantly higher Las population than the others (Table 1). In treatment of control, EMA and PMA at 20 minutes incubation period, the Ct value and Las population (cell/g of plant tissue) of control (none-chemical treated), EMA and PMA treatments were 21.15 ± 1.03 and 5.70×10^6, 22.47 ± 2.11 and 2.30×10^6 and 24.12 ± 2.34 and 0.80×10^6, respectively. Control treatment gave significantly higher Las population than the others (Table 1). Further calculation of % live Las cells at 5 minutes incubation period, control treatment was unable to distinguish that number whereas EMA and PMA treatment were able to distinguish them by showing % live Las cells of 6.72% and 6.56%, respectively which was not significantly different by DMRT ($P < 0.05$) (Table 1). Considering the calculation of % live Las cells at 20 minutes incubation period, control treatment was unable to distinguish that number whereas EMA and PMA treatments was able to distinguish them by showing % live Las cells of 40.35% and 13.14%, respectively which was significantly different by DMRT at $P < 0.05$. (Table 1) Interestingly, the live Las cells from EMA treated for 20 minutes was a suitable incubation period. Therefore, this incubation period was selected and applied for further experiment.

Comparison of Plant Tissue Preparation with Ethidium Monoazide (EMA) to evaluation Live Cells of '*Candidatus* Liberibacter asiaticus' (Las) Using Quantitative Real-Time PCR (qPCR)

Three methods of citrus tissue preparation consisted of soaking, pulverizing and pulverizing with grinding in liquid nitrogen (N_2) were compared to find out a suitable and reliable practice to provide high efficacy to detect efficient detection of live Las in different citrus tissues by EMA-qPCR technique. EMA was selected to use in the experiment since EMA was better effective detection live Las than PMA in which non-treated EMA was used as control to calculate % Live Las population. In non-treated EMA (EMA-), the Ct value and Las population of soaking, pulverizing and pulverizing with grinding in liquid N_2 treatments were 24.22 ± 4.88 and 7.56×10^6, 28.01 ± 4.38 and 0.61×10^6 and 29.63 ± 3.37 and 0.21×10^6, respectively.

Soaking method gave significantly higher Las population than the others (Table 2). In the treated-EMA (EMA+), Ct value and Las population % live Las cells of soaking, pulverizing and pulverizing with grinding in liquid N_2 treatments were 26.61 ± 3.78 and 1.56×10^6, 32.12 ± 5.70 and 0.40×10^6 and 33.47 ± 4.46 and 0.01×10^6, respectively. Further calculation of % live Las cells from soaking, pulverizing and pulverizing with

grinding in liquid N_2 found that soaking method was significantly higher of % live cells (20.63%) than pulverizing (6.56%) and pulverizing with grinding in liquid N_2 (4.76%) (Table 2). Therefore, soaking citrus tissue in EMA solution for 20 minutes was selected as a standard EMA-qPCR technique to quantify live Las population in citrus tissues.

Table 1 Efficiency of Ethidium Monoazide (EMA) and Propidium Monoazide (PMA) treated citrus tissues for 5 and 20 minutes on quantification of live cells of '*Candidatus* Liberibacter asiaticus' (Las) by quantitative real-time PCR (qPCR)

Treatment	C_t value (± SD)[1/]	Las population[2/] (cell/g of plant tissue)	%Live cells[3/]
5 minutes			
Control[4/]	21.04 ± 0.50[a5/]	6.10×10^6	UC[6/]
EMA	25.14 ± 0.21[b]	0.41×10^6	6.72[c]
PMA	25.17 ± 0.18[b]	0.40×10^6	6.56[c]
20 minutes			
Control	21.15 ± 1.03[a]	5.70×10^6	UC
EMA	22.47 ± 2.11[ab]	2.30×10^6	40.35[a]
PMA	24.12 ± 2.34[bc]	0.80×10^6	13.14[b]

[1/] Ct values were obtained from the qPCR detection of Las with 16S rRNA gene using HLBaspr primer. The Ct values from qPCR data are the mean value from nine replications followed by the standard deviation (SD) of the mean.

