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ORIGINAL ARTICLE

Detection of *Xanthomonas citri* pv. *viticola* on grapevine by real-time PCR and BIO-PCR using primers designed from pathogenicity and xanthomonadin gene sequences

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Abstract Grapevine bacterial canker caused by *Xanthomonas citri* pv. *viticola* (*X. campestris* pv. *viticola*) (Xcv), was detected in Brazil in 1998 and is currently regarded as a quarantine disease with limited distribution in the country. To improve sensitivity and speed in the detection of Xcv in asymptomatic grapevines, two pairs of primers were designed, targeting sequences of a pathogenicity gene (*hrpB)* and the xanthomonadin coding cluster. Both pairs were tested in conventional PCR (cPCR) and real-time PCR (qPCR) formats. Primers targeting the *hrpB* gene showed cross reactions with other *Xanthomonas* spp. but were effective for use in both cPCR and qPCR, whereas primers for the xanthomonadin gene were highly specific for Xcv but showed low efficiency in qPCR. Enrichment of plant extracts in semi-selective medium before qPCR allowed a significant increase in sensitivity when compared to total DNA extraction, making it possible to detect as low as 10^1 CFU ml^{-1}. Under natural infection conditions, symptomatic and asymptomatic grapevines were tested by qPCR with *hrpB* primers and cPCR with xanthomonadin primers. In both cases, plant extracts were enriched for 36-72h. Xcv was detected in all symptomatic samples by qPCR and the result was confirmed by cPCR. For the asymptomatic samples, Xcv was detected in 93.4% with qPCR and in 89.5% with cPCR. These two methods offer advantages in terms of sensitivity and specificity, and they could be useful in quarantine programs, certification of grapevine propagating material and detection of inoculum sources in alternative hosts, contributing to the prevention of pathogen spread to disease-free areas.

Keywords Grapevine bacterial canker · *Vitis vinifera* · *Xanthomonas campestris* pv. *viticola.* PCR-based diagnosis · qPCR

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Introduction

Grapevine bacterial canker was detected in northeastern Brazil in 1998, affecting plants of the table grape variety Red Globe in the irrigated areas of the São Francisco river valley, (Malavolta Junior et al. 1999; Lima et al. 1999). The causal agent was identified as *Xanthomonas campestris* pv. *viticola* (Nayudu) Dye. Several studies

based on multilocus sequencing and whole genome analysis (Parkinson et al. 2009; Midha and Patil 2014; Ferreira et al. 2019) indicated that this pathovar belongs to the *X. citri* species, and a proposal for reclassification as *X. citri* pv. *viticola* (Xcv) has recently been suggested (Gama et al. 2018). Xcv is a regulated pathogen and subject to an official control program according to Brazilian legislation (MAPA 2014).

Early diagnosis of plant diseases is a crucial step for disease control and protection of agriculture. The diagnosis of bacterial canker is based on the evaluation of symptoms, isolation of the pathogen in culture medium followed by its identification using a series of tests that may include biochemical and nutritional tests, pathogenicity on grapevine, hypersensitivity reaction on tomato leaves, serology, rep-PCR, and/or species-specific PCR (Araújo et al. 2005; Trindade et al. 2005, 2007). However, these methods rely on culturing and have low sensitivity for early detection of Xcv in asymptomatic grapevine material due to low populations present in plant tissues (Trindade et al. 2007).

In recent decades, rapid, specific and sensitive DNA-based methods have been made available for the detection of pathogens in plant material (Vincelli and Tisserat 2008). PCR and its variations, such as BIO-PCR and real-time PCR (qPCR), have become in many cases the standard methods for the detection and identification of phytopathogenic bacteria (Palacio-Bielsa et al. 2009; Bull and Koike 2015; Loreti et al. 2018).

BIO-PCR (Schaad et al. 1995) consists of the biological amplification of the target organism followed by DNA amplification by PCR, which increases sensitivity when compared to conventional PCR (cPCR). However, the efficiency of this method depends on the specificity of the selective medium and primers. Some advantages associated with BIO-PCR are the elimination of PCR inhibitors and the detection of viable and culturable cells only. Its usefulness for detecting seed-borne pathogens has been demonstrated in a number of cases (Hassankiadeh et al. 2011; Kim et al. 2012; Singh et al. 2014).

Real-time PCR (qPCR) is a sensitive, robust and reproducible method for quantitative measurements (Schaad and Frederick 2002). qPCR has been widely used for the detection of phytopathogenic bacteria in various formats, including important *Xanthomonas* pathogens (Mavrodieva et al. 2004; Vandroemme et al. 2008; Palacio-Bielsa et al. 2011; Robène et al. 2015).

For the detection and identification of Xcv, Trindade et al. (2007) developed a PCR-based method using primers Xcv1F/3R and/or RST2/Xcv3R, which amplify a partial sequence of the *hrp* cluster (*hrpB6*/*hrcN* gene) that encodes the type III secretion system (TTSS), required for the pathogenicity of several plant pathogenic bacteria. These primers were tested with different sample types: (i) purified DNA, (ii) cell suspensions, (iii) macerated extracts from previously inoculated grapevine leaves, (iv) leaf or fruit washes followed by enrichment in liquid, or (v) solid culture medium. Detection of Xcv, however, was not possible when plant tissue extracts were used directly in the reactions, but amplifications were positive when an enrichment step was used prior to PCR, so called BIO-PCR (Trindade et al. 2007).

Efficient control of bacterial plant diseases requires a combination of methods including the use of healthy plant material and cultivation practices (Janse and Wenneker 2002). In Brazil, grapevine cultivation and winemaking are in full expansion and are spreading to other regions with no viticulture tradition, such as the southeastern and midwestern regions. Thus, the use of healthy and tested material is essential to avoid the introduction and dissemination of bacterial canker in new areas. Given the need for early detection of Xcv in asymptomatic plant material, the present study aimed to develop new protocols for rapid, specific and sensitive detection of Xcv, including its presence in latent form, applying both cPCR and qPCR.

