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Nota/Note

Comparison of whole-tissue maceration and sap extraction for isolation of '*Candidatus* Liberibacter asiaticus'

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SUMMARY

'*Candidatus* Liberibacter spp.' are fastidious alphaproteobacteria associated with Huanglongbing (HLB), a highly destructive disease for citrus production around the world. So far, no axenic culture of these bacteria have been established, limiting its study. However, the first step to cultivate plant pathogenic bacteria is to obtain viable cells. So, the aim of this study was to compare two methods to extract viable cells of '*Ca*. L. asiaticus' from diseased plant tissue of infected citrus, periwinkle and dodder by using the PMA-qPCR technique. We applied two methods for extraction of bacterial cells, the whole-tissue maceration procedure for citrus, periwinkle and dodder and the sap extraction method by using the Scholander pressure pump for citrus and periwinkle. The extraction of '*Ca*. L. asiaticus' cells by tissue maceration method provided a larger number of viable and non-viable bacterial cells when compared to the Scholander pressure pump method in both, citrus and periwinkle. The largest amount of bacteria extracted by the Scholander pressure pump was in periwinkle plants. However, the amount of cells obtained from dodder plants using maceration was higher than those obtained from the other plants by the same method, pointing out that dodder could be an excellent inoculum source for '*Ca*. L. asiaticus' isolation tests. In this study, the two methods provided sufficient amounts of '*Ca.* L. asiaticus' to perform bacterial isolation tests, and may be used in further studies on HLB bacteria and its disease.

Index terms: propidium monoazide, greening, extraction method, pressure bomb.

Comparação da maceração de tecido inteiro e da extração de seiva para isolamento de '*Candidatus* Liberibacter asiaticus'

RESUMO

'*Candidatus* Liberibacter spp.' são alfaproteobactérias fastidiosas associadas ao *huanglongbing* (HLB), uma doença destrutiva para a citricultura mundial. Até o momento, não foi possível o estabelecimento de culturas axênicas dessas bactérias, o que limita seu estudo. Entretanto, o primeiro passo para o cultivo de bactérias fitopatogênicas é a obtenção de células viáveis. Assim, o objetivo desse estudo foi comparar dois métodos de extração de células viáveis de '*Ca*. L. asiaticus' de plantas de citros, vinca e cuscuta infectadas utilizando a técnica de PMA-qPCR. Os métodos utilizados foram por bomba de pressão de Scholander para extração da seiva das plantas de citros e de vinca, e

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maceração de tecido para plantas de citros, vinca e cuscuta. O método de extração de células de '*Ca*. L. asiaticus' por maceração apresentou o maior número de células bacterianas, viáveis e não viáveis, quando comparado ao método da bomba de pressão de Sholander tanto em citros quanto em vinca. A maior quantidade de bactérias extraídas pela bomba de pressão de Sholander foi em plantas de vinca. Todavia, a quantidade de células obtidas de plantas de cuscuta utilizando a maceração foi superior à obtida nas demais plantas pelo mesmo método, podendo a cuscuta ser excelente fonte de inóculo para testes de isolamento de '*Ca.* L. asiaticus'. Os dois métodos de extração forneceram quantidades suficientes de células de '*Ca.* L. asiaticus' para realização de estudos de isolamento da bactéria, podendo também ser empregados em diferentes estudos sobre a bactéria e a doença por ela causada.

Termos de indexação: propídio monoazida, *greening*, método de extração, bomba de pressão.