[2/] Las population was calculated from $Y = 13.82\text{-}0.2866X$, where X is the mean Ct value and Y is the log concentration of the target DNA copies by expressed as cell/g of plant tissue.

[3/] %Live cells was calculated from proportion number of Las population from the treated EMA or PMA with non-treated control samples.

[4/] Treatment control was represented non-treated EMA or non-treated PMA in the samples.

[5/] Different letters in the same column indicate statistically significant differences by DMRT at $P < 0.05$.

Table 2 Comparison of three citrus tissue preparations treated with Ethidium Monoazide (EMA) to evaluate efficient detection of live cells of '*Candidatus* Liberibacter asiaticus' (Las) by EMA-qPCR

Treatment	C_t value (± SD)[1/]	Las population[2/]	%Live cells[3/]
EMA(-)[4/]			
Soaking	24.22 ± 4.88[a5/]	7.56×10^6	UC[6/]
Pulverizing	28.01 ± 4.38[bc]	0.61×10^6	UC
Pulverizing w/t Grinding	29.63 ± 3.75[c]	0.21×10^6	UC
EMA(+)			
Soaking	26.61 ± 3.78[b]	1.56×10^6	20.63[a]
Pulverizing	32.12 ± 5.70[d]	0.04×10^6	6.56[b]
Pulverizing w/t Grinding	29.63 ± 3.75[c]	0.21×10^6	4.76[c]

[1/] Ct values were obtained from the qPCR detection of Las with 16S rRNA gene using HLBaspr primer. The Ct values from qPCR data are the mean value from nine replications followed by the standard deviation (SD) of the mean.

[2/] Las population was calculated from $Y = 13.82\text{-}0.2866X$, where X is the mean Ct value and Y is the log concentration of the target DNA copies by expressed as cell/g of plant tissue.

[3/] %Live cells was calculated from proportion number of Las population from the sample was treated EMA (EMA+) with non-treated EMA (EMA-) samples.

[4/] Treatment control was represented non-treated EMA in the samples.

[5/] Different letters in the same column indicate statistically significant differences by DMRT at $P < 0.05$.

[6/] UC was represented unable to calculated live Las cells in non-chemical cross-linking treatment.

Quantification Live Cells of '*Candidatus* Liberibacter asiaticus' (Las) by EMA qPCR technique on Symptomatic and Asymptomatic Leaves

This experiment was carried out to compare an efficiency of EMA-qPCR technique for quantitative detection of live Las cells from asymptomatic and symptomatic citrus leaves of three citrus cultivars consisted of pummelo (*Citrus maxima* (Burm.) Merr.), sour orange (*Citrus aurantium* L.) and sweet orange (*Citrus sinensis* Osbeck) which were maintained in Picos farm at the USDA-ARS Horticultural Research Laboratory, Fort Pierce, FL, USA. The EMA-qPCR technique was performed by using petioles of asymptomatic and symptomatic leaves (Figure 1e). In non-treated EMA (EMA-) of asymptomatic leave of and symptomatic leave of pummelo, sour orange and sweet orange, the Ct value and Las population (cell/g of plant tissue) in asymptomatic samples were 29.34 ± 1.42 and 2.58 × 10^5 (pummelo), 29.24 ± 2.43 and 2.75 × 10^5 (sour orange) and 26.47 ± 1.17 and 17.12 × 10^5(sweet orange) whereas in symptomatic samples were 28.60 ± 0.63 and 4.19 × 10^5 (pummelo), 23.24±1.84 and 114.24 × 10^5 (sour orange) and 30.00±1.92 and 1.66 × 10^5 (sweet orange), respectively (Table 3). In the treated-EMA (EMA+) of asymptomatic leave of and symptomatic leave of pummelo, sour orange and sweet orange, the Ct value and Las population (cell/g of plant tissue) in asymptomatic samples were 31.63±0.63 and 0.58 × 10^5 (pummelo), 31.58 ± 1.79 and 0.58 × 10^5 (sour orange), 31.75±1.01 and 0.58 × 10^5 (sweet orange) whereas in symptomatic samples were 30.08 ± 0.44 and 1.58 × 10^5 (pummelo), 25.70 ± 3.81 and 28.46 × 10^5 (sour orange) and 32.12 ± 2.12 and 0.41 × 10^5 (sweet orange), respectively (Table 3). Further calculation of % live Las cells from asymptomatic leave of pummelo, sour orange and sweet orange were 22.09%, 21.09% and 3.38%, respectively whereas % live Las cells from symptomatic leave of pummelo, sour orange and sweet orange were 37.71%, 24.92 and 24.70%, respectively (Table 3). Apparently, the mean of %live Las cells from symptomatic leaves of all citrus cultivars was higher significantly higher different ($P < 0.05$) than asymptomatic leaves.

