Trop. plant pathol. (2015) 40:251–259
DOI 10.1007/s40858-015-0020-1

PCR-based methods for detection of *Erwinia psidii* on guava

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Received: 19 January 2015 / Accepted: 14 May 2015 / Published online: 11 June 2015
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Abstract *Erwinia psidii* causes bacterial blight of guava (*Psidium guajava*), one of the most important diseases of this crop in Brazil. Control measures are not effective, and dissemination often occurs through contaminated but asymptomatic propagating plant material. Considering the need for a reliable and sensitive method for detecting the pathogen in asymptomatic plant material, *E. psidii*-specific PCR primers were designed from a 355-bp fragment of the recombinase A gene (*rec*A) amplified from *E. psidii* type strain. Primer pair Ep2L/2R only amplified DNA from *E. psidii* and its detection limit was 10^{-5} ng/μL of purified DNA and 10 CFU (colony forming units) of bacterial cell suspension/mL. Three methods, conventional PCR, IC-PCR, and BIO-PCR were evaluated with the selected primers for their potential to detect *E. psidii* on guava leaves. BIO-PCR and conventional PCR were more sensitive and less time-consuming than IC-PCR. The detection limits on extracts of macerated guava leaves spiked with bacterial suspensions at different concentrations were 10 and 10^3 CFU/mL for BIO-PCR and conventional PCR, respectively. The PCR method here described could be useful for developing a protocol for early detection of this pathogen in asymptomatic guava plants.

Keywords *rec*A · *Psidium guajava* · Immuno-capture PCR · BIO-PCR

Section Editor: Bernardo Halfeld-Vieira

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Introduction

Bacterial blight caused by *Erwinia psidii* Rodrigues Neto, Robbs and Yamashiro is one of the most important diseases of guava (*Psidium guajava* L.) in Brazil. The bacterium affects branches and twigs of guava trees, causing dieback. Leaves, blossom and green fruits are also affected. The disease can significantly reduce yield, and chemical control measures are not effective or may lead to other problems such as phytotoxicity on developing fruits (Rezende et al. 2008). *E. psidii* was first detected in 1982 in Valinhos and Pindamonhangaba counties of São Paulo state, Brazil, and described in 1987 as a new species belonging to the 'amylovora' group species (Rodrigues Neto et al. 1987). Later it was also detected in several other regions in the state, which suggested pathogen dissemination through contaminated material. The disease was reported in Minas Gerais state in 1993 (Romeiro et al. 1993), Espírito Santo and Paraná in 2000 (Oliveira et al. 2000; Coelho et al. 2002) and Distrito Federal in 2001 (Junqueira et al. 2001). Until recently, *E. psidii* had been registered only in Brazil, but in 2011 it was described as the causal agent of a new disease affecting shoots and stems of *Eucalyptus* species in Argentina and Uruguay (Coutinho et al. 2011), suggesting a host shift event. *E. psidii* was also identified as the causal agent of die-back and wilt of *Eucalyptus* spp. in Brazil (Arriel et al. 2013), a disease observed in eucalyptus stands since 2009. Worldwide bacterial blight could be of importance to the major guava-growing countries such as India, Egypt, Mexico and Pakistan (Janse 2012).

Erwinia psidii is one of the most important pathogens affecting guava orchards in central Brazil. It causes severe yield losses, limiting the availability of fruit in local markets during the rainy season (October–March) leading, in many cases, to growers abandoning the crop. Yield losses of up to 85 % were reported (Junqueira et al. 2001). In 2001, surveys were carried

out in orchards in Distrito Federal and, in 56 % of the properties, the presence of *E. psidii* was confirmed (Coelho et al. 2002; Marques et al. 2007).

Typical symptoms of the disease are shoot wilting and blight, yellowing, water soaking of the area neighboring the leaf veins, and necrosis and mummification of immature inflorescence and fruit (Rodrigues Neto et al. 1987; Coelho et al. 2002; Marques et al. 2007). However, the lack of symptoms does not guarantee the acquisition of pathogen-free plant material. Asymptomatic young plants will show symptoms approximately 2 years after their installation in the orchard, which is the time required for fructification (Gonzaga Neto et al. 2001). Therefore, more sensitive and accurate methods for pathogen detection on asymptomatic guava plants are necessary (Marques et al. 2007; Teixeira et al. 2008).