Material and methods

Bacterial strains

The bacterial strains used in the study are listed in Table 1. Xcv strains were previously identified by PCR using the Xcv1F/3R primers (Trindade et al. 2007). All strains were preserved in sterile distilled water at room temperature and in 30% glycerol at -20 °C and grown in semi-selective NYDAM (Peixoto et al. 2006) composed of 3 g of beef extract, 5 g of peptone, 10 g of glucose, 5 g of yeast extract, 18 g of agar, 0.1 g of ampicillin and distilled water to 1 liter, or in 523 medium (Kado and Heskett 1970) at 28 °C for 2-3 days. In all PCR assays, DNA or cell suspension from Xcv strain UnB 1188 (SISGEN A99F2F2) was used as a positive control. For the detection assays of Xcv in

Table 1 Bacterial strains used in this study

Strains	Host	Origin*	Collection year
Xanthomonas citri pv. *viticola*			
NCPPB 2475 (LMG 965)	*Vitis vinifera*	India	1972
A2, A3, A11, A12	*Vitis vinifera*	Petrolina - PE	2010
AR1, AR2	*Vitis vinifera*	Petrolina - PE	2012
AM 1, AM 2, AM 3	*Amaranthus* sp.	Petrolina - PE	2012
P1S5, P1S6 (CFBP 7764), P1S9, P1S16,	*Vitis vinifera*	Petrolina - PE	2012
P2S1, P2S2, P2S4, P2S6	*Vitis vinifera*	Petrolina - PE	2012
RS 2, RS 8, RS 10, RS 11	*Vitis vinifera*	Curaçá, BA	2012
TR 1, TR 3	*Vitis vinifera*	Petrolina - PE	2012
UnB 1183, UnB 1184, UnB 1188, UnB 1189, UnB 1190, UnB 1192, UnB 1193, UnB 1194	*Vitis vinifera*	Petrolina - PE	1998
UnB 1205	*Vitis vinifera*	Sobradinho - BA	2000
UnB 1307, UnB 1309, UnB 1313, UnB 1315	*Vitis vinifera*	Petrolina - PE	2005
UnB 1318	*Vitis vinifera*	na**	2006
UnB 1429	*Vitis vinifera*	Petrolina - PE	2016
Xcv 2	*Vitis vinifera*	Lagoa Grande - PE	2008
Agrobacterium tumefaciens UnB 1138	*Daucus carota*	na	1996
Acidovorax citrulli UnB 1232	*Cucumis melo*	Petrolina - PE	2000
Burkholderia cepacia UnB 1134	*Allium sativum*	na	1996
Clavibacter michiganensis subsp. *michiganensis* UnB 1391	*Solanum lycopersicum*	Monte Alto - MG	2014
Curtobacterium flaccumfaciens pv. *flaccumfaciens* UnB 1376	*Phaseolus vulgaris*	Alto Paraíso - GO	2013
Dickeya chrysanthemi UnB 336	*Beta vulgaris* var. *cicla*	na	1983
Erwinia psidii IBSBF 435	*Psidium guajava*	Valinhos - SP	1982
Pectobacterium carotovorum subsp. c*arotovorum* UnB 1036	*Solanum lycopersicum*	Planaltina - DF	1992
Pseudomonas cichorii UnB 1387	*Gerbera* sp.	Brazlândia - DF	2013
Pseudomonas corrugata UnB 1142	*Solanum lycopersicum*	na	1996
Pseudomonas syringae pv. *tomato* UnB 853	*Solanum lycopersicum*	Vargem Bonita - DF	1991
Ralstonia solanacearum UnB 1173	*Solanum lycopersicum*	Planaltina - DF	1998
Xanthomonas axonopodis pv. *manihotis* UnB 1111	*Manihot esculenta*	Paranavaí - PR	1996
Xanthomonas axonopodis pv. *passiflorae* UnB 1395	*Passiflora edulis*	Vargem Bonita - DF	2016
Xanthomonas axonopodis pv. *phaseoli* UnB 187	*Phaseolus vulgaris*	Brasília - DF	1981
Xanthomonas axonopodis pv. *ricini* UnB 607	*Ricinus communis*	Ibiapina - CE	1985
Xanthomonas campestris pv. *campestris* UnB 1394	*Brassica oleracea*	Vargem Bonita - DF	2016
Xanthomonas campestris pv. *vitians* UnB 845	*Lactuca sativa*	Vargem Bonita - DF	1991
Xanthomonas citri pv. *citri* UnB 92	*Citrus* sp.	na	na
Xanthomonas citri pv. *fuscans* UnB 773	*Phaseolus vulgaris*	na	1990
Xanthomonas citri pv. *malvacearum* UnB 87	*Gossypium* sp.	Planaltina - DF	1980
Xanthomonas citri pv. *mangiferaeindicae* UnB 764	*Mangifera indica*	Planaltina - DF	1990
Xanthomonas cucurbitae UnB 1080	*Cucumis sativus*	Vargem Bonita- DF	1995
Bacteria associated to grapevine (non-xanthomonads)			
2 strains	*Vitis vinifera*	Brasília - DF	2016
29 strains	*Vitis vinifera*	Petrolina - PE	2016
10 strains	*Vitis* spp.	Petrolina - PE	2016

* PE, state of Pernambuco; BA, Bahia; MG, Minas Gerais; GO, Goiás; SP, São Paulo; DF, Federal District; PR, Paraná.

** information not available

grapevine, healthy detached leaves of 'Thompson Seedless' plants, kept in a greenhouse, were used.

DNA extraction and templates for PCR

PCR templates used in the detection assays were: (i) bacterial total genomic DNA, (ii) bacterial cell suspension, (iii) DNA from grapevine leaves, and (iv) DNA purified from grapevine leaf extract spiked with bacterial suspension. The concentration of the Xcv cell suspensions was determined by spectrophotometry (A550 = 0.575, using a digital spectrophotometer UV-1203 [Shimadzu Corporation, Japan]) corresponding to 10^8 CFU ml^{-1} and confirmed by colony counting after 48 h growth at 28° C on the semi-selective NYDAM medium. Genomic DNA was extracted from 48h-cultures, using the PureLink extraction kit (Invitrogen), according to the manufacturer's recommendations. The CTAB method was used for extracting DNA from grapevine leaves (Doyle and Doyle 1990). Purification of DNA from grapevine leaf extracts spiked with bacterial suspension was performed according to the protocol described by Llop et al. (1999). DNA integrity and purity were verified by 1% agarose gel electrophoresis and by the NanoVue spectrophotometer (GE Healthcare - Life Sciences). DNA was diluted to 10 ng μl^{-1} and stored at -20 °C until used.

Primer design

New primers were designed based on the partial sequences of the *hrpB6 (hrcN)* gene (Xcv primers) (Trindade et al. 2007) and the xanthomonadin biosynthetic pathway genes (Xpig primers) (Table S1). The lack of colony pigmentation in Xcv, different from most *Xanthomonas* spp., can be used as a phenotypic marker, and to genotypically differentiate Xcv from other yellow and white *Xanthomonas* species. For that, sequences of the xanthomonadin biosynthetic gene cluster from Xcv (CBZT010000000) and *Xanthomonas citri* pv. *mangiferaeindicae* (KF991092), were downloaded from GenBank and aligned using the MAFFT plugin (Katoh et al. 2002) in Geneious R8 (Kearse et al. 2012). The region selected for primer design was between an intergenic zone and the 3-oxoacyl-[acyl-carrier-protein] reductase, absent in *Xanthomonas citri* pv. *mangiferaeindicae* due to the presence of a transposon (Fig. S1). Furthermore, the reverse primer (Xpig 1R) was designed inside a polymorphic region in which *Xanthomonas citri* pv. *citri* differs from the pathotype strain of Xcv (LMG 965) as described by Midha and Patil (2014). Primers were designed with the Primer3 plugin (Rozen and Skaletsky 2000) also in Geneious R8 and checked for %GC, hairpin, self-dimer and pair dimer. The designed primers were tested *in silico* against the entire genome sequence of Xcv and *Xanthomonas citri* pv. *mangiferaeindicae*. Primers were synthesized by Invitrogen® (Life Technologies).

