Assessment of *Xanthomonas arboricola* pv. *juglandis* Bacterial Load in Infected Walnut Fruits by Quantitative PCR

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Abstract

Xanthomonas arboricola pv. *juglandis* is the etiologic agent of important walnut (*Juglans regia* L.) diseases, causing severe fruit drop and high economic losses in walnut production regions. Rapid diagnostics and knowledge of bacterial virulence fitness are key to hinder disease progression and apply timely phytosanitary measures. This work describes an *X. arboricola* pv. *juglandis*-specific real-time quantitative PCR (qPCR) using *X. arboricola* pv. *juglandis*-specific DNA markers to quantify the bacterial load in infected walnut plant tissues. Method validation was achieved using calibration curves obtained with serial dilutions of *X. arboricola* pv. *juglandis* chromosomal DNA and standard curves obtained from walnut samples spiked with *X. arboricola* pv. *juglandis* cells. High correlations ($R^2 > 0.990$ and > 0.995) and low limits of detection (35 chromosomes/qPCR reaction and 2.7 CFU/qPCR reaction) were obtained for both markers

considering the calibration and standard curves, respectively. Assessment of qPCR repeatability, reproducibility, and specificity allowed us to demonstrate the reliability and consistency of the method. Furthermore, in planta quantification of *X. arboricola* pv. *juglandis* bacterial load using infected walnut fruit samples showed a higher detection resolution compared with standard PCR detection. By allowing quantification of virulence fitness of distinct *X. arboricola* pv. *juglandis* strains in planta, the proposed qPCR method may contribute to assertive risk assessment of walnut diseases caused by *X. arboricola* pv. *juglandis* and ultimately help to improve phytosanitary practices.

Keywords: DNA markers, qPCR, virulence, walnut diseases, *Xanthomonas arboricola* pv. *juglandis*

Xanthomonas arboricola pv. *juglandis* is the etiological agent of walnut bacterial blight, the most important bacterial disease of walnut (*Juglans regia* L.) (Frutos 2010). *X. arboricola* pv. *juglandis* has also been associated with brown apical necrosis (Moragrega et al. 2011) and vertical oozing canker diseases (Hajri et al. 2010), altogether causing major economic losses attributable to its worldwide distribution and persistence (Frutos and López 2012; Lamichhane 2014).

In the last decade, several studies emphasized the genetic diversity of *X. arboricola* pv. *juglandis* populations, contributing to improve our knowledge on the epidemic behavior of this pathogen (Cesbron et al. 2014; Fischer-Le Saux et al. 2015; Hajri et al. 2012; Ivanović et al. 2014). In addition, nonpathogenic *X. arboricola* lineages were isolated from symptomatic walnut trees. These genetically diverse

strains were shown to group separately from the well-defined clusters of pathogenic *X. arboricola* pv. *juglandis* strains (Cesbron et al. 2015; Essakhi et al. 2015), also supporting differences in virulence (Giovannardi et al. 2016). Considering the high genetic diversity of *X. arboricola* strains and the isolation of related nonpathogenic bacteria, there is a need to characterize walnut-colonizing xanthomonads, taking into account their pathogenicity and virulence, to make robust risk assessments and contribute to implementation of timely and scientifically based phytosanitary practices.

Pathogenicity and virulence are often inaccurately used as synonyms and are sometimes inconsistently used in the scientific literature, leading to debates regarding the most appropriate definitions of these terms (Bos and Parlevliet 1995; Casadevall and Pirofski 1999; Shapiro-Ilan et al. 2005; Thomas and Elkinton 2004). Despite these disputes, the thorough revision made by Shapiro-Ilan et al. (2005) provides a coherent understanding of these two concepts, defining pathogenicity as the ability to cause disease, whereas virulence refers to pathogen fitness or its aptitude to colonize the host. Thus, although pathogenicity is a qualitative trait, virulence refers to the pathogen's capabilities to thwart the host's defenses, ultimately leading to a disease phenotype. Aligned with these definitions, some authors define virulence as inversely proportional to the number of microorganisms required to trigger infection (Casadevall and Pirofski 2001; Shapiro-Ilan et al. 2005). These and other related concepts frequently used in phytopathology were revisited in a thoughtful pedagogic essay addressing the above definitions (Surico 2013). Whatever the semantic understanding of pathogenicity and virulence, the interest in quantifying the bacterial load in infected tissues in order to determine the pathogen fitness of a given infective strain is irrefutable. Currently, most of the studies to determine virulence of phytopathogenic bacteria are based on a gradient scale of symptoms (Bandi et al. 2015; Frutos and López 2012). Although this procedure may provide a semiquantitative measure of virulence and might distinguish a poorly virulent strain from a highly virulent strain, it is dependent on a visual or phytopathometrical evaluation of the symptoms and is not suitable to accurately quantify the bacterial load.

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Quantitative PCR (qPCR) is a frequently used method in environmental microbiology to measure target DNA from environmental samples, using a standard curve plotted from known concentrations of target DNA (Pérez et al. 2013). Despite the existence of numerous real-time PCR methods established for several phytopathogens, the quantification of plant pathogenic microorganisms in infected plant material or in environmental matrices such as soils still lacks adequate optimization to assist plant disease management practices (Alemu 2014; Lievens et al. 2006).

Real-time PCR protocols have been widely used to detect and identify several members of the *Xanthomonas* genus, namely *X. campestris* (Berg et al. 2006), *X. fragariae* (Vandroemme et al. 2008; Weller et al. 2007), *X. axonopodis* pv. *citri* (Golmohammadi et al. 2007), *X. oryzae* pv. *oryzae* (Cho et al. 2011), *X. arboricola* pv. *pruni*

(Palacio-Bielsa et al. 2011, 2015) *X. campestris* pv. *campestris* (Laala et al. 2015), and *X. axonopodis* pv. *allii* (Robène et al. 2015). Despite the importance of these contributions, the qPCR potential beyond detection and identification of *Xanthomonas* species remains poorly explored. One exception has been a qPCR method developed to distinguish resistant and susceptible cultivars based on quantification of *X. albilineans* (Garces et al. 2014).