Detection and identification of *E. psidii* is traditionally carried out by isolation on culture medium followed by several nutritional/physiological tests (Coelho et al. 2002). Marques et al. (2007) proposed a minimal list of physiological and nutritional tests for pathogen identification, which were: Gram stain, oxidation/fermentation (O/F) test, catalase and urease activity, pectate degradation, hypersensitive reaction on tobacco, acid production from mannitol and raffinose, and nitrate reduction. Those tests allow the differentiation of *E. psidii* from other species in the genus *Erwinia* complex, including species now reclassified in *Brenneria, Lonsdalea* and *Pantoea*. Moreover, a method of inoculation using detached shoots was described, which proved to be useful to confirm pathogenicity and was less time-consuming than plant inoculations (Marques et al. 2007). Bacterial detection using serology with a specific polyclonal antibody has been described as well (Teixeira et al. 2008); however, due to its low sensitivity, an enrichment step in plant extracts was necessary to detect bacterial cells in small numbers.

PCR, either conventional or real time, has been broadly employed for successful detection and diversity studies of plant pathogenic bacteria, including *Pantoea stewartii* (Wensing et al. 2010), *Erwinia amylovora* (Powney et al. 2011; Dreo et al. 2012; Gehring and Geider 2012; Wensing et al. 2012; Hannou et al. 2013) and *E. piriflorinigrans* (Barbé et al. 2014). Several genomic regions, including PCR fragments obtained from rep-PCR profiles (Rico et al. 2008), plasmidial sequences (Bereswill et al. 1992; Barbé et al. 2014) and draft whole genome sequences (Pritchard et al. 2013), have been used for the development of specific identification tools for plant pathogenic enterobacteria. The recombinase A (*rec*A) gene is a valuable marker for the major bacterial groups (Eisen, 1995) and for differentiating species in *Erwinia* and related genera (Waleron et al. 2002; Young and Park 2007; Parkinson et al. 2009; Wensing et al. 2010). Despite the high degree of sequence conservation in the *rec*A gene, the existing sequence polymorphism was useful for differentiating 19 species in *Erwinia* (Waleron et al. 2002). In that study, only the type strain of *E. psidii* isolated from guava in Brazil was included. In a preliminary study of 50 strains of *E. psidii*, from Distrito Federal and three Brazilian states (SP, ES and PR), we compared their *Alu* I-restriction profiles of a 730 bp fragment of the *rec*A gene. The banding patterns were identical for all strains, regardless of their geographic origin (Torres et al. 2008). Therefore, this gene was chosen as a good candidate for the design of specific primers for *E. psidii.* Several PCR protocols have been described for *E. amylovora* and other plant-associated *Erwinia* (Taylor et al. 2001; Powney et al. 2011; Gehring and Geider 2012), but a specific test for *E. psidii* has not yet been reported.

The objectives of this study were to design and select *E. psidii*-specific primers based on the partial sequencing of the *rec*A gene, and to develop a PCR-based method that could be used for early detection of the pathogen on asymptomatic plant material.

Materials and methods

Bacterial strains and culture conditions

Fifty-nine strains of *E. psidii* were analyzed in this study. These strains were obtained from the Collection of Plant Pathogenic Bacteria at Instituto Biológico (IBSBF), Campinas, SP, Brazil, and from collections maintained at the Plant Pathology Department of the Universidade de Brasília and Laboratório de Quarentena Vegetal, Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil (Table 1). The strains were recovered in 523 culture medium (Kado and Heskett 1970), and cultivated for 48 h at 30 °C in duplicates. All strains were maintained in sterile distilled water at 4–6 °C. The isolation of bacterial flora from guava leaves was performed by pressing the upper and lower surface of the leaves against the culture medium. For leaves and stems, isolation was also carried out by macerating tissue in sterile water followed by plating on Kado's medium 523. Colonies with different cultural aspects were selected, and pure cultures were established and subjected to biochemical tests of catalase activity and oxidation/fermentation, according to standard protocols. Gram reaction was determined by a potassium hydroxide (3 %) solubility test. A guava fruit isolate of *Colletotrichum gloeosporioides* kindly provided by L.E.B Blum, University of Brasilia, was also used for the specificity tests.

DNA extraction

DNA from *E. psidii* was extracted from 48 h-old cultures using the Wizard genomic DNA extraction kit (Promega) according to the manufacturer's instructions. DNA extraction from other plant pathogenic bacteria and resident bacteria from guava leaves and stems followed the protocol described

Table 1 Brazilian *Erwinia psidii* strains from guava (*Psidium guajava*) used in this study