Table 3 Efficacy of EMA-qPCR technique on quantification of live cells of '*Candidatus* Liberibacter asiaticus' (Las) from asymptomatic and symptomatic leaves of pummelo, sour orange and sweet orange grown in Picos Farm at USDA-ARS Horticultural Research Laboratory, Fort Pierce, FL, USA

Source	C_t value (± SD)[1/]		Las population[2/] (cell/g of plant tissue)		%Live cells[3/]
	EMA (-)	EMA (+)	EMA (-)	EMA (+)	
Asymptomatic leave					
Pummelo	29.34 ± 1.42^{c}	31.63 ± 0.63^{b}	2.58 × 10^5	0.58 × 10^5	22.09^{bc}[4/]
Sour orange	29.24 ± 2.43^{c}	31.58 ± 1.79^{b}	2.75 × 10^5	0.58 × 10^5	21.09^{c}
Sweet orange	26.47 ± 1.17^{b}	31.75 ± 1.01^{b}	17.12 × 10^5	0.58 × 10^5	3.38^{d}
Symptomatic leave					
Pummelo	28.60 ± 0.63^{bc}	30.08 ± 0.44^{b}	4.19 × 10^5	1.58 × 10^5	37.71^{a}
Sour orange	23.24 ± 1.84^{a}	25.70 ± 3.81^{a}	114.24 × 10^5	28.46 × 10^5	24.92^{b}
Sweet orange	30.00 ± 1.92^{c}	32.12 ± 2.12^{b}	1.66 × 10^5	0.41 × 10^5	24.70^{b}

[1/] Ct values were obtained from the qPCR detection of Las with 16S rRNA gene using HLBaspr primer. The Ct values from qPCR data are the mean value from nine replications followed by the standard deviation (SD) of the mean.

[2/] Las population was calculated from $Y = 13.82\text{-}0.2866X$, where X is the mean Ct value and Y is the log concentration of the target DNA copies by expressed as cell/g of plant tissue.

[3/] %Live cells was calculated from proportion number of Las population from the sample was treated EMA (EMA+) with non-treated EMA (EMA-) samples.

[4/] Different letters in the same column indicate statistically significant differences by DMRT at $P < 0.05$

Efficacy of EMA-qPCR Technique to Quantify Live Cells of '*Candidatus* Liberibacter asiaticus' (Las) on Mature and Immature Seeds with EMA-qPCR