Conventional PCR (cPCR)

The primers designed for Xcv detection were initially tested in cPCR. Cell suspensions (10^7 CFU ml^{-1}) from seven Xcv strains were used. The negative control consisted of sterilized distilled water. The assays were repeated twice with three replicates per sample. Primers were selected following these criteria: amplification of the product of expected size, absence of nonspecific bands, reproducibility and positive amplification for all Xcv strains tested. The reactions were carried out in a thermal cycler (My Cycler, Bio-Rad) programmed to 2 min at 95°C, followed by 30 cycles of 30 s at 95 °C, 30 s at 64 °C (Xcv primers) or 68 °C (Xpig primers), and 15 s at 72 °C, followed by a final extension of 10 min at 72 °C. Reactions contained: 0.1 mM of each dNTP, 1.5 mM $MgCl_2$, 0.5 μM (Xcv primers) or 0.2 μM (Xpig primers) of each primer, 1X PCR buffer (20 mM Tris HCl pH 8.4, 50 mM KCl), 1 U of Taq polymerase (Invitrogen), 2 μl of DNA template, and sterile distilled water to a final volume of 12 μl. PCR products were analyzed by agarose (1.5%) gel electrophoresis in 0.5X TBE buffer (50 mM Tris, 1 mM Na_2EDTA, 50 mM boric acid [pH 8.3]), stained with ethidium bromide (0.5 μg ml^{-1}) and registered using the digital system L-PIX ST® (Loccus Biotechnology).

Real time PCR (qPCR)

The primer pairs selected in cPCR were tested in qPCR. The criteria for choosing the best combination were: reaction efficiency (100 ± 10%), sensitivity and absence of dimers and nonspecific products. Efficiency and sensitivity in real-time PCR were evaluated by generating standard curves by serial dilutions (1:10) of Xcv genomic DNA (1.5 ng μl^{-1} to 150 fg μl^{-1}). Ultra-Pure ™ DNase/RNase-Free (Invitrogen) distilled water was included as negative control. The assays were repeated twice and for each dilution eight replicates were

performed. The reactions were performed in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) thermocycler using the SYBR® Green system for fluorescence detection. Amplification reactions were prepared to a final volume of 15 μl containing 1 μl of DNA template, 1X Master Mix SYBR Green (Applied Biosystems) and 0.05 μM (Xcv primers) or 0.04 μM (Xpig primers). The cycling conditions were: 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 64 °C (Xcv primers) or 67 °C (Xpig primers) and a final extension of 1 min at 60 °C. To verify the absence of primer dimers or nonspecific products, a melting curve was generated for all reactions.

Primer specificity and sensitivity

Cell suspensions (10^7 CFU ml^{-1}) from 40 strains of Xcv, 11 strains belonging to different *Xanthomonas* species, 12 strains of other phytopathogenic bacteria, and 41 strains of epiphytic/endophytic bacteria associated with nine grape varieties (Table 1) were used to confirm primer specificity. To obtain bacteria associated with grapevine tissue, leaf fragments were macerated with a few droplets of water, followed by plating in 523 medium. Pure cultures of these strains were established and characterized phenotypically, according to standard protocols (Schaad et al. 2001). Purified DNA from grapevine leaves was also tested to prevent false positives.

The sensitivity of detection of Xcv by cPCR was determined using genomic DNA and bacterial cell suspensions. Serial dilutions (1:10) were performed to obtain concentrations ranging from 1.5 ng μl^{-1} to 150 fg μl^{-1} for genomic DNA and 10^8 CFU ml^{-1} to 1 CFU ml^{-1} for bacterial suspensions. The assays were repeated twice, and for each dilution three reactions were performed. Negative and positive controls were sterile distilled water and purified genomic DNA from Xcv strain UnB 1188, respectively.

The efficiency and sensitivity of detection of Xcv by qPCR were determined by generating standard curves by serial dilutions (1:10) of genomic DNA (1.5 ng μl^{-1} to 150 fg μl^{-1}) and bacterial suspensions (10^8 to 1 CFU ml^{-1}). The slope of the generated standard curve was used to estimate the efficiency of the reaction. Negative and positive controls were Ultra-Pure ™ DNase/RNase-Free water (Invitrogen) and genomic DNA purified from Xcv strain UnB 1188, respectively. The assays were repeated twice with eight replicates for each dilution.

Detection limit of Xcv in plant tissue by total DNA extraction and PCR

To determine the detection limit of Xcv in plant material, we used total DNA extraction of grapevine leaf extracts spiked with bacterial suspensions at different concentrations. To prepare the extract, 1 g (fresh weight) of leaf tissue was macerated with 15 ml of sterilized distilled water. One hundred microliters of suspensions at concentrations from 10^8 to 10^1 CFU ml^{-1} were added to 900 μl of leaf extract. Thus, each sample contained decreasing amounts of cells, ranging from 10^7 to 1 CFU ml^{-1}. Bacterial suspensions at the same concentrations were tested as positive controls and the negative control consisted of healthy leaf extracts. DNA of the extracts was purified and used in cPCR and qPCR, as previously described. The assay was repeated twice, with four independent replicates for each inoculum concentration and two amplification reactions each, in total eight reactions per treatment. As positive and negative PCR controls, Xcv purified genomic DNA and sterilized distilled water were used.