Strain	Origin (city-state)	Year of collection
IBSBF[a] 435 (type strain), (CFBP 3627, NCPPB 3555, ATCC 49406)	SP	1982
IBSBF 446	SP	1982
IBSBF 452	Valinhos-SP	1983
IBSBF 453	Valinhos-SP	1983
IBSBF 454	Valinhos-SP	1983
IBSBF 493	Itariri-SP	1984
IBSBF 954	SP	1992
IBSBF 1347	Brazlândia-DF	1997
IBSBF 1461	Urupês- SP	1999
IBSBF 1480	Santa Tereza-ES	2000
IBSBF 1523	Carlópolis-PR	2000
IBSBF 1574	SP	2001
IBSBF 1575	Brazlândia-DF	2000
IBSBF 1576	Brazlândia-DF	2001
IBSBF 1577	Brazlândia-DF	2001
IBSBF 1578	Brazlândia-DF	2001
IBSBF 1579	Brazlândia-DF	2001
Emb[b]. A18-7	Brazlândia-DF	2000
Emb. C76.1; 081.3; 082.2 133.1; 134.1;142.2; 148.2; 150.1 151.2; 153.1; 1576;294.1; 294.2 295.3; 296.1; 299.3;338.2 342.1; 343.2; 344.2; 345.2 400.3; 421.2; 424.1;432.1; 433.2; 434.2; 434.3;435.2; 435.3; 436.3 438.2; 439.3	Brazlândia-DF	2002
Emb. C441.3; 442.1	Brazlândia-DF	2004
UnB[c] 1285; 1286; 1287; 1288; 1289; 1290	Brazlândia-DF	2005

SP São Paulo state, *PR* Paraná state, *DF* Distrito Federal, *ES* Espírito Santo state

[a] Phytobacteria Culture Collection of Instituto Biológico, Campinas, SP, Brazil

[b] Embrapa Recursos Genéticos e Biotecnologia

[c] Departamento de Fitopatologia, Universidade de Brasília, Brasília DF, Brazil

by Ausubel et al. (1995). Extractions from guava leaves and from mycelia of the fungus *Colletotrichum gloeosporioides* yielded DNA of better quality with the CTAB method of Doyle and Doyle (1987). DNA was dissolved in sterile distilled water to a final volume of 50 μL and stored at −20 °C until used. The quantification of DNA concentration was performed by agarose gel electrophoresis with High Mass DNA ladder (Invitrogen) as reference followed by dilutions to 10 ng /μL.

*rec*A gene amplification

For the amplification of the *rec*A gene, reactions were made for a final volume of 50 μL, containing: 100 ng DNA, 1 X buffer (50 mM of KCl, 10 mM Tris HCl), 1 μM primer *rec*A F (5′GGT AAA GGG TCT ATC ATG CG3′) and 1 μM primer *rec*A R (5′CCT TCA CCA TAC ATA ATT TGG A3′) (Waleron et al. 2002), 2.5 mM $MgCl_2$, 250 μM each of dNTPs, 1 U of *Taq* DNA polymerase. The PCR program was as follows: initial denaturation at 95 °C/3 min, followed by 32 cycles of 94 °C/1 min, 47 °C/1 min and 72 °C/2 min, and a final extension at 72 °C for 5 min in a thermocycler model PTC-100 (MJ Research, Inc.). Amplification products (5 μL added to 1 μL of loading buffer) were separated by electrophoresis on 1 % agarose gels in 0.5 X TBE buffer. The gels were stained in an ethidium bromide solution (0.5 mg/mL for 5 min), washed in distilled water for 10 min, and visualized using a digital system LPix–STI (Loccus Biotecnologia).

Partial sequencing of the *rec*A gene and primer selection

The amplification products corresponding to a *rec*A gene fragment from *E. psidii* type strain IBSBF 435 and five other strains (UnB 1287 and 1288, Emb.C76.1, C134.1 and

C150.1) were precipitated with 25 μL sodium acetate (7.5 M) and 125 μL of absolute ethanol, for 12 h at −20 °C, followed by centrifugation for 40 min at 14,000 rpm. The pellets were washed with 100 μL of 70 % ethanol, centrifuged for 5 min at 5000 rpm at room temperature, air dried, dissolved in ultrapure water (15 μL) and kept at 4 °C for 2 h. For each *E. psidii* strain, two sequencing reactions were prepared, one with each forward and reverse primer at 10 μM and 5 μL of the precipitated PCR product. The products were sequenced according to the fluorescent labeling protocol (Dynamic ET Terminator, Amersham Biosciences). Sequencing was performed using MegaBace automated sequencer (Pharmacia Biotech), at the Laboratory of Molecular Biology, Universidade de Brasília, Brasilia, DF, Brazil.

Analysis of sequences and design of primers

The sequences were edited and aligned using BioEdit (Hall 1999) and analyzed with BLASTN (Altschul et al. 1990). Selection of primers was performed using *Primer* 3 program (Rozen and Skaletsky 2000), with the primers located in positions of most divergence among the comparable *rec*A gene sequences of *E. psidii* strains, including the Genbank sequences of the type strain (AY217065) and *E. mallotivora* (AY217064), *E. tracheiphila* (DQ859879), *E. persicina* (DQ859883), *Pectobacterium cypripedii* (DQ859876), *Pantoea aglomerans* (AY219007), and *Pantoea stewartii* (AY219003). The designed primers were synthesized commercially at IDT (Integrated DNA Technologies, Inc.).