Advances concerning a framework to select taxon-specific DNA markers (i.e., DNA signatures) successfully validated for PCR-based detection of *Xanthomonas* species in infected plant samples (Albuquerque et al. 2012) further empower the development of qPCR approaches to determine the bacterial load in several host plants. Nine *X. arboricola* pv. *juglandis*-specific DNA markers (XAJ1 to XAJ9) were selected and

Table 1. List of bacteria strains used in this study

Strain ^z	Geographic origin	Year of isolation
<i>Xanthomonas arboricola</i> pv. <i>juglandis</i>		
CFBP 176	France	1961
CFBP 877	France	1966
CFBP 2564	Italy	1985
CFBP 2632	Spain	1984
CFBP 5252	France	2000
CFBP 6556	Italy	1993
CFBP 6557	Italy	1999
CFBP 7072	Spain	1993
CFBP 7179 ^{GSS}	France	2002
CFBP 7244	France	1978
LMG 745	The Netherlands	1978
LMG 746	United Kingdom	1955
LMG 747 ^{T;GSS}	New Zealand	1956
LMG 748	New Zealand	1956
LMG 749	New Zealand	1957
LMG 751 ^{GSS;*}	Romania	1962
LMG 752	United Kingdom	1964
LMG 8047	The Netherlands	1979
CPBF 1480	Azeitão, Portugal	2014
CPBF 1486	Alcobaça, Portugal	2014
CPBF 1492	Alcobaça, Portugal	2014
CPBF 1502	Alcobaça, Portugal	2014
CPBF 1527	Estremoz, Portugal	2014
CPBF 100	Ponte da Barca, Portugal	2015
CPBF 1514	Estremoz, Portugal	2014
<i>X. arboricola</i> strains not belonging to the <i>juglandis</i> pathovar		
<i>X. arboricola</i> pv. <i>populi</i> CFBP 3123 ^{PRS}	The Netherlands	1979
<i>X. arboricola</i> pv. <i>pruni</i> LMG 852 ^{PRS}	New Zealand	1953
<i>X. arboricola</i> pv. <i>celebensis</i> LMG 677 ^{PRS}	New Zealand	1960
<i>X. arboricola</i> pv. <i>corylina</i> LMG 689 ^{PRS}	United States	1939
<i>X. arboricola</i> pv. <i>fragariae</i> LMG 19145 ^{PRS}	Italy	1993
<i>X. arboricola</i> CPBF 122	Ponte da Barca, Portugal	2015
<i>X. arboricola</i> CPBF 1530	Estremoz, Portugal	2014
Other <i>Xanthomonas</i> strains		
<i>X. axonopodis</i> pv. <i>citri</i> LMG 9322 ^T	United States	1989
<i>X. axonopodis</i> pv. <i>dieffenbachiae</i> LMG 695 ^{PRS}	Brazil	1965
<i>X. axonopodis</i> pv. <i>phaseoli</i> LMG 7455 ^{PRS}	United States	1986
<i>X. campestris</i> pv. <i>campestris</i> LMG 568	United Kingdom	1957
<i>X. euvesicatoria</i> LMG 922	United States	1939
<i>X. fragariae</i> LMG 708 ^T	United States	1960
<i>X. gardneri</i> LMG 962 ^{GSS;T}	Yugoslavia	1953
<i>X. oryzae</i> pv. <i>oryzae</i> LMG 5047 ^{PRS}	India	1965
<i>X. oryzae</i> pv. <i>oryzicola</i> LMG 797 ^{PRS}	Malaysia	1964
<i>X. perforans</i> NCPPB 4321 ^T	United States	1991
<i>X. translucens</i> pv. <i>translucens</i> LMG 876 ^{GSS;PRS}	United States	1933
<i>X. vesicatoria</i> LMG 911 ^{GSS;T}	New Zealand	1955
<i>Xanthomonas</i> sp. CPBF 367	Loures, Portugal	2016
<i>Xanthomonas</i> sp. CPBF 98	Ponte da Barca, Portugal	2015
<i>Xanthomonas</i> sp. CPBF 1488	Alcobaça, Portugal	2014

^z CFBP = French Collection for Plant-Associated Bacteria, Institut National de la Recherche Agronomique, Angers, France; GSS = genome sequenced strain; LMG = Belgian Coordinated Collections of Microorganisms/LMG Bacteria Collection, Universiteit Gent Laboratorium voor Microbiologie, Gent, Belgium; T = type strain; NCPPB = National Collection of Plant Pathogenic Bacteria, Fera Science Ltd., York, United Kingdom; CPBF = Coleção Portuguesa de Bactérias Fitopatogênicas, Oeiras, Portugal; and PRS, pathovar reference strain. The asterisk in LMG 751^{GSS;*} indicates other collection no. NCPPB 1447.

validated in 2017 (Fernandes et al. 2017). Three of these markers (XAJ1, XAJ6, and XAJ8) were shown to efficiently detect *X. arboricola* pv. *juglandis* in naturally infected walnut leaves and fruits using a multiplex PCR approach (Fernandes et al. 2017), raising the opportunity to use these DNA markers to measure *X. arboricola* pv. *juglandis* virulence fitness from symptomatic walnut leaves and fruits.

In this work, we developed a qPCR method using two *X. arboricola* pv. *juglandis*-specific DNA markers able to quantify *X. arboricola* pv. *juglandis* bacterial load from infected walnut plant tissues and measure the virulence of distinct *X. arboricola* pv. *juglandis* strains. This method may contribute to the rapid identification of highly virulent *X. arboricola* pv. *juglandis* lineages or strains in walnut orchards, which is essential to take timely phytosanitary action and perform epidemiological risk assessments of *X. arboricola* pv. *juglandis*-causing diseases.

Materials and Methods

Bacterial cultures and DNA extraction. The bacterial strains used in this study included 25 *X. arboricola* pv. *juglandis* strains, seven *X. arboricola* strains not belonging to the *juglandis* pathovar, and 15 strains of other *Xanthomonas* species (Table 1). Single colonies were grown in nutrient broth (2 g/liter of yeast extract, 5 g/liter of peptone, 5 g/liter of NaCl, 0.45 g/liter of KH_2PO_4 , and 2.29 g/liter of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) and DNA was extracted using the E.Z.N.A. Bacterial DNA Kit (Omega Bio-Tek, Norcross, GA), following the manufacturer's instructions. DNA quantification was carried out using the Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA).