INTRODUCTION

Huanglongbing (HLB) is the most important disease for citrus production around the world (Bové, 2006). Three species of bacteria are associated with HLB, '*Candidatus* Liberibacter asiaticus', '*Candidatus* Liberibacter africanus', and '*Candidatus* Liberibacter americanus' (Garnier et al., 1984; Jagoueix et al., 1994; Teixeira et al., 2005). '*Ca.* Liberibacter spp.' are Gram negative and fastidious bacteria that colonize the phloem of the hosts causing severe damage to plants (Jagoueix et al., 1994). These three '*Ca*. Liberibacter spp.' species infecting citrus can be transmitted naturally between Rutaceous plants by psyllids, as well as by grafting of infected propagative material (Bové, 2006). Despite that, these bacteria have also been reported infecting other plants besides the Rutaceous, such as periwinkle (*Catharanthus roseus*) (Garnier & Bové, 1983), tomato (*Lycopersicum esculentum*) (Duan et al., 2008) and tobacco (*Nicotiana tabacum* cv. Xanthi) (Francischini et al., 2007), through dodder transmission (*Cuscuta* spp.).

The first attempts to isolate the HLB organisms were by the 1970s and 1980s (Ghosh et al., 1971; Garnett, 1984). More recently, studies concerning the isolation and cultivation of '*Ca.* Liberibacter spp.' have been published (Davis et al., 2008; Sechler et al., 2009). However, these results could not be reproduced independently and the Koch's postulates were also not fulfilled, thus the HLB bacteria remain considered as a non-cultivable organism.

The establishment of '*Ca.* Liberibacter spp.' in pure cultures is of utmost importance to carry out several different studies on bacteria and its interactions with host plants and insect vetors. However, the bacteria isolation require several steps, such as the establishment of appropriate methods for extracting viable bacteria from plant and insect vetors tissues, development of culture media for isolation and cultivation, and suitable storage conditions. Nevertheless, inappropriate methods for bacteria extraction from host tissues can irreparably compromise the integrity of the pathogen cells and, thereby, preclude isolation and cultivation of bacteria in pure culture.

A technique for HLB bacteria extraction from host tissues should allow to obtain sufficient amount of viable bacterial cells to establish cultures in artificial culture medium. Here, we analyze the viability of '*Ca*. L. asiaticus' cells present in saps extracted from tissue of plants infected with HLB bacteria by using propidium monoazide (PMA) associated with quantification by qPCR (real-time quantitative Polymerase Chain Reaction) technique. PMA is a dye that binds only to the DNA of cells that have damaged membrane (Nocker et al., 2006; Hu et al., 2013, 2014) and, thus inhibits DNA amplification by PCR from non-viable cells. Furthermore, the qPCR technique associated with PMA allow us to quantify the amount of viable '*Ca*. L. asiaticus' cells present in the extracts used for bacteria isolation. Thus, the objective of this study was to establish efficient protocols to extract the largest number of viable cells of '*Ca*. L. asiaticus' from tissues of different host plants.

MATERIALS AND METHODS

Plant material

In this study, we used samples of HLB symptomatic citrus (*Citrus sinensis* (L.) Osbeck cv. 'Pera'), periwinkle (*Catharanthus roseus*) and dodder (*Cuscuta* sp.) infected with '*Ca*. L. asiaticus' bacterium. The presence of bacteria in samples of all three plants was confirmed by PCR using the A2/J5 primers (Hocquellet et al., 1999). The HLB bacteria was extracted from the different plant tissues by two methods, the tissue maceration procedure and the sap extraction by using the Scholander pump. The bacterium was extract from dodder samples only by the tissue

maceration procedure due to technical difficulties to use the Scholander pump in this case.

Tissue maceration procedure

Three samples of citrus, periwinkle and dodder plants were processed by using the protocol described by Trivedi et al. (2009), with minor modifications, to evaluate the extraction of viable HLB bacterial cells by the tissue maceration procedure. Bark of citrus or periwinkle stems with, approximately, 5 mm in diameter were removed and scraped with an aid of a scalpel. These tissue pieces were macerated in a mortar with 15 mL of sterile distilled water. The dodder tendrils were also macerated in 15 mL of sterile distilled water. The macerated tissue was transferred to a 50 mL tube and centrifuged for 2 min at 1,200 g. The supernatant was aliquoted into 1.5 mL tubes and centrifuged for 5 min at 12,000 g and then discarded. The resulting pellet was weighed and about 40 mg was resuspended in 1 mL of sterile distilled water. After this step, half of the samples were subjected to PMA treatment as described below.