Primer specificity

Four selected primers were used in three combinations: Ep 1R/1L, Ep2L/2R and Ep 2L/1R and checked for specificity through PCR assays with purified DNA from *E. psidii* strains (Table 1), 20 plant pathogenic bacteria (Table 2) and 17 resident bacteria isolated from guava leaves and stems. The primers were also tested with purified DNA from *C. gloeosporioides* and with DNA extracted from guava leaves variety "Pedro Sato". All amplification reactions were composed of 1X buffer (50 mM of KCl, 10 mM Tris HCl), 1.25 mM of $MgCl_2$, 0.1 mM of dNTPs, 0.5 μM of each primer, 0.5 U of *Taq* DNA polymerase and 30 ng of DNA. The volume was adjusted with sterile water to a final volume of 25 μL. The program used for amplification was: denaturing at 95 °C/3 min, followed by 32 cycles of 94 °C/1 min, 64 °C/1 min, 72 °C /2 min and final extension at 72 °C for 5 min. Primer specificity was also checked electronically using the Primer-Blast tool (Ye et al. 2012) available at NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast).

Table 2 Plant pathogenic bacteria tested for selecting specific primers for *Erwina psidii*

Strain	Species	Host
UnB 1232	*Acidovorax citrulli*	Melon
UnB 646.2	*Acidovorax citrulli*	Melon
UnB 1138	*Agrobacterium tumefaciens*	Carrot
UnB 1151	*Clavibacter michiganensis* subsp. *michiganensis*	Tomato
UnB 1253	*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*	Bean
UnB 336	*Dickeya chrysanthemi*	Chard
UnB 1036	*Pectobacterium carotovorum*	Turnip
UnB 1142	*Pseudomonas cichorii*	Tomato
UnB 1147	*Pseudomonas syringae* pv. *tomato*	Tomato
UnB 1273	*Ralstonia solanacerum*	Tomato
UnB 1159	*Xanthomonas axonopodis* pv. *manihotis*	Cassava
FAL	*Xanthomonas axonopodis* pv. *passiflorae*	Passion fruit
UnB 1079	*Xanthomonas axonopodis* pv. *vitians*	Lettuce
UnB 97	*Xanthomonas axonopodis* pv. *vitians*	Lettuce
UnB 845	*Xanthomonas axonopodis* pv. *vitians*	Lettuce
UnB 1318	*Xanthomonas axonopodis* pv. *viticola*	Grapevine
IBSBF 2579	*Xanthomonas citri* pv. *anacardii*	Cashew
IBSBF 2586	*Xanthomonas citri* pv. *mangiferaeindicae*	Mango
IBSBF 2585	*Xanthomonas citri* pv. *spondiae*	*Spondias dulcis*
UnB 828	*Xanthomonas campestris* pv. *campestris*	Kale

IBSBF, Phytobacteria Culture Collection of Instituto Biológico, Campinas, SP, Brazil; UnB, Departamento de Fitopatologia, Universidade de Brasília, Brasília DF, Brazil, and FAL, Fazenda Agua Limpa, Universidade de Brasília, Brasília DF, Brazil

Primer sensitivity

In order to determine the detection threshold of the selected primers sets, genomic DNA of the type strain IBSBF 435, diluted from 10 to 10^{-6} ng/μL DNA, was used. The detection limit was also determined with a suspension of cells obtained from a 48 h culture, and adjusted to 8 % transmittance in a spectrophotometer (model V-530 UV–VIS, JASCO), which corresponds to 10^8 CFU/mL. Serial dilutions in sterile distilled water were carried out to a final concentration of 10 CFU/mL. After dilutions, 3 μL was added to each amplification reaction, for both DNA and cell suspensions.

Erwinia psidii detection by PCR, IC-PCR and BIO-PCR

Conventional PCR

Detached guava leaves cv. Pedro Sato were inoculated by spraying with bacterial suspensions of strain IBSBF 435, adjusted from 10^6 to 10 CFU/mL, as described above. Following inoculation, the leaves were air dried for 4 h at room temperature. Leaves were cut into small 1 × 1 cm pieces and then washed in 5 mL of sterile distilled water, under agitation at 200 rpm for 1 h. The washing was centrifuged at 13,200 rpm for 10 min, the supernatant was discarded and the pellet suspended in 250 μL of cold buffer from Pathoscreen kit (Agdia), followed by two dilutions, 1:10 and 1:100, with the same buffer. PCR was performed with Ep 2L/2R primers and 3 μL of each sample and each dilution in the reaction. As negative controls, reactions with only water and reactions with extracts of healthy guava leaves macerated with water were included. For positive controls, we used purified DNA and a cell suspension at 10^6 CFU/mL of *E. psidii* type strain IBSBF 435. The amplification reactions using primers Ep 2L/2R were prepared as previously described with the same cycling program.