Walnut fruit inoculation with *X. arboricola* pv. *juglandis* and bacterial DNA extraction. Immature walnut fruits from a single walnut tree (phenophases Gf + 30 and Gf + 45, Mikulic-Petkovsek et al. 2011) without symptomatic lesions were used in this study. The fruits were disinfected by immersion in 70% ethanol for 30 s and were thoroughly washed with sterile distilled water. Five groups of four walnut fruits each were tested. The fruits were inoculated by immersion in an *X. arboricola* pv. *juglandis* LMG 747^T bacterial suspension of approximately 10^8 CFU/ml for up to 30 min at room temperature with slow shaking. As negative controls, the fruits were immersed in sterile distilled water for 30 min, following the same conditions as described above. After inoculation, the fruits were incubated under controlled environmental conditions (16-h/8-h photoperiod and a temperature of 24°C/18°C) in sterile individual plastic containers to ensure high humidity. After 2 weeks, all fruits inoculated with *X. arboricola* pv. *juglandis* LMG 747^T showed clear disease symptoms. One fruit from each group was then divided into eight identical parts by two longitudinal sections passing through the fruit apical scar (micropile) followed by an equatorial section (Fig. 1). For DNA extraction, the outer pericarp layer (external fruit

green husk containing the lesions) of each selected fruit fraction was excised with a sterile scalpel, the weight of tissue recovered was registered, and the fraction was then macerated with 5 ml of sterile distilled water in extraction bags (Bioreba AG, Reinach, Switzerland). Then 2 ml of the extract was centrifuged at $16,000 \times g$ for 10 min to obtain a bacterial pellet used for DNA extraction using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Selection of *X. arboricola* pv. *juglandis*-specific DNA markers for qPCR. Eighteen fully sequenced genomes of *X. arboricola* pv. *juglandis* were surveyed for the presence of *X. arboricola* pv. *juglandis*-specific DNA markers (XAJ1 to XAJ9) previously characterized by Fernandes et al. (2017): NCPBP 1447 (NZ_AJTL000000000.1), CFBP 2528 (NZ_JZEF000000000.1), CFBP 7179 (JZEG01000001.1), CFSAN033077 (NZ_LHBK000000000.1), CFSAN033081 (NZ_LHBO000000000.1), CFSAN033086 (NZ_LHBT000000000.1), J303 (NZ_LSGZ000000000.1), 417 (CP012251.1), CFSAN033078 (NZ_LHBL000000000.1), CFSAN033079 (NZ_LHBM000000000.1), CFSAN033080 (NZ_LHBN000000000.1), CFSAN033082 (NZ_LHBP000000000.1), CFSAN033083 (NZ_LHBQ000000000.1), CFSAN033084 (NZ_LHBR000000000.1), CFSAN033085 (NZ_LHBS000000000.1), CFSAN033087 (NZ_LHBU000000000.1), CFSAN033088 (NZ_LHBV000000000.1), and CFSAN033089 (NZ_LHBW000000000.1) (Supplementary Fig. S1). The occurrence of these nine *X. arboricola* pv. *juglandis*-specific markers was further evaluated in 108 *X. arboricola* pv. *juglandis* isolates (Fernandes et al. 2018) (Supplementary Fig. S2). The matrices assembled based on the data gathered showed that four markers (XAJ1, XAJ4, XAJ6, and XAJ8) were present in most of the *X. arboricola* pv. *juglandis* strains, but only XAJ1 and XAJ6 were present in all *X. arboricola* pv. *juglandis* strains analyzed and therefore were chosen as the most promising for qPCR.

PCR-based confirmation of walnut fruit infection by *X. arboricola* pv. *juglandis*. Infection of fruits by *X. arboricola* pv. *juglandis* LMG 747^T was confirmed by PCR using the primer pairs XAJ1F/XAJ1R and XAJ6F/XAJ6R to target XAJ1 and XAJ6 DNA markers as previously described (Fernandes et al. 2017) (Table 2). PCR reactions were carried out in 20 μl reactions containing 1 $\times$ DreamTaq Buffer (Thermo Scientific, Vilnius, Lithuania), 0.2 mM of each dNTP (Thermo Scientific), 0.2 μM of each primer (STAB Vida, Lisbon, Portugal), 1 U of DreamTaq DNA Polymerase (Thermo Scientific), and 2 μl of DNA extracted from infected fruit fractions. PCR was performed with an initial 5 min denaturation at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 61°C, and 30 s of extension at 72°C, with a final extension step of 10 min at 72°C. PCR products were separated on 0.8% agarose gels stained with GreenSafe Premium (Nzytech, Lisbon, Portugal).

Fig. 1. Walnut fruit symptoms of *Xanthomonas arboricola* pv. *juglandis* infection. Each fruit was divided into eight parts (numbered 1 to 8). Arrows indicate the fruit apical scar.

qPCR calibration curves with XAJ1 and XAJ6 markers. Two new primer pairs (XAJ1qPCR_Fwd/XAJ1qPCR_Rev and XAJ6qPCR_Fwd/XAJ6qPCR_Rev) targeting a smaller fragment of *X. arboricola* pv. *juglandis*-specific markers XAJ1 (100 bp) and XAJ6 (101 bp) were designed for qPCR amplification (Table 2) using Geneious software (version 7.1.2; Biomatters, Auckland, New Zealand). Optimal annealing temperatures were determined based on the melting curves using both pairs of primers (Supplementary Fig. S3). qPCR calibration curves were constructed using serial dilutions (1 to 10^{-7}) of *X. arboricola* pv. *juglandis* LMG 747^T DNA by plotting the quantification cycle (Cq) versus the number of DNA marker copies. Each dilution was assessed in triplicate and the number of *X. arboricola* pv. *juglandis* chromosomes per microliter was calculated using the Science Primer copy number calculator for real-time PCR (<http://scienceprimer.com>). Each real-time PCR reaction contained 1× Sso Advanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA), 0.35 µM of each primer, 1 µl of each DNA template dilution, and nuclease-free water to a total volume of 20 µl. Reactions were carried out in hard-shell PCR plates (96-well, white shell/clear well), sealed with Microseal B Adhesive Sealer (Bio-Rad). qPCR cycle conditions were as follows: initial denaturation step of 98°C for 3 min, 39 cycles of 98°C for 10 s and 63°C for 30 s, and a final melting curve analysis from 65 to 95°C, with 0.5°C increments for 5 s per step. A nontemplate control was included

in each run. Reactions were carried out in a CFX Connect Real-Time Detection System (Bio-Rad) and results were analyzed using CFX Manager software (version 3.1; Bio-Rad).