Detection limit of Xcv in plant tissue after an enrichment step (BIO-PCR)

To determine the detection limit of Xcv in plant material employing an enrichment step, leaf extracts spiked with bacterial suspensions were prepared as described above. For each sample, 100 μl of the extract were streaked onto one NYDAM plate. Plates were incubated at 28 °C for 72 h. The same volume of bacterial suspensions (10^8 to 10^1 CFU ml^{-1}) was plated as positive controls, and the negative control consisted of healthy leaf extracts. After 72h, each plate was washed with 1 ml of sterile distilled water, and the resulting cell suspension was diluted 1:100 and used as templates for both cPCR and qPCR. The assay was repeated twice, each one with three replicates (plates) for each bacterial concentration and three amplification reactions for each replicate, in total nine reactions per treatment (bacterial concentration). Positive and negative PCR controls were used as described in the previous assay.

qPCR and cPCR for Xcv detection in bacterial canker-infected grapevines

Leaves, stems, or petioles of 17 grapevine varieties and leaves of *Amaranthus* sp. were collected in Petrolina,

Table 2 Specificity of primers designed from the pathogenicity gene *hrpB* and the xanthomonadin cluster of *Xanthomonas citri* pv. *viticola*

Strains	Xcv1F/13R	Xcv8F/9R	Xcv10F/3R	Xcv16F/17R	Xcv18F/19R	Xcv20F/21R	Xpig1F/1R	Xpig2F/1R
Xanthomonas citri pv. *viticola*[a]	+[b]	+	+	+	+	+	+	+
Xanthomonas axonopodis pv. *manihotis* UnB 1111	-[c]	-	-	-	-	-	-	-
Xanthomonas axonopodis pv. *passiflorae* UnB 1395	-	-	-	-	+	-	-	-
Xanthomonas axonopodis pv. *phaseoli* UnB 187	-	-	-	-	+	-	-	-
Xanthomonas axonopodis pv. *ricini* UnB 607	-	+	+	-	+	+	-	-
Xanthomonas campestris pv. *campestris* UnB 1394	+	-	+	-	-	-	-	-
Xanthomonas campestris pv. *vitians* UnB 845	-	-	-	-	-	-	-	-
Xanthomonas citri pv. *citri* UnB 92	+	+	+	+	+	+	-	-
Xanthomonas citri pv. *fuscans* UnB 773	+	-	+	-	+	+	-	-
Xanthomonas citri pv. *malvacearum* UnB 87	-	-	-	-	-	-	-	-
Xanthomonas citri pv. *mangiferaeindicae* UnB 764	+	+	+	+	+	+	-	-
Xanthomonas cucurbitae UnB 1080	-		-	-	-	-	-	-
Agrobacterium tumefaciens UnB 1138	-	-	-	-	-	-	-	-
Acidovorax citrulli UnB 1232	-	-	-	-	-	-	-	-
Burkholderia cepacia UnB 1134	-	-	-	-	-	-	-	-
Clavibacter michiganensis subsp. *michiganensis* UnB 1391	-	-	-	-	-	-	-	-
Curtobacterium flaccumfaciens pv. *flaccumfaciens* UnB 1376	-	-	-	-	-	-	-	-
Dickeya chrysanthemi UnB 336	-	-	-	-	-	-	-	-
Erwinia psidii IBSBF 435	-	-	-	-	-	-	-	-
Pectobacterium carotovorum subsp. *carotovorum* UnB 1036	-	-	-	-	-	-	-	-
Pseudomonas cichorii UnB 1387	-	-	-	-	-	-	-	-
Pseudomonas corrugata UnB 1142	-	-	-	-	-	-	-	-
Pseudomonas syringae pv. *tomato* UnB 853	-	-	-	-	-	-	-	-
Ralstonia solanacearum UnB 1173	-	-	-	-	-	-	-	-
Grapevine bacteria (non xanthomonads)	-	-	-	-	-	-	-	-

[a] All Xcv strains as in Table 1

[b] (+) positive amplification

[c] (-) no amplification

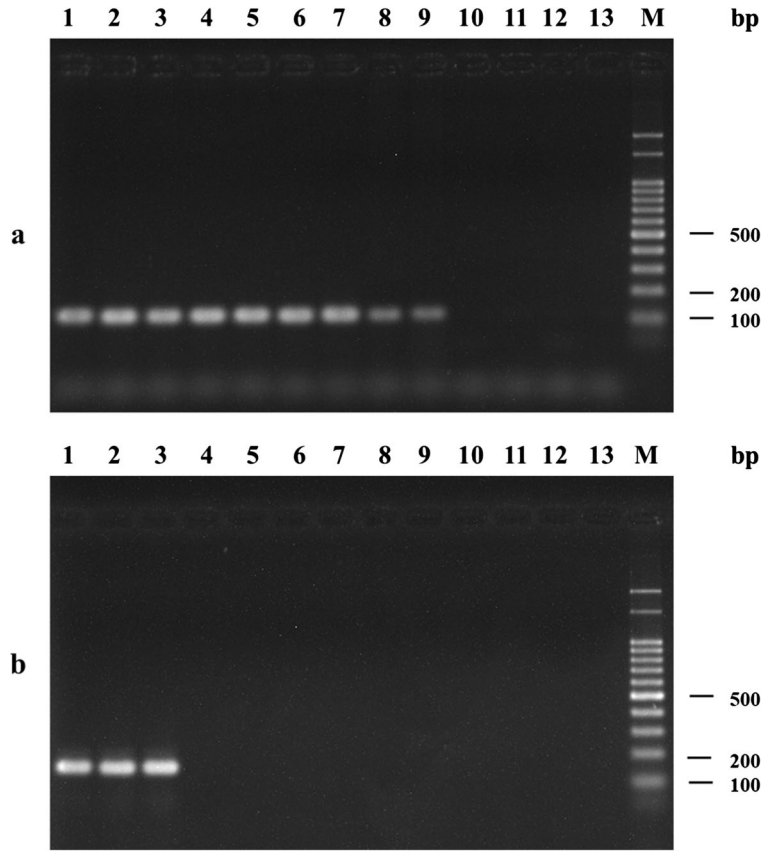


Fig. 1 Specificity of primer pairs (**a**) Xcv18F/19R and (**b**) Xpig2F/1R for detection and identification of *Xanthomonas citri* pv. *viticola*. Lanes 1: NCPPB 2475; 2: UnB 1188; 3: UnB 1205; 4: UnB 92; 5: UnB 764; 6: UnB 773; 7: UnB 607; 8: UnB 1395; 9: UnB 187; 10-11: endophytic/ epiphytic bacteria from grape leaves; 12: DNA from grapevine; 13: PCR water control; M: 100-bp ladder (Ludwig®) Lanes 1-3: *Xanthomonas citri* pv. *viticola*; 4: *X. citri* pv. *citri*; 5: *X. citri* pv. *mangiferaindicae*; 6. *X. citri* pv. *fuscans*; 7: *X. axonopodis* pv. *ricini*; 8: *X. axonopodis* pv. *passiflorae*; 9: *X. axonopodis* pv. *phaseoli*

state of Pernambuco, Brazil, in a table grape vineyard showing high incidence of bacterial canker. Seventy-five leaf samples were collected, 25 showing typical bacterial canker symptoms and 50 symptomless. In addition, 12 samples were collected from roots of Red Globe plants showing severe canker symptoms. Samples from different hybrids, one rootstock variety (IAC 313), and cv. Red Globe plants growing in a greenhouse were also tested. They were originated from a commercial nursery and were naturally infected. A total of 25 leaf/petiole samples were collected, 11 with symptoms and 14 with no visible symptoms. All samples were washed under tap water, dried on paper towels, and an extract was obtained after maceration (1 g) in 15 ml of sterile distilled water followed by plating (100 μl) onto NYDAM medium. Plates were incubated at 28 °C for 36 h (symptomatic samples) or 72 h (symptomless samples). After washing and dilution (1:100), aliquots from the plate washes were used for cPCR with primers Xpig2F/1R and qPCR with Xcv18F/19R. Each sample was analyzed in triplicates, and positive and negative controls were added as previously described.