The method developed in this study was applied to determine Las population with the possible correlation between mature seed and immature seed based on the hypothesis that concentration of Las cell in mature seed is lower than immature seed by using EMA-qPCR technique to quantify bacterial live cells in citrus plant tissue. The mature seed was collected from citrus fruits during the period April, 2014. The immature seed was collected from young citrus fruit on May, 2014 from Picos farm at the USDA-ARS Horticultural Research Laboratory, Fort Pierce, FL, USA. Vascular bundle of citrus in mature and immature seed were isolated and determined live Las population by EMA-qPCR technique. In non-treated EMA (EMA-), the Ct value and Las population (cell/g of plant tissue) of mature and immature seeds were 24.16 ± 0.70 and 7.86×10^5 and 24.72 ± 0.25 and 5.43×10^5, respectively (Table 4). Mature seed gave significantly higher Las population than immature seed. In the treated-EMA (EMA+), the Ct value and Las population (cell/g of plant tissue) of mature and immature seed were 34.35 ± 2.17 and 0.01×10^5 and 25.36 ± 0.09 and 3.56×10^5, respectively (Table 4). Further calculation of % live Las cells from mature seed was significantly lower % live cells (12.73%) than immature seed (65.56%), respectively which was significantly different by DMRT at $P < 0.05$ (Table 4).

Table 4 Detection and assessment of live cells of '*Candidatus* Liberibacter asiaticus' (Las) from vascular bundle of immature and mature seeds by EMA-qPCR technique

Vascular bundle	C_t value (± SD) [1/]		Las population[2/] (cell/g of plant tissue)		%Live cell[3/]
	EMA (-)	EMA (+)	EMA (-)	EMA (+)	
Mature Seed	24.16 ± 0.70[a]	34.35 ± 2.17[a]	7.86×10^5	0.01×10^5	12.73[b4/]
Immature Seed	24.72 ± 0.25[a]	25.36 ± 0.09[b]	5.43×10^5	3.56×10^5	65.56[a]

[1/] Ct values were obtained from the qPCR detection of Las with 16S rRNA gene using HLBaspr primer. The Ct values from qPCR data are the mean value from nine replications followed by the standard deviation (SD) of the mean.

[2/] Las population was calculated from $Y = 13.82\text{-}0.2866X$, where X is the mean Ct value and Y is the log concentration of the target DNA copies by expressed as cell/g of plant tissue.

[3/] %Live cells was calculated from proportion number of Las population from the sample was treated EMA (EMA+) with non-treated EMA (EMA-) samples.

[4/] Different letters in the same column indicate statistically significant differences by DMRT at $P < 0.05$.

Efficacy of Quantitative Real-Time PCR (qPCR) and Ethidium Monoazide (EMA) with Quantitative Real-Time PCR (EMA-qPCR) for Detection of Las in Citrus Petioles

Comparison on the efficacy of qPCR and EMA-qPCR techniques for detection of Las in smooth flat seville cultivar (SFS-12-20A) (*Citrus paradisi*, L.) from pith petiole was carried out in the experiment. The Ct value and Las population (cell/g of plant tissue) from qPCR and EMA-qPCR were 20.01 ± 1.04 and 12.17×10^7 and 22.48 ± 1.15 and 2.38×10^7, respectively which were not statistically significant by DMRT at $P < 0.05$ (Table 5). Considering live cells and dead cells of Las bacteria, qPCR technique was unable to distinguish that number whereas EMA-qPCR technique was able to distinguish them by showing 19.57% of live cells. This number can be used to calculate percentage of dead cells from the total (100%) of Las cells minus the percentage of live cells (100-19.57=80.43%) which was achieved at 80.43% of dead cells (Table 5).

Table 5 Efficiency of quantitative real-time PCR (qPCR) and Ethidium Monoazide (EMA) with quantitative Real-time PCR (EMA-qPCR) for detection of '*Candidatus* Liberibacter asiaticus' (Las) on petioles of smooth flat seville cultivar

Type of PCR	Ct value (± SD)[1/]	Las population[2/]	%Live cells[3/] (cell/g of plant tissue)	%Dead cells[4/]
qPCR	20.01 ± 1.04[a5/]	12.17×10^7	UC[6/]	UC
EMA-qPCR	22.48 ± 1.15[a]	2.38×10^7	19.57	80.43

[1/] Ct values were obtained from the qPCR detection of Las with 16S rRNA gene using HLBaspr primer. The Ct values from qPCR data are the mean value from nine replications followed by the standard deviation (SD) of the mean.