Validation of qPCR with spiked walnut leaf samples. Spiked walnut leaf samples were used for qPCR validation and inferred the presence of possible PCR inhibitors in plant extracts. *X. arboricola* pv. *juglandis* LMG 747^T cells were grown in nutrient agar (NA) medium (Difco Laboratories, Detroit, MI) to an optical density of 0.135 at $\lambda = 600$, corresponding to approximately 10^8 CFU/ml as estimated by plating serial dilutions of the bacterial culture in NA at 28°C. Spiked walnut leaf samples were prepared by mixing *X. arboricola* pv. *juglandis* LMG 747^T cells recovered from 2 ml of each dilution (10^7 CFU/ml to 10^0 CFU/ml) after a centrifugation at $7,000 \times g$ for 5 min with 2 ml of bacteria-free plant extract. DNA from each spiked sample (i.e., sample processing control [SPC]) was extracted using the Qiagen DNeasy Plant Mini Kit as mentioned above and amplified in triplicate to construct a calibration curve for each marker (XAJ1 and XAJ6). The limit of detection (LOD) was determined as the CFU value of the dilution corresponding to the last linear point of the standard curve (Marancik and Wiens 2013). qPCR reactions and data analysis were carried out as described in the previous section.

qPCR repeatability, reproducibility, and analytical specificity. qPCR repeatability (intra-assay variance) and reproducibility (interassay variance) were assessed according to Bustin et al. (2009). Intra-assay

Table 2. *Xanthomonas arboricola* pv. *juglandis*-specific markers used in this study^z

DNA marker	Primer	Sequence (5' to 3')	Amplicon length (bp)	Source
XAJ1	XAJ1qPCR_Fwd	AGACGCGTCACTTCGGAAGAG	100	This study
	XAJ1qPCR_Rev	CCAGCCTGTTCTAGGTGGTTTG		
	XAJ1F	GTTGTCAAGGTCGCTACTGGACGC	758	
	XAJ1R	CGACAGGTACCGAAAGTGGACG		
XAJ6	XAJ6qPCR_Fwd	AGAGATTGATGAGCGATGCGAG	101	This study
	XAJ6qPCR_Rev	GCACAGCGGGAAGTAATAGCAA		
	XAJ6F	AAGTCAGATGCGAAGCGAAAGG	436	
	XAJ6R	GCACAGCGGGAAGTAATAGCAAAC		

^z F = forward and R = reverse.

Table 3. DNA marker XAJ1 repeatability and reproducibility of the quantitative PCR assay^y

Sample ^z	CFU/µl	Intra-assay CV				Interassay CV			
		Assays (n)	Mean Cq	SD	% CV	Assays (n)	Mean Cq	SD	% CV
SPC-0	2.70×10^5	3	17.41	0.10	0.58	3	17.22	0.23	1.36
SPC-1	2.70×10^4	3	20.73	0.14	0.68	3	20.50	0.27	1.32
SPC-2	2.70×10^3	3	23.73	0.14	0.58	3	23.59	0.19	0.79
SPC-3	2.70×10^2	3	27.36	0.17	0.60	3	27.01	0.35	1.31
SPC-4	2.70×10^1	3	30.55	0.43	1.39	3	30.35	0.55	1.80
SPC-5	2.70×10^0	3	35.36	0.25	0.72	3	34.20	1.26	3.67
SPC-6	2.70×10^{-1}	3	35.68	n/a	n/a	3	35.48	0.57	1.62
SPC-7	2.70×10^{-2}	3	n/a	n/a	n/a	3	35.71	n/a	n/a

^y CV = coefficient of variation, Cq = quantification cycle, SPC = sample processing control, and n/a = not amplified.

^z The SPC dilutions within the linear range of the standard curve are shown in bold.

Table 4. DNA marker XAJ6 repeatability and reproducibility of the quantitative PCR assay^y

Sample ^z	CFU/µl	Intra-assay CV				Interassay CV			
		Assays (n)	Mean Cq	SD	% CV	Assays (n)	Mean Cq	SD	% CV
SPC-0	2.70×10^5	3	16.79	0.03	0.16	3	16.85	0.26	1.54
SPC-1	2.70×10^4	3	20.06	0.12	0.60	3	20.03	0.22	1.08
SPC-2	2.70×10^3	3	23.31	0.10	0.43	3	23.31	0.24	1.05
SPC-3	2.70×10^2	3	26.67	0.04	0.14	3	26.66	0.14	0.54
SPC-4	2.70×10^1	3	29.84	0.18	0.61	3	29.78	0.37	1.23
SPC-5	2.70×10^0	3	31.66	0.14	0.43	3	31.81	0.49	1.55
SPC-6	2.70×10^{-1}	3	34.89	0.53	1.53	3	33.80	1.02	3.02
SPC-7	2.70×10^{-2}	3	35.05	0.23	0.64	3	34.00	1.10	3.22

^y CV = coefficient of variation, Cq = quantification cycle, SPC = sample processing control, and n/a = not amplified.

^z The SPC dilutions within the linear range of the standard curve are shown in bold.

variation was calculated considering three replicates in a single run/plate and interassay variation was calculated using three independent experiments with three replicates each (Table 3 and 4). Coefficients of variation (CVs) were calculated according to the following formula: $CV = \text{standard deviation Cq values} / \text{average Cq}$ (Robène et al. 2015).

Analytical specificity evaluates the detection of target DNA relatively to nontarget DNA sequences (Bustin et al. 2009) and was validated by taking into consideration the Cq value variations and/or differences between target (i.e., *X. arboricola* pv. *juglandis*) and

nontarget bacteria (i.e., other *X. arboricola* strains not belonging to the *juglandis* pathovar and other *Xanthomonas* species). Accordingly, for this analysis, DNA from reference *Xanthomonas* strains isolated from different geographic regions was used, including 25 *X. arboricola* pv. *juglandis* strains, seven *X. arboricola* strains not belonging to the *juglandis* pathovar, and 15 strains of other *Xanthomonas* species (Table 5). DNA extraction and quantification was carried out as described above, and 20 ng of DNA was used for each qPCR reaction. Melting curves to confirm primer pair specificity for both markers (XAJ1 and XAJ6) and for each tested strain were

Table 5. Quantitative PCR assay specificity for XAJ1 and XAJ6 markers: Comparative analysis of quantification cycle (Cq) values obtained for target groups (*Xanthomonas arboricola* pv. *juglandis* strains) and nontarget groups (other *X. arboricola* strains and other *Xanthomonas* strains)^z