IC-PCR

For IC-PCR, we used the polyclonal antibody As 14.1 produced against *E. psidii* type strain IBSBF 435 (Teixeira et al. 2008). The antibody was diluted 1:200 in a coating buffer (1.59 g/L $NaCO_3$; 2.93 g/L $NaHCO_3$). To each well of a polypropylene plate, 100 μL of the diluted antibody was added and incubated at 4 °C for 16 h. Young leaves of guava "Pedro Sato" were collected, weighed (1 g) and placed in plastic bags, where they were macerated with 2 mL of bacterial suspensions in water at concentrations from 10^7 to 10 CFU/mL. The wells were washed 3 times with PBST buffer (Phosphate buffered saline with Tween 2 %) and 100 μL of the macerated leaves mixed with bacterial suspension were added into each well, with three replicates per concentration. The plates were incubated at 4 °C for 16 h. The suspension was discarded followed by three washes with PBST buffer. The excess buffer was drained on a paper towel. Positive and negative controls, amplification reactions (25 μL added to each well) and cycling program were as previously described.

BIO-PCR

Cell suspensions from a 48 h-culture of *E. psidii* IBSBF 435 were adjusted to 8 % transmittance and 10-fold serially diluted to obtain suspensions up to 10 CFU/mL. Young guava leaves of variety Pedro Sato (1 g) were collected and washed in distilled water and dried with paper towel. The leaves were placed in plastic bags and 2 mL of bacterial suspensions were added at 10^7 to 10 CFU/mL. The leaves were macerated inside the plastic bags, and 100 μL of the extract was inoculated on Kado's 523 medium, with three replicates for each dilution. Plates were incubated for 24 or 48 h, followed by washes of each plate with 2 mL of sterile water. Each plate wash was then diluted 1:10. From this suspension, 3 μL was used for PCR with primers Ep 2L/2R. Negative and positive controls and conditions for PCR were as described above. All PCR tests (Conventional, IC and BIO) were repeated once.

Results

Partial sequencing of *rec*A gene and primer selection

PCR products corresponding to a 730 bp-fragment of the *recA* gene were sequenced and a conserved region among *E. psidii* strains spanning 355 bp was chosen for primer selection. The sequences were aligned with the sequence of type strain IBSBF 435. Primers were designed to anneal to the sequences that contained the most variable sites among *E. psidii* and other species and genera, and high identity (over 85 %) among strains of *E. psidii*. Two pairs of primers for specific amplification were designated Ep1L (5′ AGGCGTTGGAAATCTG TGAC3′) and Ep1R (5′GCAGGGTATTGGACTGCTTC 3′), and Ep2L (5′CCAAAAAGCTTGGTGTGGAT3′) and Ep2R (5′ CATGTGCGAGTCACCAATTT3′), with expected amplification products of 197 and 182 bp, respectively. PCR was carried out with both primer sets with an initial concentration of 2.5 mM $MgCl_2$. While primer pair Ep 2L/2R gave one single fragment of approximately 200 bp for all tested guava strains, the other pair, Ep 1R/1L, under the same reaction conditions, did not amplify the DNA of some of the strains, for which only smearing could be seen on the gels after several attempts. Reduction of the magnesium concentration to 1.25 mM led to the amplification of all tested strains of *E. psidii* for both primer sets but, still, with primer pair Ep 1L/1R, nonspecific products were obtained with some of the

strains. Therefore, a third primer combination, Ep 2L/1R, was tested for specificity.

Primer specificity and sensitivity

The two primers sets (Ep 2L/2R and Ep 2L/1R) gave positive and single signals with all *E. psidii* strains tested. These two primers sets did not amplify DNA from 20 phytopathogenic bacteria, resident bacteria from guava leaves or stems, guava leaves, and from the fungal pathogen *C. gloeosporioides* (Fig. 1). Using the Primer Blast tool, primer pair Ep 2L/2R was reported as specific to the input template (*E. psidii* AY 217065) as other targets containing no mismatches were not found in the selected NCBI database (limited to Bacteria). Regarding sensitivity thresholds, Ep 2L/2R was 10-fold more sensitive than Ep 2L/1R with both purified DNA and bacterial cell suspension (Table 3).