Results

Primer design and selection

In silico searches of the new primer and amplicon sequences revealed no significant homology with the sequences available in the Genbank database. Based on previously established criteria for cPCR, six out of 10 pairs of primers targeting the *hrpB* gene (Xcv primers), and the two pairs targeting the xanthomonadin coding cluster (Xpig primers) were selected (Table S1). All selected primers were tested in qPCR. Based on the screening via qPCR, the pair Xcv18F/19R showed the best results. The reactions showed high efficiency, and the melting curves showed a single peak indicating the amplicon`s melting temperature (Tm), without interfering signals, indicating the amplification only of the desired amplicon. The Xpig primers showed low efficiency in qPCR, probably because of the longer amplicon (above 150 bp) compared to Xcv18F/19R (Table S1).

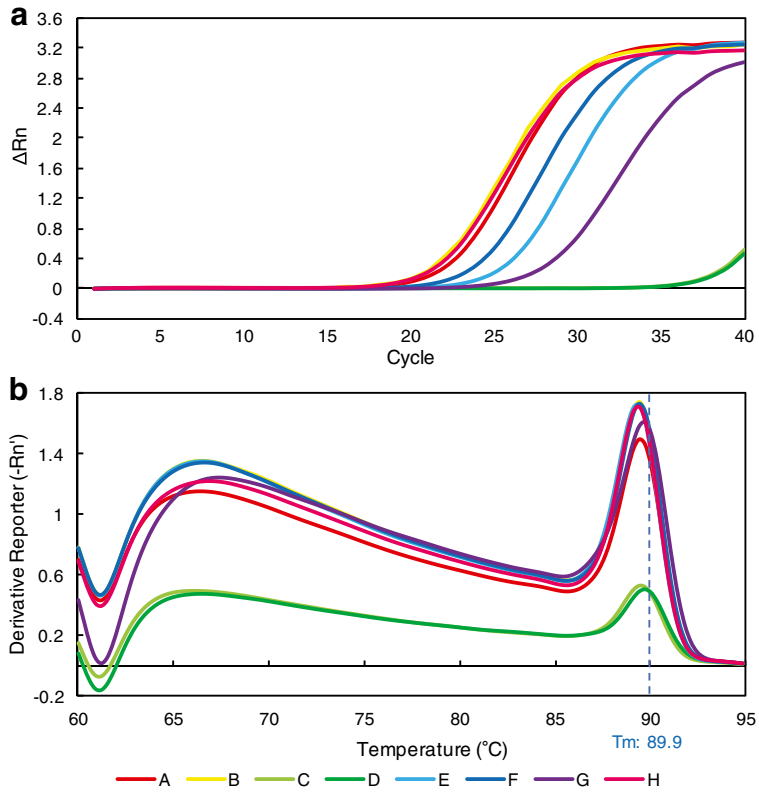


Fig. 2 Amplification (**a**) and melting (**b**) curves of real-time PCR with primers Xcv18R/19F and purified genomic DNA (5 ng μl^{-1}) from different species of *Xanthomonas*. Color legend: A - *X. citri* pv. *viticola* (UnB 1188); B - *X. citri* pv. *viticola* (NCPPB 2475); C - *X. axonopodis* pv. *passiflorae* (UnB 1395); D - *X. axonopodis* pv. *phaseoli* (UnB 187). E - *X. axonopodis* pv. *ricini* (UnB 607); F - *X. citri* pv. *magiferaindicae* (UnB 764); G - *X. citri* pv. *fuscans* (UnB 773); H - *X. citri* pv. *citri* (UnB 92)

Primer specificity

Specificity tests performed with cPCR showed that all selected primers amplified DNA from all 40 strains of Xcv, but not from healthy, non-contaminated grape leaves or from 41 strains of epiphytic/endophytic bacteria associated with those leaves. Xpig primers were highly specific for Xcv, since the amplification was negative with all tested strains of other phytopathogenic bacteria. However, reactions using the primers for the *hrpB* gene (Xcv primers) showed positive amplifications for some *Xanthomonas* strains (Table 2, Fig. 1). The six *Xanthomonas* strains (*X. axonopodis* pv. *passiflorae*, *X. citri* pv. *citri*, *X. citri* pv. *fuscans*, *X. citri* pv. *mangiferaeindicae*, *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *ricini*) with positive amplification using primers Xcv18F/19R, were also tested in qPCR, to verify any differences in Ct (cycle thresholds) and Tm in relation to Xcv. It was observed that the amplicons from these species all had very close Ct and Tm values. The exceptions were *X. axonopodis* pv. *passiflorae* (Ct = 35.09) and *X. axonopodis* pv. *phaseoli* (Ct = 36.06), which showed significantly higher Cts at the same DNA concentration (5 ng μl^{-1}) of Xcv (Ct = 18.40) (Fig. 2).

cPCR and qPCR: sensitivity

The sensitivity of Xcv detection by cPCR was assessed using the Xcv18F/19R and Xpig2F/1R primer pairs. The Xcv18F/19R pair was chosen because of its efficiency for both cPCR and qPCR (Table 3) and the Xpig2F/1R pair because of its high specificity for Xcv. The sensitivity with cPCR was the same for both primers: 15 pg μl^{-1} for purified DNA and 10^3 CFU ml^{-1} for bacterial suspension (Table 4). Sensitivity of qPCR with Xcv18F/19R primers was higher and the detection limits were 1.5 pg μl^{-1} for genomic DNA and 10^2 CFU ml^{-1} for cell suspension (Table 4). The regression and amplification curves obtained by plotting Ct values versus values of the initial amount of template in the reaction are shown in Fig. 3 and S2.

Detection limit of Xcv in plant tissue

The detection limit of Xcv in leaf extracts spiked with bacterial suspensions was evaluated by two methods, simple DNA extraction or extract enrichment in NYDAM medium (BIO-PCR), followed by cPCR with both sets of primers, and qPCR with the Xcv primers (18F/19R) only. The detection limit by the simple DNA extraction method was 10^3 CFU ml^{-1} in cPCR, with no difference between the primers used. In qPCR, the limit was 10^2 CFU ml^{-1} with an average Ct of 35.83 (Table 5). Using BIO-PCR the detection limit in cPCR was from an initial concentration of 10^2 CFU ml^{-1}, with no difference between the primers used. However, using the real-time format, the detection limit decreased 10-fold, with an initial concentration of only 10^1 CFU ml^{-1} (Ct = 34.42) (Table 5). Therefore, the combination of bacterial

Table 3 Efficiency of real-time PCR (qPCR) with primer pair Xcv18F/19R, evaluated from calibration curves obtained with genomic DNA (1.5 pg μl^{-1} to 150 fg μl^{-1}) and bacterial suspensions (10^8 to 1 CFU ml^{-1}) of *Xanthomonas citri* pv. *viticola* (Xcv)

Template	[a]E (%)	[b]R^2	[c]Slope	[d]Y = int
gDNA Xcv	99.16	0.996	3.342	25.827
gDNA Xcv	94.98	0.990	3.448	26.678
Cell suspension Xcv	92.87	0.995	3.506	44.310
Cell suspension Xcv	92.54	0.994	3.515	44.428

[a] E = PCR efficiency, 100% is the maximum theoretical value, which means perfect doubling of molecules at each cycle.