Sap extraction by using the Scholander pump

Three samples of citrus or periwinkle were used to evaluate the extraction of viable cells of '*Ca*. L. asiaticus' from infected plant shoots using the Scholander pressure pump (Soil Moisture Equipment Corp., Santa Barbara, CA, USA). For higher efficiency in the extraction process with the Scholander pump, considerable care was taken to maintain the integrity of the stems, avoiding the removal of leaves and the use of broken shoots. Stems of approximately 15 cm in length and maximum thickness of 4 mm in diameter were individually placed inside the Scholander pump, leaving around 3 cm of the end out of the pump. The total phloem and xylem saps of plant samples were forced out of the branch by applying a pressure of approximately 3,000 to 4,000 kPa. The sap was collected with micropipette and deposited in 1.5 mL microcentrifuge tube. For each sample, 2 mL of sap were collected and equally distributed in two microcentrifuge tubes. After this step, half of samples were subjected to PMA treatment as described below. The other half of samples were used for DNA extraction according to Murray & Thompson (1980). The DNA obtained from each sample was resuspended in 50 µL of Milli-Q water.

Pre-treatment with PMA

The extracts obtained by each procedure were treated with PMA (Biotium Inc., Haward, CA, USA), according to the protocol described by Hu et al. (2013). Initially, a stock solution of 10 mg mL^{-1} of PMA (dissolved in sterile distilled water) was prepared and this solution was kept protected from light at 4 °C. The PMA solution (50 µM) was added to the tubes containing plant extracts samples at a final concentration of 25 µg mL^{-1}. The tubes were incubated in the dark for 5 min at room temperature, with occasional inversions to allow PMA to penetrate into the cells with damaged membrane and to bind to the bacterial DNA. After the incubation period, the tubes were briefly vortexed for 5 sec and placed on ice. To activate the PMA reaction, the tubes were opened and subjected to halogen light (650 W) at 20 cm of distance for 2 min. After treatment with PMA, samples were centrifuged for 5 min at 12,000 g. The supernatant was discarded and 700 µL of CTAB buffer was added to the pellet. DNA extraction was performed following the protocol of Murray & Thompson (1980) and resuspended in 50 µL of Milli-Q water.

qPCR test for HLB bacterium quantification

Samples treated and not treated with PMA, obtained by the different extraction methods, were submitted to quantification of '*Ca*. L. asiaticus' bacterium by qPCR technique. A TaqMan® probe detection system for a specie-specific 16S rDNA gene region of bacterium was used. The qPCR reaction was prepared for a final volume of 15 µL, containing 7.5 µL of 2× ABI TaqMan Universal Master Mix (Life Technologies), 10-20 ng of DNA, 0.25 µM of the HLBas and HLBr primers, 0.15 µM of the probe HLBp labeled at the 5' terminus with the reporter dye 6-carboxyfluorescein (FAM) and at the 3' terminus with Quencher (BHQ), and nuclease free water (Ambion™, Invitrogen, Carlsbad, CA, USA) to adjust the volume. The reaction conditions for amplification were 2 min of incubation at 60 °C, 10 min of denaturation at 95 °C, followed by 40 cycles of 15 sec at 95 °C and 1 min at 58 °C. Fluorescence signals were collected after one minute at 58 °C during each cycle (Li et al., 2006). Amplification and detection were performed on an ABI Viia 7 qPCR apparatus (Applied Biosystem, Foster City, CA, USA). All reactions were performed in duplicate and, at each run, negative and positive controls were