Detecting *Erwinia psidii* by PCR, IC-PCR and BIO-PCR

Conventional PCR

Amplification with primer pair Ep 2L/2R was positive and reproducible only when employing the extraction buffer with anti-oxidants (sodium sulfite, sodium azide, polyvinylpyrrolidone and albumin) to dilute (1:10) the sediment from guava leaf washes. After centrifugation, the sediment was suspended in 250 μL of cold extraction buffer and diluted 1:10 with the same buffer. At all concentrations, amplification from undiluted extracts gave negative or inconsistent signals. Using this procedure, it was possible to amplify *E. psidii* from guava leaf washes from initial concentrations of 10^6 to 10^3 CFU/mL (Fig. 2). Below 10^3 CFU/mL, the signals were of low intensity and inconsistent between replicates.

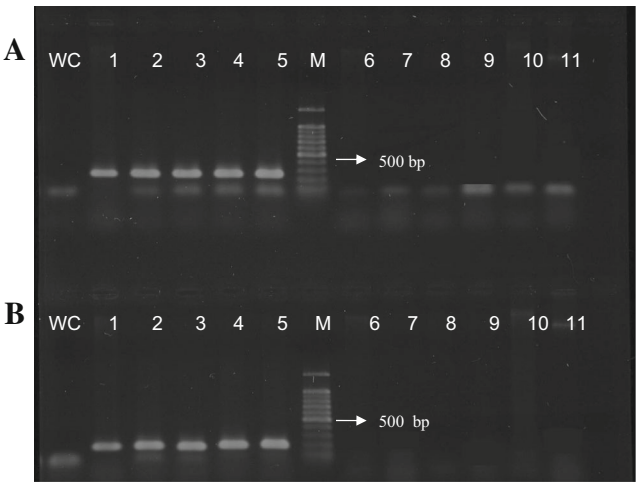

Fig. 1 Specificity of primers for *Erwnia psidii*: PCR products with primer pair Ep 2L/1R (**a**) and Ep 2L/2R (**b**) on agarose gel (1 %). *WC*—negative water control, *1–5*: *Erwinia psidii* strains: *1*-IBSBF 435, *2*-IBSBF 1579, *3*-IBSBF 1575, *4*-Emb. B81.3, *5*-Emb. C338.2, *M*—100 bp-DNA ladder (Promega), *6–7*: bacteria isolated from guava leaves, *8*—guava DNA extracted from young leaves, *9*—*Colletotrichum gloeosporioides, 10*—*Pectobacterium carotovorum*, *11*—*Xanthomonas axonopodis* pv. *vitians*

Table 3 Sensitivity of primer sets Ep 2L/2R and Ep 2L/1R tested with purified DNA and bacterial cell suspensions of *Erwinia psidii* type strain IBSBF 435

Concentration								
Purified DNA (ng/μL)								
Primers	10	1	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Ep 2L/2R	+	+	+	+	+	+	+	−
Ep 2L/1R	+	+	+	+	+	+	−	−
Suspension (CFU/mL)								
Primers	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10
Ep 2L/2R	+	+	+	+	+	+	+	+
Ep 2L/1R	+	+	+	+	+	+	+	−

(+) PCR products detected; (−) PCR products not detected on ethidium-bromide stained agarose gels

IC-PCR

Imunocapture-PCR with the antibody As 14.1 and primer set Ep 2L/2R gave positive amplifications up to 10^4 CFU/mL concentration of bacterial cells added to macerated leaf extracts. With this starting concentration the amplified fragment was visible on agarose gels, but it was of very low intensity. A cell suspension of strain IBSBF 435 at 10^6 CFU/mL, used as a positive control, gave a much stronger signal than the leaf extracts with a similar cell concentration (Fig. 3).

BIO-PCR

There were no differences in the amplification results, employing 24 or 48 h of enrichment of the bacterial population on 523 culture medium, indicating that the BIO-PCR detection protocol for *E. psidii* may be performed successfully in 24 h, thus reducing the time for disease diagnosis. PCR amplification was positive at all concentrations tested, from 10^7 to 10 CFU/mL (Fig. 4).

Discussion

This study describes the selection of PCR primers, specific to *E. psidii,* and their use in three PCR formats. The primers were then evaluated for their sensitivity in detecting the pathogen on guava leaves.