[b] R^2 is a measure of data linearity among technical replicates of the same and different serial dilutions; 1 is the best fit.

[c] The slope is the angular coefficient (a) of the equation for the standard curve (y=ax+b).

[d] Y = int represents the value of Ct where the curve crosses the y-axis.

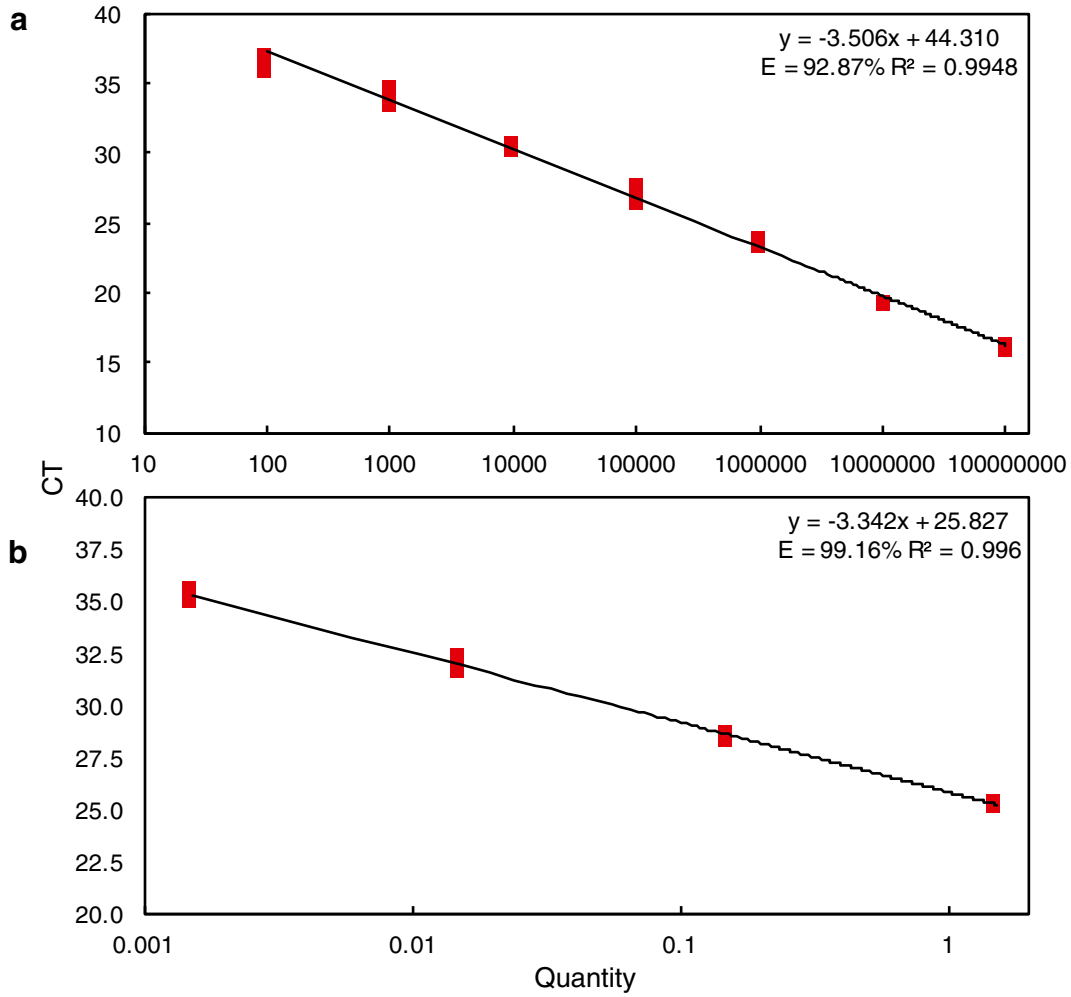
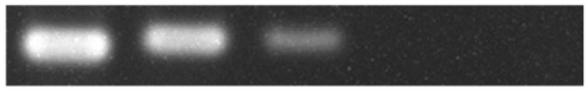
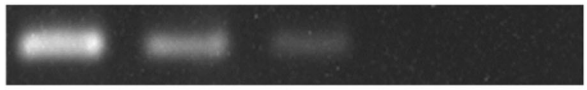
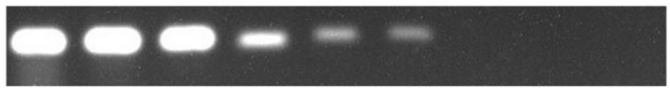
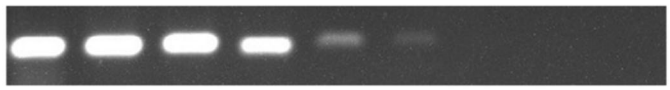


Fig. 3 Real-time PCR (SYBR® Green) standard curves with primers Xcv18F/19R for detection of (**a**) genomic DNA (1.5 ng μl^{-1} to 150 fg μl^{-1}) and (**b**) cell suspension (10^8 to 1 CFU ml^{-1}) from *Xanthomonas citri* pv. *viticola*

Table 4 Sensitivity of conventional PCR (cPCR) and real-time PCR (qPCR) for detecting genomic DNA or cell suspensions of *Xanthomonas citri* pv. *viticola*. For qPCR, Ct values are indicated (the numbers presented are the means of two independent experiments)

		DNA ([ ] μl^{-1})				
PCR	**Primers**	**1.5 ng**	**150 pg**	**15 pg**	**1.5 pg**	**150 fg**
qPCR	Xcv 18F/19R	25.63	29.06	32.54	35.56	-
cPCR	Xcv18F/19R					
	Xpig2F/1R					

		UFC ml^{-1}							
PCR	**Primers**	10^8	10^7	10^6	10^5	10^4	10^3	10^2	10^1	1
qPCR	Xcv 18F/19R	16.20	19.21	23.59	27.38	30.54	33.90	36.59	-	-
cPCR	Xcv18F/19R									
	Xpig2F/1R									

enrichment in culture media with qPCR would allow the highest sensitivity for pathogen detection. In that case, cycle threshold values ≤ 34.42 were considered as the cut-off for a true positive result.