Strain	XAJ1		XAJ6	
	Cq	Mean $\pm$ SD	Cq	Mean $\pm$ SD
<i>X. arboricola</i> pv. <i>juglandis</i>				
CFBP 176	13.76		13.43	
CFBP 2564	14.06		13.42	
CFBP 2632	13.74		13.46	
CFBP 5252	13.81		13.76	
CFBP 6556	13.68		13.28	
CFBP 6557	13.22		13.09	
CFBP 7072	13.69		13.07	
CFBP 7179	13.66		13.16	
CFBP 7244	13.91		13.56	
CFBP 877	13.92		13.60	
LMG 745	14.47		13.80	
LMG 746	13.91	13.38 $\pm$ 1.00	13.70	13.12 $\pm$ 0.84
LMG 747	11.02		11.56	
LMG 748	13.22		12.68	
LMG 749	13.67		13.11	
LMG 751	13.36		12.73	
LMG 752	13.20		12.92	
LMG 8047	13.75		13.45	
CPBF 1480	10.17		9.89	
CPBF 1486	14.31		13.80	
CPBF 1492	13.69		13.77	
CPBF 1502	13.33		13.09	
CPBF 1527	13.96		13.51	
CPBF 100	13.43		13.12	
CPBF 1514	11.63		32.98	
<i>X. arboricola</i> strains not belonging to the <i>juglandis</i> pathovar				
<i>X. arboricola</i> pv. <i>populi</i> CFBP 3123	29.56		29.21	
<i>X. arboricola</i> pv. <i>pruni</i> LMG 852	n/a		n/a	
<i>X. arboricola</i> pv. <i>celebensis</i> LMG 677	12.22		35.56	
<i>X. arboricola</i> pv. <i>corylina</i> LMG 689	19.96		37.69	
<i>X. arboricola</i> pv. <i>fragariae</i> LMG 19145	35.75		n/a	
<i>X. arboricola</i> CPBF 122	32.48		31.47	
<i>X. arboricola</i> CPBF 1530	37.63		36.00	
Other <i>Xanthomonas</i> strains				
<i>X. axonopodis</i> pv. <i>citri</i> LMG 9322	35.32		31.97	
<i>X. axonopodis</i> pv. <i>phaseoli</i> LMG 7455 ^{PRS}	36.78		39.78	
<i>X. campestris</i> pv. <i>campestris</i> LMG 568	33.03		34.41	
<i>X. fragariae</i> LMG 708	37.82		n/a	
<i>X. gardneri</i> LMG 962	34.84		34.84	
<i>X. vesicatoria</i> LMG 911	35.56		n/a	
<i>X. axonopodis</i> pv. <i>dieffenbachiae</i> LMG 695	n/a		35.70	
<i>X. euvesicatoria</i> LMG 922	n/a		35.56	
<i>X. oryzae</i> pv. <i>oryzae</i> LMG 5047 ^{PRS}	n/a		n/a	
<i>X. oryzae</i> pv. <i>oryzicola</i> LMG 797	n/a		35.58	
<i>X. perforans</i> NCPPB 4321	n/a		35.10	
<i>X. translucens</i> pv. <i>translucens</i> LMG 876	n/a		36.71	
<i>Xanthomonas</i> sp. CPBF 98	12.94		29.96	
<i>Xanthomonas</i> sp. CPBF 1488	31.04		30.36	
<i>Xanthomonas</i> sp. CPBF 367	37.66		36.30	

^z CFBP = French Collection for Plant-Associated Bacteria, Institut National de la Recherche Agronomique, Angers, France; LMG = Belgian Coordinated Collections of Microorganisms/LMG Bacteria Collection, Universiteit Gent Laboratorium voor Microbiologie, Gent, Belgium; CPBF = Coleção Portuguesa de Bactérias Fitopatogênicas, Oeiras, Portugal; n/a = not amplified; PRS, pathovar reference strain; and NCPPB = National Collection of Plant Pathogenic Bacteria, Fera Science Ltd., York, United Kingdom.

determined in duplicate (Supplementary Fig. S4). DNA extraction and quantification was carried out as described above, and 20 ng of DNA was used for each qPCR reaction. Melting curves to confirm primer pair specificity for both markers (XAJ1 and XAJ6) and for each tested strain were determined in duplicate. qPCR reaction efficiency was calculated according to the following curve fitting method (Kralik and Ricchi 2017): Percent efficiency = $(10^{(-1/m)} - 1) \times 100$, where m is the slope of the linear regression equation of the standard curve.

qPCR quantification of *X. arboricola* pv. *juglandis* in infected walnut fruits. To estimate the number of *X. arboricola* pv. *juglandis* cells by qPCR in infected walnut fruit tissues, standard curves obtained from serial dilutions of SPCs for both XAJ1 and XAJ6 markers were generated as described above.

The qPCR reaction for each *X. arboricola* pv. *juglandis*-infected one-eighth fruit part contained 1× Sso Advanced Universal SYBR Green Supermix (Bio-Rad), 0.35 μM of each primer, 2 μl of template DNA, and nuclease-free water to a total volume of 20 μl. qPCR reactions were carried out in triplicate and in parallel with the SPC standard curves. CFX Manager software (version 3.1) was used to analyze the data and to calculate the efficiency of the qPCR reaction and the correlation coefficient (R^2) of SPC standard curves. The absolute copy number for each marker in each fruit sample was calculated considering that each marker (XAJ1 and XAJ6) occurs as a single copy locus in *X. arboricola* pv. *juglandis* genomes and using the following formula:

$$\text{Copy number} = 10^{((Cq - b)/m)}.$$

This formula is derived from the linear regression equation of the standard curve:

$$Cq = m(\log \text{ copy number}) + b,$$

where Cq is the quantification cycle value obtained for the tested sample, m is the slope, and b is the y-intercept of the linear

Fig. 2. Calibration curves for **A**, XAJ1 and **B**, XAJ6 DNA markers obtained from serial dilutions of *Xanthomonas arboricola* pv. *juglandis* strain LMG 747 DNA. The linear portion of the curve ranged from 3.5×10^6 to 35 chromosomes/μl. The efficiency (E) of the qPCR assays was 98.7 and 96.4% for XAJ1 and XAJ6, respectively. The correlation efficiency (R^2) was 0.990 for XAJ1 and 0.990 for XAJ6. Standard deviations at each point are represented in the figure. Cq = quantification cycle.

regression equation of the calibration curve. The results were expressed as the number of *X. arboricola* pv. *juglandis* chromosomes or the cell number (*X. arboricola* pv. *juglandis* load) per gram of fruit fresh weight for each one-eighth fruit part. *X. arboricola* pv. *juglandis* bacteria load per fruit was determined as the average *X. arboricola* pv. *juglandis* load per gram of fresh weight obtained for the eight fruit parts and the corresponding standard deviation.

Statistical analysis. IBM SPSS Statistics software (version 25) was used to perform all statistical analyses. Two different tests were performed on qPCR data to compare the Cq values obtained: the Student t test was carried out to compare Cq values between two markers, whereas one-way analysis of variance was used for the comparison of Cq values between fruits, and the means were compared using the Tukey test at the $P < 0.05$ level.