included. To generate a standard curve, DNA fragments of 16S rDNA of '*Ca*. L. asiaticus' obtained by previous PCR were cloned into competent *Escherichia coli* cells with TOPO TA plasmid insert (Invitrogen, Carlsbad, CA, USA). Known concentrations of these plasmids carrying the target DNA, obtained from cloned libraries, were diluted serially, starting from the concentration of 10 ng μL^{-1} under ten serial dilutions in the ratio of 1:2 for higher resolution and encompassing most unknown samples concentration matches in the standard curve midpoints (Sow et al., 2009; Gallup & Ackermann, 2006). The threshold cycle numbers (Ct) reported were used to establish a linear regression based on copy number of DNA samples to generate a standard curve. The Ct values of the samples were interpolated in a standard curve to determine the number of copies of the gene in the samples (Li et al., 2006). The data were submitted to analysis of variance using the SASM-Agri software (Canteri et al., 2001). The means were compared by the Tukey test with a significance level of 5%.

RESULTS

Tissue maceration procedure

The tissue maceration method yielded extracts with total '*Ca*. L. asiaticus' concentrations ranging from 5.77×10^{11} bacterial cells per milligram of tissue of periwinkle and up to 1.57×10^{16} bacterial cells per milligram of tissue of dodder (Table 1). Using PMA-qPCR test to estimate viable cells of '*Ca*. L. asiaticus', we found concentrations in citrus, periwinkle and dodder samples ranging from 1.21×10^{11} to 5.88×10^{12}, 5.86×10^{10} to 1.80×10^{14} and 1.21×10^{15} to 3.43×10^{15} cells per milligram of tissue, respectively (Table 1). Therefore, dodder plants provided the recovery of the largest number of total and viable cells of '*Ca*. L. asiaticus' compared to periwinkle and citrus (Table 1). Further, more than 90% of the cells of '*Ca*. L. asiaticus' recovered were viable ones for all kind of plant tissue tested (Table 1).

Sap extraction by using the Scholander pump

Extraction of the '*Ca*. L. asiaticus' by using the Scholander pump was only carried out for citrus and periwinkle samples, as it was not possible to test this method in dodder due to the structure of the tendrils. The amount of total cells of bacteria obtained from the sap of citrus and periwinkle tissue ranged from 2.34×10^7 up to 1.15×10^{11} cells per milliliter of sap, respectively (Table 1). Regarding the amount of viable cells of '*Ca*. L. asiaticus', we found it ranging from 1.36×10^7 up to 3.18×10^{10} cells per milliliter of sap of citrus and periwinkle, respectively, based on the PMA-qPCR test (Table 1). The amount of total and viable bacterial cells in the sap of citrus and periwinkle plants obtained using the Scholander pump did not differ significantly (Table 1). For citrus, approximately 94% of '*Ca*. L. asiaticus' cells were viable ones based on the PMA-qPCR test, whereas for periwinkle cell viability was 97% (Table 1).

Table 1. PMA-qPCR analysis and quantification of '*Candidatus* Liberibacter asiaticus' extracted by different methods from different host plants.

Extraction method/host plant	Log of '*Ca*. L. asiaticus' genomes per milligram of tissue or milliliter of sap		CV (%)[1]
	Without PMA	With PMA	
Maceration			
Citrus	13.4[2] ± 0.5 a[3] (9)	12.4 ± 0.2 b (9)	1.4
Periwinkle	13.7 ± 1.3 a (9)	12.6 ± 1.4 b (9)	10.6
Dodder	16.1 ± 0.1 a (9)	15.4 ± 0.1 b (9)	1.5
Scholander pressure pump			
Citrus	12.87 ± 0.5 a (3)	12.55 ± 0.5 a (3)	5.22
Periwinkle	13.73 ± 0.9 a (3)	13.46 ± 0.6 a (3)	9.92

[1]CV(%), coefficient of variation; [2]Values represent mean and standard deviation (n = 3 or n = 9, different sample size due to the amount of infected tissue obtained); [3]Means followed by the same letter in the line do not differ statistically by the Tukey test at the 5% level of significance