Erwinia psidii is a relatively poorly studied plant pathogen, due to its restricted geographic distribution and narrow host

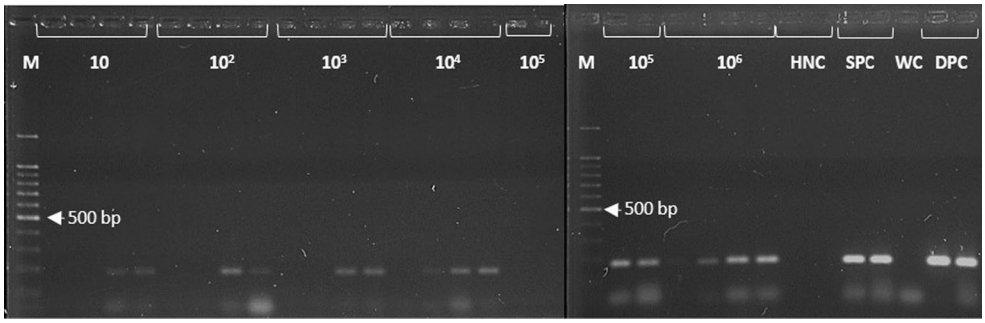

Fig. 2 Conventional PCR products with *Erwinia psidii* primers Ep 2L/2R separated by agarose gel electrophoresis, obtained from increasing concentrations of bacterial suspensions (CFU/mL) spiked on healthy guava leaves. For each concentration, samples in duplicates of undiluted (left) and diluted 1:10 (right) extracts from macerated leaves were used in the reactions. HNC: healthy guava leaf negative control; SPC: guava leaf spiked with cell suspension of *E. psidii* IBSBF 435 at 10^7 CFU/mL; WC: PCR water negative control, and DPC: purified DNA of *E. psidii* IBSBF 435 as PCR positive control

range, limited to species in the Myrtaceae family (Rodrigues Neto et al. 1987; Coutinho et al. 2011; Arriel et al. 2013). A comparison of genomic profiles obtained by rep-PCR among 42 strains, originating from four states in Brazil and collected over a number of years, from 1982 to 2005, revealed homogeneity among strains (Teixeira et al. 2009). Strains from different origins shared identical rep-PCR profiles. This analysis also generated a number of potential target DNA fragments, present in all strains, which could be used for the development of an identification tool. However, the primers derived from an ERIC-amplified sequence generated several nonspecific bands, in addition to the target fragment. Therefore, we searched for an alternative strategy, based on coding sequences of the *rec*A gene, which has been previously shown to contain enough polymorphism to differentiate *E. psidii* from its close relatives *E. mallotivora* and *E. tracheiphila* (Waleron et al. 2002; Arriel et al. 2013).

In this study, two combinations of primers were selected (Ep 2L/2R and Ep 2L/1R) to amplify a fragment of the *rec*A gene. Positive signals were detected with all *E. psidii* strains and the potential of false positives due to amplification of DNA from other bacteria was checked with other plant pathogenic bacteria and bacteria found in the same host. The combination of nucleotide polymorphism in the *rec*A sequences with higher annealing temperature allowed the desired specificity. *RecA* gene sequences have also been used for primer design for the identification of *Pectobacterium carotovorum* subsp. *brasiliensis* (El Tassa and Duarte 2006), however, for this pathogen, even under different annealing temperatures, the desirable specificity was not achieved.

Comparisons between the detection limits of the two primer sets showed a 10-fold increase in the sensitivity of primers Ep 2L/2R with both purified DNA and bacterial cell suspension. Positive amplification with Ep2L/2R was achieved even at the lowest concentration tested (10 CFU/mL). A comparable PCR detection limit for *E. amylovora* cells in water and in plant material was observed (Stoger et al. 2006).

The primers were combined with a polyclonal antibody in an IC-PCR format and were tested with different

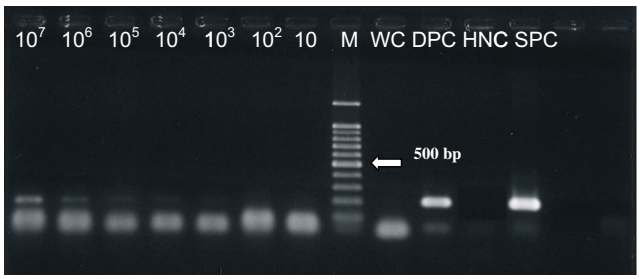

Fig. 3 Immuno-capture-PCR (IC-PCR) products obtained from cell suspensions (10^7 to 10 CFU/mL) of *Erwinia psidii* IBSBF 435 with primer pair Ep2L/2R separated by agarose gel electrophoresis. M—100 bp-DNA Ladder (Promega). WC—PCR water negative control; DPC—positive DNA control from strain IBSBF 435; HNC—healthy guava leaves negative control; SPC—cell suspension of strain IBSBF 435 at 10^6 CFU/mL as a PCR positive control