[2/] Las population was calculated from $Y = 13.82\text{-}0.2866X$, where X is the mean Ct value and Y is the log concentration of the target DNA copies by expressed as cell/g of plant tissue.

[3/] %Live cells was calculated from proportion number of Las population from the sample was treated EMA with non-treated EMA samples.

[4/] %Dead cells was calculated from the total percentage of Las cells (100%) minus with percentage of live Las cells.

[5/] Different letters in the same column indicate statistically significant differences by DMRT at $P < 0.05$.

[6/] UC was unable to calculate live cells in treatment of bacteria.

Discussion

In this study, we have successfully developed a method for quantification live cells of '*Candidatus* Liberibacter asiaticus' (Las) in petiole and vascular bundle of citrus using qPCR assay with the aid of Ethidium Monoazide (EMA) which was designated as EMA-qPCR technique. The EMA-qPCR has been used for qualitative DNA-based live/dead differentiation of bacteria in pure monoculture models (Nogva et al., 2003). Photo-induced cross-linking was reported to inhibit PCR amplification of DNA from dead cells. EMA cross-linking to DNA actually rendered the DNA insoluble and led to its loss together with cells debris during genomic DNA extraction. Dead cells of *Escherichia coli* strain 0157:H7 was removed with EMA prior extraction DNA (Nocker and Camper, 2006). The unbound EMA was remained free in solution, is concurrently inactivated by reacting with water molecules (DeTraglia et al., 1978). The resulting hydroxylamine is no longer capable of covalently binding to DNA (Kell et al., 1998). DNA from viable cells was protected from reactive EMA before light exposure by an intact cell membrane/cell wall which was not affected by the inactivated EMA after cell lysis. EMA was used in combination with real-time PCR (EMA-qPCR) for quantification of viable and dead cells of *Campylobacter jejuni* in food-borne with mixed bacterial population (Rudi et al., 2005). Ethidium Monoazide (EMA) and Propidium Monoazide (PMA) were compared to differentiation between live and dead bacterial cells which was found that the application of EMA is hampered by the fact that the chemical can also penetrate live cells of some bacterial species. Transport pumps actively export EMA out of metabolically active cells, but the remaining of EMA causing loss of DNA. PMA was applied for avoiding DNA loss (Nocker et al., 2006). In this work, EMA and PMA were used to compare quantify live Las cell in citrus petioles which was found that EMA has higher efficacy to quantify live cell than PMA. This was our reason to select EMA to future study on differentiation live and dead cell in EMA-qPCR technique (Table 1). Incubation period at 20 minutes has maximum effective to lead EMA penetrate into plant tissue due to its hardness structure to penetrate to binding bacterial DNA inside. Therefore, at 5 minutes incubation period is not sufficient to optimum EMA penetration. However, incubation period at 30 minutes (data not show) was not effective to quantify live cells as compared to at 20 minutes.

In this present study, we developed a sensitive, efficient and reliable method for quantification of live Las bacterial cells using qPCR assays in petiole with the aid of EMA treated on plant tissue preparation samples consisted of soaking, pulverizing and pulverizing with grinding in liquid nitrogen (N_2). Soaking the tissue in EMA solution which is simply and high efficacy method than pulverizing and pulverizing with grinding in liquid nitrogen (N_2) (Table2). Selection of plant tissues used in this study were petiole and vascular bundle in accordance with recent report that '*Candidatus* Liberibacter asiaticus' was distributed in bark tissue, leaf midrib, roots and different floral and fruit parts of infected citrus trees (Tatineni et al., 2008). Therefore, soaking method is appropriate sample preparation that allows chemical to penetrate dead cells with compromised cell walls and bind to their DNA with simple, quickly and reliable. This was our reason to select soaking citrus tissue in the EMA solution to future study on differentiation live and dead cell for standard method in our developed EMA-qPCR technique.