DISCUSSION

Isolation and cultivation of plant pathogenic bacteria are important to allow different kind of studies such as the mechanisms of bacteria survival, dissemination and infection. Further, this information is crucial to define proper strategies for prevention and control of the diseases caused by these plant pathogens (Fuente & Burdman, 2011). In the present study, both methods of bacteria extraction tested, tissue maceration and sap extraction with the Scholander pump, were efficient to allow to obtain viable cells of '*Ca*. L. asiaticus' from tissues of plants infected with HLB bacterium. More than 90% of the '*Ca*. L. asiaticus' cells obtained from citrus and periwinkle samples by the two methods were viable, based on the PMA-qPCR test (Table 1). Parker et al. (2014) also found viable cells of '*Ca*. L. asiaticus' in pummelo (*Citrus maxima* cv. 'Mato Buntan') seeds, used as an inoculum source. However, the bacterial population ranged from 3.3×10^4 to 9.8×10^5 per milliliter of extract, well below the amount of bacterial cells observed in our findings. The amount of total and viable HLB bacteria in the extracts was higher than 2.34×10^7 and 1.36×10^7 cells per milligram of tissue or milliliter of sap, respectively (Table 1). It should be pointed out that dodder presented the highest concentrations of viable bacterial cells obtained by the tissue maceration method (Table 1). These results confirm other studies with tissues of different plant species where variation in bacterial population was observed, but '*Ca*. L. asiaticus' concentration was also higher in dodder plants (Trivedi et al., 2009; Hu et al., 2013).

The use of the Scholander pump to extract sap from citrus and periwinkle plants showed to be an efficient method in recovering '*Ca*. L. asiaticus' viable cells. This method had already been used to extract other bacteria colonizing the vascular tissue of bean, soybean and cotton plants (Hallmann et al., 1997), as well as *Xylella fastidiosa* from grapevine and oleander (Bextine & Miller, 2004). To our knowledge, this is the first study where the Scholander pressure pump in association with the PMA-qPCR technique was used for extraction and estimation of viability of '*Ca*. L. asiaticus' cells from the sap of plants infected with HLB.

Although the isolation of '*Ca*. Liberibacter spp.' associated with HLB disease has been reported (Davis et al., 2008; Sechler et al., 2009), so far, the bacteria remains as non-cultivable one because these works have not been reproduced independently. Further, those studies did not determine the viable bacterial cells present in the inoculum source. Therefore, the amount of inoculum could be insufficient to establish pure culture of the bacteria. A large amount of viable cells of '*Ca.* Liberibacter spp.' is, for certain, the first step to succeed in isolation and establishment of axenic culture of HLB bacteria.

'*Ca*. L. asiaticus' is not uniformly distributed in the host plant tissues (Tatineni et al., 2008; Li et al., 2009; Hartung et al., 2010), however, the bark of the stems of citrus and periwinkle and the tendrils of dodder showed a high concentration of the bacterium (Table 1). For that reason, these tissues seem to be appropriate as an inoculum source for the isolation of the bacteria. In regard to dodder, the highest concentration of viable cells of '*Ca*. L. asiaticus' were obtained by using the tissue maceration extraction method from this plant (Table 1). Our findings confirm other studies, in which differences in '*Ca*. L. asiaticus' concentration were observed among different plant species (Trivedi et al., 2009; Hartung et al., 2010; Hu et al., 2013). Another interesting aspect in the use of dodder for extraction of '*Ca*. L. asiaticus' cells is the ease in the process of tissue maceration, besides the small amount of contaminating organisms (data not shown).

The PMA-qPCR technique used to determine the viability of uncultured bacterial cells is relatively recent (Hu et al., 2013; Hu et al., 2014). The results obtained in this study revealed that the PMA-qPCR technique, used to evaluate the viability of '*Ca*. L. asiaticus' present in different host plants, is a valuable technique to monitor the amount and quality of the initial inoculum in studies on bacterial isolation, as well as in other studies regarding the HLB bacterium. Further, among the studied plants, dodder seems to be an excellent inoculum source for '*Ca.* L. asiaticus' isolation.

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