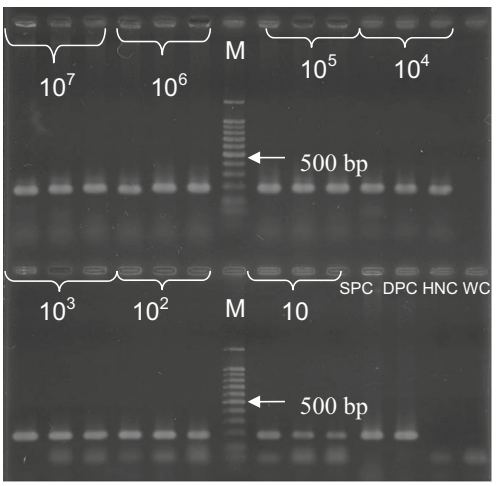

Fig. 4 BIO-PCR products with primer pair Ep2L/2R obtained from guava leaf extracts inoculated with cell suspensions (10^7 to 10 CFU/mL) of *Erwinia psidii* IBSBF 435 and separated by agarose gel electrophoresis after 24 h enrichment, three replicates per concentration. M-100 bp-DNA Ladder (Promega); reactions with: SPC, bacterial suspension of strain IBSBF 435 at 10^6 CFU/mL; DPC: purified DNA of strain IBSBF 435; HCN: healthy guava leaves negative control and WC: PCR negative water control

concentrations of bacterial cells mixed with macerated guava leaves. IC-PCR (Wetzel et al. 1992) uses a pathogen-specific antibody to concentrate target cells before PCR. This simple step confers higher sensitivity for bacterial detection, in some cases estimated as 100-fold higher than that of direct PCR (Xiao et al. 2007), and more sensitive than conventional PCR or indirect ELISA (Khoodoo et al. 2005). However, in our tests, a positive signal was observed only with a starting concentration of 10^4 cells/mL or higher. Of the three methods used for specific detection of *E. psidii* on inoculated guava leaves, this was the least sensitive in relation to BIO-PCR and conventional PCR.

When detecting bacteria directly from plant extracts, false negatives are often reported as a result of contamination of DNA samples with inhibitors such as phenolic compounds (Khoodoo et al. 2005). Guava leaves are also rich in phenolics (Marinho et al. 2008) that interfere with DNA extraction and amplification. Our preliminary results from direct amplification using crude extracts from macerated guava leaves in water were not successful. Several dilutions of the crude extract did not improve the results. The problem could be solved with the use of antioxidants in the extraction buffer followed by ten-fold dilutions of the extracts. BIO-PCR also improves sensitivity for bacterial detection and avoids the inhibitors present in various biological samples (Schaad et al. 1995). This method, which consists of promoting bacterial multiplication on culture medium before PCR, has been employed for the detection of pathogenic bacteria in plant tissue (Vanneste et al. 2008), seeds (Song et al. 2004), soil, weeds and water (Lin et al. 2009) and can be applied for detecting slow-growing bacteria in the presence of inhibitors (Fatmi et al. 2005). In this study, BIO-PCR showed the lowest detection limit (10 CFU/mL corresponding to 20 CFU/ g of leaf tissue). For *E. amylovora* the BIO-PCR protocol was capable of detecting 10^2 CFU of the pathogen per apple bud (Vanneste et al. 2008). With BIO-PCR only viable cells are detected, thus generating fewer false positives. On the other hand, the main constraint is the larger number of steps in the protocol and the time required for bacterial growth on culture.

In conclusion, our results demonstrated the use of BIO-PCR as a tool for detecting *E. psidii* that could potentially be applied to its detection in asymptomatic guava plants, since it has enough sensitivity for detecting small populations of the pathogen. For guava bacteriosis, a good strategy would be the use of BIO-PCR to test asymptomatic material, whereas conventional PCR would probably meet the requirements of sensitivity for symptomatic plants. The molecular detection methods described here, such as conventional PCR or BIO-PCR, and immunological methods described by Teixeira et al. (2008), could be integrated in a reliable and specific protocol for certification of guava planting material. This would assuredly contribute to the reduction of disease dissemination into new areas in Brazil. The occurrence of *E. psidii* in *Eucalyptus* is relatively recent, and since there is apparently no host specificity among the strains from both plants (Arriel et al. 2013), it would be interesting to test this method with strains from *Eucalyptus*.

Acknowledgments C.F. Silva was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazil. Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) provided scholarships to N. Pontes and P. Torres (PIBIC). We are grateful to Dr. Júlio Rodrigues Neto, IBSBF, Campinas, São Paulo, for kindly providing the strains, and José Cezar Castro, Universidade de Brasília, for technical assistance. This study was funded by Fundação de Amparo à Pesquisa do Distrito Federal (FAP-DF), grant 193.000.254/2007.

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