Results

Selection of *X. arboricola* pv. *juglandis*-specific DNA markers for qPCR. Nine *X. arboricola* pv. *juglandis*-specific DNA markers (XAJ1 to XAJ9) were previously described and characterized by Fernandes et al. (2017). The occurrence of these nine markers was investigated in 18 fully sequenced genomes of *X. arboricola* pv. *juglandis* strains isolated from walnut plant organs. XAJ1, XAJ4, and XAJ6 were present in all genomes, whereas XAJ8 was not present in one of the 18 genomes analyzed. Although the other markers were *X. arboricola* pv. *juglandis*-specific, they were absent from some *X. arboricola* pv. *juglandis* strains: marker XAJ9 was present in seven genomes, XAJ7 and XAJ5 in three genomes, and XAJ2 in only one genome. These results were further validated in a collection of 108 *X. arboricola* pv. *juglandis* tested for the presence of XAJ

Fig. 3. Standard curves for **A**, XAJ1 and **B**, XAJ6 DNA markers obtained from serial dilutions of sample processing control (SPC) samples. The linear portion of the curve ranged from SPC-0 (2.7×10^5 CFU/μl) to SPC-5 (2.7×10^0 CFU/μl). The efficiency (E) of the qPCR assays was $97.6 \pm 6.4\%$ for XAJ1 and $111.8 \pm 0.5\%$ for XAJ6. The correlation efficiency (R^2) was 0.999 for XAJ1 and 0.995 for XAJ6. Standard deviations at each point are represented in the figure. Cq = quantification cycle.

markers by dot blot hybridization (Fernandes et al. 2018). The data revealed that XAJ1 was present in 108 isolates (100%), XAJ2 in 19 isolates (18%), XAJ3 in 57 isolates (53%), XAJ4 in 102 isolates (94%), XAJ5 in 53 isolates (49%), XAJ6 in 108 isolates (100%), XAJ7 in 34 isolates (31%), XAJ8 in 100 isolates (93%), and XAJ9 in 70 isolates (65%). Altogether, these data allowed us to identify XAJ1 and XAJ6 as the most reliable markers to detect and identify *X. arboricola* pv. *juglandis* and were chosen for further analysis.

A BLAST analysis of the XAJ1 and XAJ6 marker sequences, obtained from *X. arboricola* pv. *juglandis* strain NCPPB 1447 (accession number PRJNA84273), showed that the best nontarget BLAST hit for qPCR marker XAJ1 (100 bp) was obtained with *X. campestris* pv. *campestris* strain 17 (E-value = $2e-04$, query coverage = 51%). Concerning the XAJ6 qPCR marker (101 bp) the best nontarget BLAST hit was obtained with *X. axonopodis* pv. *phaseoli* strain ISO18C8 (E-value = 5.4, query coverage = 14%).

qPCR calibration curves for markers XAJ1 and XAJ6. After selection of *X. arboricola* pv. *juglandis*-specific markers XAJ1 and XAJ6 and design of the qPCR primer pairs, two qPCR calibration curves for each marker were generated using 10-fold dilutions of *X. arboricola* pv. *juglandis* LMG 747^T chromosomal DNA and 10-fold dilutions of DNA extracted from walnut samples spiked with *X. arboricola* pv. *juglandis* LMG 747^T bacteria (SPCs). The qPCR calibration curves obtained with chromosomal DNA revealed an efficiency of 98.7 and 96.4% for XAJ1 and XAJ6, respectively, and an R^2 value of 0.990 for both markers (Fig. 2). Taking into account that in silico analyses showed that both markers occur as a single copy locus within the chromosome of the sequenced *X. arboricola* pv. *juglandis*, the standard curves allowed estimation of an LOD of 35 chromosomes/qPCR reaction for both markers. The SPC standard curves obtained from three independent experiments with three replicates each resulted in an average efficiency of 97.6 ± 6.4 and $111.8 \pm 0.5\%$ and R^2 values of 0.999 and 0.995 for XAJ1 and XAJ6, respectively (Fig. 3). The LOD obtained for SPC was determined as 2.7 CFU/qPCR reaction for both markers.

qPCR repeatability, reproducibility, and specificity. Repeatability (i.e., intra-assay variability) of SPCs revealed a CV of Cq values that ranged from a minimum of 0.58% (SPC-0) to a maximum of 1.39% (SPC-4) for the XAJ1 marker and from a minimum of 0.16% (SPC-0) to a maximum of 0.61% (SPC-4) for the XAJ6 marker (Tables 3 and 4). Regarding reproducibility (i.e., interassay variability), the CV values of Cq ranged from a minimum of 0.79 (SPC-2) to a maximum of 3.67% (SPC-5) for XAJ1 and from a minimum of 0.54 (SPC-3) to a maximum of 1.55% (SPC-5) for XAJ6, taking into account three independent experiments (Tables 3 and 4).

Assessment of qPCR specificity for both markers showed that all tested *X. arboricola* pv. *juglandis* strains gave identical average Cq values for XAJ1 (13.38 ± 1.00) and XAJ6 (13.12 ± 0.84) (Table 5). With only *X. arboricola* pv. *juglandis* isolate CPBF 1514, the Cq value was 32.98 for XAJ6 but 11.63 for XAJ1, which is within the Cq average obtained for this marker with all other *X. arboricola* pv. *juglandis* strains tested. On the contrary, the Cq values of *X. arboricola* strains not belonging to the *juglandis* pathovar doubled the Cq values obtained for *X. arboricola* pv. *juglandis* strains (>29.21) or showed no amplification for both markers, with the exception of *X. arboricola* pv. *celebensis* LMG 677 and *X. arboricola* pv. *corylina* LMG 689, which showed Cq values of 12.22 and 19.96 for XAJ1, respectively. In addition, the Cq values for strains of other *Xanthomonas* species were at least twofold higher (>29.96) than the average Cq values for *X. arboricola* pv. *juglandis* or revealed no amplification for both markers, except for *Xanthomonas* sp. CPBF 98 with a Cq value of 12.94 for XAJ1 (Table 5).

PCR detection and qPCR quantification of *X. arboricola* pv. *juglandis* in infected walnut fruits. To confirm walnut fruit infection with *X. arboricola* pv. *juglandis* and dismiss the occurrence of possible inhibitory PCR compounds, a preliminary detection PCR was carried out using DNA extracted from walnut fruit epicarp tissues. The results showed PCR amplification for both markers (XAJ1 and XAJ6) in at least one of the one-eighth parts of the five tested fruits (fruits A to E)

(Fig. 4), although a higher PCR efficiency was generally obtained with XAJ1. Interestingly, for some of the one-eighth fruit samples and regardless of the symptomatic evidence of infection, no amplification was observed whatever the marker, as shown with one-eighth fruit parts B2, B6, and B8 from fruit B (Fig. 4).