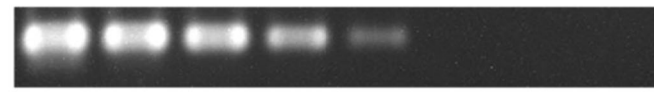
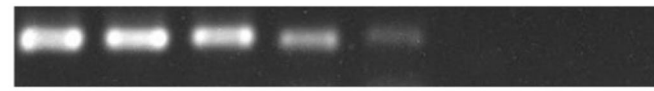
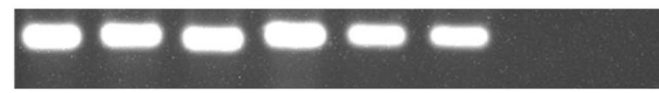
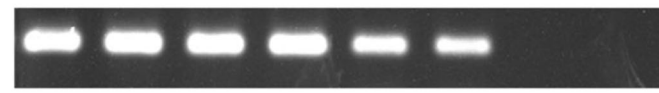
qPCR and cPCR for Xcv detection in a bacterial canker-infected area

Because of its higher sensitivity, the method that employed enrichment in culture medium was chosen for protocol validation in an area with natural occurrence of bacterial canker. In all 75 field samples (25 with symptoms and 50 with no visible symptoms), Xcv was detected by both cPCR and qPCR. For the 12 root samples, without visible symptoms, 75.0% were positive by qPCR (Ct = 28.10) and 50.0% by cPCR. From the total of 25 samples collected in the greenhouse, Xcv was detected in 100% of symptomatic and 85.7% (12/14) of asymptomatic samples. Xcv was not detected in the rootstock IAC 313 and in one of the hybrid accessions (Tables 6 and 7). Bacterial isolation on NYDAM was successful for all symptomatic samples confirming the results obtained by cPCR and qPCR.

Discussion

Grapevine bacterial canker (GBC) caused by *X. citri* pv. *viticola* is responsible for losses in Brazil and India, affecting mostly table grape production. The emergence of Xcv in northeastern Brazil in 1998 and later in other parts of the country is probably due to pathogen introduction and dispersion by propagative plant material. In São Paulo state, for example, Xcv detection in 2009 caused the eradication of approximately 4,700 plants in a table grape vineyard (Rodrigues Neto et al. 2011). Consequently, exclusion of Xcv-infected material is very important for preventing and managing GBC. For this

Table 5 Sensitivity of conventional PCR (cPCR) and real-time PCR (qPCR) for detecting *Xanthomonas citri* pv. *viticola* on leaf extracts spiked with bacterial cell suspensions, after total DNA extraction (top) or enrichment for 72 h on semi-selective medium (bottom). For qPCR, Ct values are indicated (the numbers presented are the means of two independent experiments)

		UFC ml^{-1}							
PCR	**Primers**	**10^7**	**10^6**	**10^5**	**10^4**	**10^3**	**10^2**	**10^1**	**1**
qPCR	Xcv 18F/19R	19.45	22.46	25.86	29.55	32.96	35.83	-	-
cPCR	Xcv18F/19R								
	Xpig2F/1R								
qPCR	Xcv 18F/19R	17.00	17.70	20.12	20.42	21.14	22.64	34.42	-
cPCR	Xcv18F/19R								
	Xpig2F/1R								

purpose, the development of effective detection methods that combine high specificity and sensitivity is required.

A PCR-based method for detection of Xcv in symptomatic and asymptomatic plants was already developed

Table 6 PCR detection of *Xanthomonas citri* pv. *viticola* in symptomatic grapevine and *Amaranthus* sp. leaves in a bacterial canker-infected area in Brazil

Grapevine variety/ other hosts	**Site**	**qPCR* (Ct**)**	**cPCR*****
Benitaka	Field	1/1 (20.39)	1/1
BRS Isis	Field	1/1 (18.75)	1/1
BRS Linda	Field	5/5 (21.22)	5/5
BRS Núbia	Field	2/2 (18.98)	2/2
BRS Vitória	Field	2/2 (19.74)	2/2
Crimson	Field	2/2 (19.41)	2/2
Italia	Field	1/1 (19.20)	1/1
Niagara Rosada	Field	1/1 (18.87)	1/1
Red Globe	Field	6/6 (20.32)	6/6
Sugraone	Field	1/1 (20.94)	1/1
Thompson Seedless	Field	3/3 (22.10)	3/3
Vitis spp hybrids	greenhouse	10/10 (22.57)	10/10
Red Globe	greenhouse	1/1 (19.70)	1/1
Total		**36/36**	**36/36**

* number of positive samples by real-time PCR/ total number of tested samples

** Ct (cycle threshold): means of three replicates for each positive sample

*** number of positive samples by conventional PCR/ total number of tested samples

Table 7 PCR detection of *Xanthomonas citri* pv. *viticola* in asymptomatic grapevines and *Amaranthus* sp. in a bacterial canker-infected area in Brazil

Grapevine variety/ other hosts	Site	Sample	qPCR* (Ct **)	cPCR***
Benitaka	Field	Leaf	2/2 (27.84)	2/2
Brasil	Field	Leaf	2/2 (26.05)	2/2
BRS Cora	Field	Leaf	4/4 (22.60)	4/4
BRS Isis	Field	Leaf	4/4 (23.19)	4/4
BRS Linda	Field	Leaf	2/2 (24.81)	2/2
BRS Magna	Field	Leaf	7/7 (22.47)	7/7
BRS Núbia	Field	Leaf	2/2 (22.16)	2/2
BRS Vitória	Field	Leaf	1/1 (25.70)	1/1
Crimson	Field	Leaf	1/1 (17.51)	1/1
IAC 572	Field	Leaf	2/2 (22.50)	2/2
Isabel	Field	Leaf	5/5 (28.52)	5/5
Isabel Muscat	Field	Leaf	3/3 (23.32)	3/3
Italia	Field	Leaf	2/2 (26.38)	2/2
Niagara Rosada	Field	Leaf	4/4 (26.14)	4/4
Red Globe	Field	Leaf	1/1 (19.83)	1/1
Sugraone	Field	Leaf	3/3 (20.26)	3/3
Thompson Seedless	Field	Leaf	3/3 (24.09)	3/3
Red Globe	Field	Roots	9/12 (28.10)	6/12
Amaranthus sp.	Field	Leaf	2/2 (26.54)	2/2
Vitis spp hybrids	greenhouse	Leaf	12/13 (21.16)	12/13
IAC 313	greenhouse	Leaf	0/1 (nd)****	0/1
Total			**71/76**	**68/76**