Tatineni et al. (2008) reported of quantification analysis of the HLB bacterium ranging from 14 to 137,031 cells/μg of total DNA in different tissues. However, number of total DNA is incapable to clearly demonstrate quantity of live cell or dead cell from number of bacterial population for examination the distribution and movement of the HLB pathogen which are key issues in HLB research and management. It was determined that a minimal Las concentration is required for HLB symptom development. EMA-qPCR technique is able to quantify live Las cell in symptomatic and asymptomatic HLB leave with high efficacy. All symptomatic leaves contained a higher concentration of live HLB bacterium (24.70%-37.71%) than asymptomatic leaves (3.38%- 22.09%) in all three citrus species (Table 3). This confirms the observations made using electron microscopic studies of symptomatic and asymptomatic midribs (Bové, 2006). This approach is able to demonstrate on both symptomatic and asymptomatic tissue to increasing the ability for detection of early live Las cell infection in nursery to produce disease-free citrus plants.

The EMA-qPCR technique was applied to determine the possible correlation between mature seed and immature seed based on the hypothesis that concentration of Las cell in mature seed is lower than immature seed. Result was absolutely supported the hypothesis with actual live Las concentrations in immature seed (65.56%) which was significantly higher than in mature seed (12.73%) (Table 4). This information is useful as a key to elucidate quantity of live cell on citrus seed due to citrus root stock is produced from seed therefore to understand the exact quantify of live cell is crucial to produce disease-free plant.

Detection of the HLB bacterium based on PCR methodology has achieved popularity when compared with other methods from the past due to its simplicity, sensitivity and reliability. Although quantitative real-time PCR (qPCR) with species-specific primers, TaqMan primer–probe sets specific to detection *Candidatus* Liberibacter species are developed to detect of *Candidatus* Liberibacter spp. However, the weakness of qPCR is unable to distinguish between live and dead cells or is unable to estimate live cells in their hosts. Therefore, developing the qPCR assays with EMA are trending toward to considerably detection, quantification and differentiation the population of HLB pathogen in the hosts. Our results indicated that EMA-qPCR technique gave live Las cells at 20.38×10^7 or 19.51% whereas qPCR was at 12.17×10^7 cells which was equally to 80.43% dead cells. Our EMA-qPCR technique demonstrated the advantage detection of live Las cells than normal qPCR. It was consistent similar with recently research to quantify of live '*Candidatus* Liberibacter asiaticus' using qPCR with the aid of EMA in citrus and periwinkle in which the proportion of live cells in citrus and periwinkle was ranged 17–31% and 16–28%, respectively due to the weakness of unable to detect low concentration of Las population (Trivedi et al., 2009). However, those Ct values in this experiment when calculated to Las population showed higher population in qPCR technique at 12.17 cell/g of plant tissue whereas showed lower population in EMA-qPCR technique at 2.38×10^7 cell/g of plant tissue. Therefore it is evident that qPCR usually detected both live and dead cells DNA while EMA-

qPCR detectes only live cells DNA. It was about 4 times more by qPCR than EMA-qPCR (Table 5). Therefore, EMA-qPCR technique is a sensitive and reliable technique to quantify live Las cells.

The new finding EMA-qPCR technique is trending toward to considerably detection, quantification and differentiation the live population of HLB pathogen in the hosts, prove useful for the symptomatic diagnosis of HLB disease, monitoring and identification of live Las, epidemic studies, determination of virulence mechanism and in the ultimate successful management of HLB.

Acknowledgements

This research was financially supported by the Royal Golden Jubilee Ph.D. (RGJ) program of Thailand Research Fund (TRF). We thank U.S. Department of Agriculture, Agricultural Research Service, United States Horticultural Research Laboratory (USDA-ARS-USHRL), Fort Pierce, FL, USA. for supporting of research facilities.

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