When DNA extracted from each of the one-eighth parts of the five studied walnut fruits was used as a template for qPCR, full detection efficiency was achieved, even with the fruit parts for which standard PCR using the same markers was negative (Fig. 4; Table 6). Furthermore, the Cq values obtained for XAJ1 and XAJ6 were consistent between each other and among the different fruit parts, because no statistically significant differences were observed between the Cq average values obtained for both markers within the same fruit (Table 6). When comparing the average Cq values obtained for both markers and the five fruits studied, only fruit A was shown to be significantly different relative to the Cq obtained for the other fruits (Table 6).

Discussion

Considerable advances were made in the last decade concerning amplification-based methods for detection of phytopathogenic bacteria. Specifically, the improvements associated with real-time PCR and LAMP technologies, the access to extensive genomic data, which is instrumental for identifying specific DNA markers and designing better primers, and the progress obtained with more efficient thermostable DNA polymerases led to major advances in culture-independent methods of phytopathogen detection.

Currently, real-time PCR is acknowledged as cost-effective, high throughput, and capable of providing fast and accurate diagnostics of a broad range of diseases caused by bacteria, as reviewed by Mirmajlessi et al. (2015). Because of its efficiency and specificity, real-time

Fig. 4. PCR analysis of infected walnut fruits with DNA markers XAJ1 (758 bp) and XAJ6 (436 bp) for detection of *Xanthomonas arboricola* pv. *juglandis*.

PCR is presently considered the “gold standard” method for phytopathogen detection (Alemu 2014). In fact, qPCR has been used for a panoply of solutions that are beyond detection of phytopathogenic bacteria in plant samples, namely to evaluate the growth of a pathogen in resistance studies with transgenic plants (Hanke et al. 2003; Reynold et al. 1999), after application of fireblight control agents such as prohexadione calcium (Costa et al. 2001), and in breeding programs (Norelli et al. 2003), and to estimate the extent of contamination with *Erwinia amylovora* on fruits (van der Zwet and Bell 1990). Despite these advances, not many qPCR protocols have been proposed for quantifying phytopathogenic microorganisms in general and bacteria in particular. A major difficulty in optimizing qPCR protocols to detect and quantify phytopathogenic bacteria in plant samples is mainly attributable to the difficulty in identifying short species-specific DNA signatures suitable for qPCR (i.e., generally <200 bp). This is particularly pertinent for phytopathogens such as *X. arboricola* pv. *juglandis*, which are characterized by high genetic diversity and closely related to nonpathogenic *Xanthomonas* species.

Fernandes et al. (2017) identified and validated nine *X. arboricola* pv. *juglandis*-specific DNA markers (XAJ1 to XAJ9), using a comprehensive BLAST analysis of all open reading frames (>100 bp) retrieved from the draft genome of *X. arboricola* pv. *juglandis* (NCPPB 1447, LMG 751), filtered for high and exclusive similarity toward *X. arboricola* pv. *juglandis*. The occurrence of these DNA markers in a genetically diverse collection of *X. arboricola* pv. *juglandis* strains, including 18 *X. arboricola* pv. *juglandis* genomes and 108 field isolates, allowed identification of markers XAJ1 and XAJ6 as being present in all 126 *X. arboricola* pv. *juglandis* strains analyzed and therefore as promising targets for qPCR. Although the other markers *X. arboricola* pv. *juglandis* were specific, they were absent from some *X. arboricola* pv. *juglandis* strains, which could lead to false-negative qPCR results. Interestingly, XAJ1 was not present in the two fully sequenced nonpathogenic strains CFBP 7634 and 7651, whereas XAJ6 was present in the nonpathogenic CFBP 7634 but not in 7651, which raises the question of the importance for pathogenicity and virulence of the genomic regions were these

Table 6. Quantification cycle (Cq) values of qPCR quantification of infected walnut fruits with DNA markers XAJ1 and XAJ6^v

Fruit	XAJ1				XAJ6			
	Cq ^w	Mean ± SD ^{x,y}	Bacterial load (×10 ³) ^z	Mean (×10 ³) ^x	Cq ^w	Mean ± SD ^{x,y}	Bacterial load (×10 ³) ^z	Mean (×10 ³) ^x
A	24.3		46.1		23.7		36.7	
	28.6		8.90		27.9		5.47	
	25.3		45.0		24.8		33.6	
	28.3	27.01 ± 1.98 ^a	9.86	21.1 ± 16.9	27.5	26.36 ± 1.85 ^a	6.55	15.2 ± 13.4
	26.4		19.2		25.7		14.0	
	28.2		7.63		27.6		4.76	
	25.2		28.0		24.8		17.9	
B	29.8		4.01		28.9		2.52	
	26.6		21.6		26.8		15.4	
	31.9		2.20		31.7		1.55	
	30.8		4.60		30.7		3.10	
	27.8	30.88 ± 2.66 ^b	11.2	6.22 ± 6.98	26.4	30.48 ± 2.6 ^b	15.0	5.31 ± 6.13
	31.8		2.99		31.4		2.23	
	33.5		1.60		33.2		1.09	
C	30.2		4.29		30.4		2.97	
	34.4		1.28		33.2		1.18	
	25.8		23.7		26.0		18.4	
	29.0		8.00		29.2		5.32	
	35.3		0.537		35.4		0.265	
	36.1	31.00 ± 3.49 ^b	0.553	6.38 ± 8.05	35.2	31.12 ± 3.24 ^b	0.416	4.43 ± 6.20
	31.8		1.51		32.4		0.728	
D	28.0		11.8		28.5		7.29	
	30.7		2.52		31.1		1.38	
	31.3		2.40		31.2		1.59	
	31.7		2.57		32.2		1.28	
	32.0		1.73		32.2		0.975	
	30.6		3.44		30.9		1.99	
	29.0	32.31 ± 2.03 ^b	5.12	2.20 ± 1.51	29.2	32.51 ± 1.87 ^b	3.39	1.27 ± 1.01
E	33.9		1.11		34.0		0.567	
	32.2		2.15		32.6		1.09	
	35.5		0.491		35.1		0.340	
	33.6		1.03		33.9		0.502	
	29.8		4.71		29.7		3.34	
	31.7		2.54		31.3		1.89	
	33.4		1.25		33.2		0.803	
	27.6	31.5 ± 2.49 ^b	18.7	5.36 ± 5.78	27.6	31.9 ± 2.21 ^b	14.0	3.94 ± 4.33
	34.2		1.58		33.8		1.06	
	34.8		1.57		33.6		1.44	
	29.9		6.28		29.8		4.65	
	30.6		6.21		30.5		4.26	

^v The *Xanthomonas arboricola* pv. *juglandis* quantity is expressed as chromosomes per gram of fresh weight walnut fruit tissue.