* number of positive samples by real-time PCR/ total number of tested samples

** Ct (cycle threshold): means of positive samples, each with three replicates

*** number of positive samples by conventional PCR/ total number of tested samples

****not detected

but showed limitations in specificity and sensitivity (Trindade et al. 2007) and suitability for qPCR. Preliminary tests (data not shown) with the SYBR® Green detection system showed low efficiency and dimer formation when using these primers (Xcv1F/3R). Therefore, in this study, new primers were designed targeting new sites in the same pathogenicity gene sequence for use in real-time PCR. Like the previous set of primers, the new primers failed to show improved specificity. Despite being useful to differentiate species of *Xanthomonas* (Leite Junior et al. 1994b; Roberts et al. 1996), *hrpB6* encodes an ATPase (HrcN), involved in the assembly of the TTSS apparatus (Dunger et al. 2005), which is a conserved feature in *Xanthomonas* (Leite Junior et al. 1994a). Specificity tests with the new primers designed for the *hrpB* gene showed that, in addition to Xcv, DNA from non-target *Xanthomonas* species and pathovars was also amplified. Trindade et al. (2007) also observed with Xcv 1F/3R primers DNA amplification of four strains of *X. citri* pv. *mangiferaindicae* and five strains of *X. axonopodis* pv. *passiflorae*. Since there are no reports of the natural occurrence of these pathovars affecting *Vitis vinifera* and considering that, in general, *Xanthomonas* species have a restricted host range, it is very unlikely that these results would limit their use for Xcv detection in grapevines and diagnosis of GBC. Primer pair Xcv18F/19R, although lacking high specificity towards Xcv, showed the highest efficiency and sensitivity when used in real time PCR compared to the other primer sets (data not shown). Furthermore, no amplification was detected with 41 bacterial strains from grapevine natural microbiota isolated from three different environments and varieties

and neither with grapevine plant cell extract (Tables 1 and 2).

Searching for a more specific target, we used a genome-based approach to select additional annealing sites. Xcv is a non-pigmented xanthomonad and, to our knowledge, all strains collected in Brazil produce creamy-white colonies. Thus, primers targeting that feature would eliminate or minimize the risk of false negatives in diagnostic procedures.

Synthesis of the pigment xanthomonadin depends on the expression of a gene cluster. The whole genome sequencing of the pathotype strain LMG 965 (Midha and Patil 2014) showed some special features in that cluster responsible for the lack of pigmentation. Midha and Patil (2014) showed the occurrence of a four-nucleotide deletion in the gene encoding the phosphotransferase/dehydratase enzyme in Xcv and an insertion element (IS) in pv. *mangiferaindicae*, which contains white strains as well. The Xpig primers were designed based on these differences, and our assays confirmed their specificity both *in silico* and *in planta*.

Preliminary tests using leaf extracts enriched with Xcv suspensions directly in the PCR tubes were not successful, possibly because of the presence of inhibitory compounds. These compounds, when present, affect PCR, via a direct effect on the polymerase or binding to the DNA (Palacio-Bielsa et al. 2009). Trindade et al. (2007) also observed that it was not possible to detect Xcv directly in infected plant extracts. BIO-PCR was used to overcome this problem. In addition, when compared to nested-PCR, BIO-PCR with Xcv1F/3R primers was more sensitive for detecting Xcv in asymptomatic plants (unpublished results). Here, we combined BIO-PCR with the higher sensitivity of real-time PCR and more specific primers. Using total DNA extraction of samples artificially infected with Xcv did not impair sensitivity when compared with suspensions from pure cultures. The detection limits were the same, viz. 10^3 (cPCR) and 10^2 CFU ml^{-1} (qPCR). For other *Xanthomonas* spp., pathogenic on cassava, it was shown that a DNA purification step aided the diagnosis by concentrating DNA and eliminating PCR inhibitors, thus decreasing the detection limit (Flores et al. 2019). In our hands the highest sensitivity was obtained by combining bacterial population enrichment and real-time PCR. The total DNA extraction step, which is less time-consuming, may be more suitable for detecting Xcv in symptomatic material. In this case, high sensitivity would not be required because of the large bacterial population inside plant tissues. The choice of which method to use would greatly depend on lab resources, the number of samples to be tested and whether symptoms are evident or not. A duplex PCR with both sets of primers is also feasible, since both primers have the same sensitivity in cPCR. The two amplicons differ by 48 bp, so it would simply require gels with agarose concentration above 1.5% to separate and visualize both fragments by electrophoresis.

The detection limits of Xcv in extracts of leaves spiked with bacterial suspension were as low as 10^1 CFU ml^{-1} when combining extract enrichment on the semi-selective NYDAM medium and qPCR, which has proven suitable for asymptomatic plant material. This is comparable to other real-time PCR protocols, such as the one employed for the detection of *X. arboricola* pv. *pruni* in plum (Ballard et al. 2011). Previously, using leaf washes followed by plating, it was not possible to detect Xcv in 100% of the replicates at concentrations below 10^5 CFU ml^{-1} (unpublished results). With our newly developed real-time PCR method, Xcv was detected in all replicates, even at lower concentrations, when leaves were spiked.

The protocol was validated with field samples and both PCR formats, since the qPCR technique may not be accessible to many laboratories in Brazil. The results showed that our protocol can be used for the detection of Xcv in symptomatic and asymptomatic-grapevine samples and asymptomatic samples of alternative hosts. The presence of Xcv was detected by qPCR in 100% of symptomatic samples and 93.4% of the asymptomatic ones. For leaf samples, there was no difference between the techniques used for detection; that is, the positive samples by qPCR were also positive by cPCR. However, for the root samples, qPCR was more efficient, detecting the bacteria in all samples. Xcv colonizes grapevine plants systemically and could be detected in aerial plant parts, grape berries and seeds using light and scanning electron microscopy along with immunogold labeling, and a specific antibody (Tostes et al. 2014). In rootstocks Xcv has already been detected by isolation. Here we report its presence in the roots of a severely infected Red Globe vine. cPCR with Xpig primers followed by agarose gel electrophoresis confirmed the results in all leaf samples; however, for some samples with Ct above 27.0, DNA bands on agarose gels were less intense.

The methods developed in this study proved to be useful for Xcv detection in asymptomatic samples and they could be valuable tools for the detection and identification of Xcv in different situations, both for research purposes and in quarantine routines, although they may not be suitable for large-scale monitoring programs where low cost, rapidity and portability are requirements (Chiriacò et al. 2018). The choice of method would depend on laboratory resources, and whether plant material shows symptoms or not. A suggested sequence for screening a large number of samples would be performing cPCR with the specific Xpig primers (low false positive rate), followed by confirmation of the negative samples with the more sensitive (low risk of false negatives), but costly and not specific qPCR. Isolation of the pathogen with further identification, including a pathogenicity test, should also be included in critical cases such as quarantine and trade issues.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed consent All authors have reviewed the manuscript and approved its submission to European Journal of Plant Pathology.

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