^w Cq values from qPCR reactions are mean values from samples run in triplicate (technical replicates).

^x Data are means ± SD of Cq values obtained from all fruit parts for each fruit.

^y The independent-samples *t* test was used to determine statistically significant differences between the Cq values obtained with markers XAJ1 and XAJ6 for each fruit. One-way analysis of variance was applied to ascertain statistically significant differences between Cq values for each fruit for the same marker. Values followed by different superscript letters indicate statistically significant differences in Cq among different fruits for *P* < 0.05.

^z Bacterial load of *X. arboricola* pv. *juglandis*.

markers are located. Although marker XAJ1 was assigned to an intergenic region, marker XAJ6 is part of a putative coding region annotated as a hypothetical protein (Fernandes et al. 2017), which currently makes it difficult to relate these markers with genetic determinants of pathogenicity and virulence.

Conventional PCR amplification of markers XAJ1 (758 bp) and XAJ6 (436 bp) from DNA extracted from infected fruit samples did not reveal the presence of PCR inhibitors co-extracted during DNA extraction, confirming results previously reported (Fernandes et al. 2017). Furthermore, although it was not possible to detect the presence of each marker in all one-eighth fruit parts of the five fruits assayed by conventional PCR, higher efficiency was generally observed for XAJ1 compared with XAJ6, as described by Fernandes et al. (2017).

Aiming to optimize a qPCR method to quantify *X. arboricola* pv. *juglandis* in infected walnut plant samples and to make these markers suitable to ensure a high qPCR efficiency, a set of primer pairs was designed to amplify a smaller region of each marker (namely, 100 bp for XAJ1 and 101 bp for XAJ6). These qPCR targets are within the size range recommended for qPCR (Bustin et al. 2009). The calibration curves obtained resulted in an efficiency > 96% and a R^2 value of 0.99 for both markers, regardless of the DNA template (i.e., *X. arboricola* pv. *juglandis* chromosomal DNA or DNA extracted from walnut spiked samples), indicating high stability of the qPCR assay (Johnson et al. 2013). The calibration curves allowed us to determine the LOD as 35 chromosomes/qPCR reaction for both markers, using serial dilutions of *X. arboricola* pv. *juglandis* chromosomal DNA and considering that both markers occur as single copies in the *X. arboricola* pv. *juglandis* genome, whereas the LOD for spiked samples was as low as 2.7 CFU/qPCR reaction. qPCR repeatability (i.e., intra-assay variation) and reproducibility (i.e., interassay variation) of three independent experiments revealed maximum CV values of 1.39 and 3.67% for intra-assay and interassay variation, respectively, for both markers. These data indicate consistent calibration curves, which is particularly important to accurately determine the bacterial load of environmental samples (Almeida et al. 2018).

Aside from the technical validations addressed above, good qPCR specificity is essential to ensure high confidence, particularly when the microbial target is in a complex matrix such as plant samples, frequently characterized by rich microbiota (Brader et al. 2017). In addition, the high genetic diversity of *X. arboricola*, characterized by several pathovars and the existence of closely related other *Xanthomonas* species, calls for particular attention to achieve a reliable validation of a qPCR procedure for *X. arboricola* pv. *juglandis*. In this regard, specificity assays were carried out in 25 *X. arboricola* pv. *juglandis* strains, seven *X. arboricola* strains not belonging to the *juglandis* pathovar, and 15 strains of other *Xanthomonas* species.

Blastn analysis of XAJ qPCR markers showed that the best off-target values were obtained with *X. campestris* pv. *campestris* strain 17 (E-value = $2e-04$, query coverage = 51%) for XAJ1, and with *X. axonopodis* pv. *phaseoli* strain ISO18C8 (E-value = 5.4, query coverage = 14%) for XAJ6. Because these bacteria are unlikely to share the same ecological niche as *X. arboricola* pv. *juglandis*, it is not expected that they might lead to false-positive results when assaying infected walnut plant samples. Importantly, given that the present qPCR method is based on two markers (XAJ1 and XAJ6) and that the off-targets are different for both markers, the risk of a false-positive result is negligible.

Regarding the experimental specificity validation, the Cq values obtained for the target *X. arboricola* pv. *juglandis* (XAJ1 $\approx 13.38 \pm 1.00$ and XAJ6 $\approx 13.12 \pm 0.84$) contrasted with the lack of amplification or statistically different higher Cq values recorded for nontarget species. Interestingly, two of the tested strains (*X. arboricola* pv. *juglandis* CPBF 1514 and *Xanthomonas* sp. CPBF 98) showed Cq values for XAJ1 within the average range obtained for other *X. arboricola* pv. *juglandis* strains (11.63 and 12.94, respectively) but high Cq values for XAJ6 (32.98, and 36.71, respectively), characteristic of off-target xanthomonads. These results confirm the presence of

XAJ1 and absence of XAJ6 as shown by dot blot hybridization of these two strains (Fernandes et al. 2017). Multilocus sequence analysis genotyping studies showed that both strains are outside the main clusters of *X. arboricola* pv. *juglandis* and closer to other *X. arboricola* strains not belonging to the *juglandis* pathovar or atypical and nonpathogenic walnut *X. arboricola* isolates (Cesbron et al. 2015; Essakhi et al. 2015; Fernandes et al. 2018).

When comparing qPCR and PCR results of infected walnut fruit parts, the former method was shown to be more efficient in *X. arboricola* pv. *juglandis* detection than conventional PCR, because it was able to detect bacteria in fruit samples where no amplification was observed by conventional PCR (Fig. 4).

In this work, we validated a qPCR approach using two DNA markers able to determine the bacterial load of *X. arboricola* pv. *juglandis* in infected plant material. Quantification of *X. arboricola* pv. *juglandis* infecting host plant tissues within a small time frame will allow investigators to measure the virulence fitness of different strains and will support the adequacy of this method to evaluate the tolerance and/or resistance of distinct walnut genotypes to diseases caused by *X. arboricola* pv. *juglandis*, altogether contributing to improved risk assessment analysis. Taking into account the genetic diversity of *X. arboricola* pv. *juglandis* and its broad distribution, the rapid identification of highly virulent *X. arboricola* pv. *juglandis* strains will be particularly useful to inform the implementation of suitable phytosanitary containment measures.

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