Multiple DNA Markers for Identification of *Xanthomonas arboricola* pv. *juglandis* Isolates and its Direct Detection in Plant Samples

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Abstract

Xanthomonas arboricola pv. *juglandis* (*Xaj*) is the etiological agent of walnut (*Juglans regia* L.) bacterial blight (WBB), and has been associated to other walnut emerging diseases, namely brown apical necrosis (BAN) and vertical oozing canker (VOC), altogether severely affecting the walnut production worldwide. Despite the research efforts carried out to disclose *Xaj* genetic diversity, reliable molecular methods for rapid identification of *Xaj* isolates and culture-independent detection of *Xaj* in infected plant samples are still missing. In this work, we propose nine novel specific DNA markers (XAJ1 to XAJ9) selected by dedicated in silico approaches to identify *Xaj* isolates and detect these bacteria in infected plant material. To confirm the efficacy and specificity of these markers, dot blot hybridization was carried out across a large set of xanthomonads. This analysis, which confirmed the pathovar specificity of these markers, allowed to identify four broad-range markers (XAJ1, XAJ4, XAJ6, and XAJ8) and five narrow-range markers (XAJ2, XAJ3, XAJ5, XAJ7, and XAJ9), originating 12 hybridization patterns

(HP1 to HP12). No evident relatedness was observed between these hybridization patterns and the geographic origin from which the isolates were obtained. Interestingly, four isolates that clustered together according the *gyrB* phylogenetic analysis (CPBF 1507, 1508, 1514, and 1522) presented the same hybridization pattern (HP11), suggesting that these nine markers might be informative to rapidly discriminate and identify different *Xaj* lineages. Taking into account that a culture-independent detection of *Xaj* in plant material has never been described, a multiplex PCR was optimized using markers XAJ1, XAJ6, and XAJ8. This triplex PCR, besides confirming the dot blot data for each of the 52 *Xaj*, was able to detect *Xaj* in field infected walnut leaves and fruits. Altogether, these nine *Xaj*-specific markers allow conciliating the specificity of DNA-detection assays with typing resolution, contributing to rapid detection and identification of potential emergent and acutely virulent *Xaj* genotypes, infer their distribution, disclose the presence of this phytopathogen on potential alternative host species and improve phytosanitary control.

Xanthomonas arboricola (Vauterin et al. 1995) is a bacterial species responsible for economically relevant diseases in several important fruit trees (Frutos 2010; Lamichhane 2014). According to host specificity, several pathovars of *X. arboricola* are presently acknowledged (Fischer-Le Saux et al. 2015; Janse et al. 2001; Vauterin et al. 1995). Among these, pathovar *juglandis* has been considered as one of the most serious threats to walnut production worldwide (Belisario et al. 2002; Hajri et al. 2010; Lamichhane 2014; Moragrega and Ozaktan 2010). *X. arboricola* pv. *juglandis* (*Xaj*) was first described in the beginning of the 20th century (Pierce 1901), and it was since identified as the etiological agent of walnut bacterial blight (WBB), the most well-known disease of walnut (*Juglans* sp.), often recognized by the presence of necrotic spots on leaves and fruits (Smith et al. 1912). *Xaj* has been also frequently associated to brown apical necrosis (BAN) characterized by apical necrotic lesions near the blossom end of the nut, which has also been associated with other phytopathogens (Belisario et al. 2002; Moragrega and Ozaktan

2010; Moragrega et al. 2011), and vertical oozing canker (VOC), consisting of unusual symptoms, including vertical cankers, deformations, and brown exudates, which appear mainly on the trunk of walnut trees (Hajri et al. 2010). While different parts of the plant can be infected, leading to a decrease of tree vigor, the main economic impact of diseases attributed to *Xaj* is the substantial yield losses, due to a premature fruit drop and a decrease of the quality of marketable nuts (Lamichhane 2014; Scortichini 2010).

Occurrence of *Xaj* infection has been observed worldwide (Frutos 2010) with a recent increase in the number of outbreaks reported in different walnut production regions (Hajri et al. 2010; Lamichhane 2014; Moragrega et al. 2011). The impact of more frequent and severe outbreaks affecting the main producing countries is still unknown. Thus, further understanding of pathogen biology and epidemiology is crucial for the effective control of the disease (Lamichhane 2014; Scortichini 2010). Studies have shown that *Xaj* can multiply epiphytically, and penetrate and invade walnut trees during favorable conditions, mainly through stomata structures, leaf-scars, or plant wounds (Lamichhane 2014; Scortichini 2010). It has also been described that dormant buds are privileged overwintering sites for *Xaj* (Lindow et al. 2014; Mulrean and Schroth 1982). Furthermore, the existence of alternative host reservoirs of *Xaj*, such as asymptomatic plant species, might also be relevant for the dissemination and transmission of *Xaj* to walnut plants (Allen et al. 2009; Lamichhane 2014).

Currently, diagnostic procedures for *Xaj* rely on traditional methods based on bacteria isolation using differential or semiselective media, species-specific biochemical assays, and pathogenicity tests (Janse 2010; Moragrega 2012). Despite the numerous DNA-based typing

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary table and three supplementary figures are available online.

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methods for genetic characterization of *Xaj* populations that have been proposed in recent years (Burokiene and Pulawska 2012; Essakhi et al. 2015; Giovanardi et al. 2016; Hajri et al. 2010; Ivanović et al. 2015; Kałużna et al. 2014; Marcelletti et al. 2010), reliable molecular methods for rapid identification of *Xaj* isolates and culture-independent detection of *Xaj* in infected plant samples are still missing. Nonetheless, there are a few studies that report identification of *Xaj* isolates by PCR amplification of a single marker (Burokiene and Pulawska 2012; Gironde et al. 2009; Ivanović et al. 2015; Kałużna et al. 2014; Moragrega 2012), but neither the sequence of the marker nor the primers were made available, making difficult a broad assessment of this method.

To overcome some of these limitations, this study focused on the selection and validation of unique and discriminative *Xaj* genomic regions. These DNA markers, besides providing the tools for culture-independent detection of *Xaj* in infected plant material, and for the reliable identification of isolates, will certainly be useful to disclose the presence of this phytopathogen on potential alternative host species, contributing to detail the life cycle of these bacteria and helping to design the most suitable phytosanitary strategies for prevention and containment of diseases caused by *Xaj*.

Materials and Methods

Selection of specific DNA markers for *Xaj*. The identification of *Xaj*-specific DNA regions was carried out through the analysis of all possible ORFs (open reading frames) retrieved from the concatenated contigs of *Xaj* strain NCPPB 1447 draft genome (accession PRJNA84273) using Geneious v. 7.1.2 software (Biomatters, Auckland, New Zealand). A BLAST analysis (Altschul et al. 1990) was performed in order to infer the *Xaj* specificity of each ORF. Sequences with the best BLAST specificity results were chosen as the most promising regions for marker design (Table 1).

For DNA marker selection, the *Xaj*-specific ORFs were further screened for their putative function and for their suitability to design primer pairs using the Vector NTI 10 software (Invitrogen, Carlsbad, CA) favoring high PCR stringency, in order to prevent unspecific amplification and facilitate multiplexing. These premises resulted in nine putative *Xaj*-specific markers and corresponding primers pairs (XAJ1 to XAJ9), which were chosen for further experimental validation using bacterial reference strains and isolates (Table 1).

Bacterial strains, culture conditions, and DNA extraction. The bacterial strains used for validation of the nine putative *Xaj*-specific markers are listed in Table 2, and include 18 strains of *Xaj*, five strains representing the pathovars *celebensis*, *corylina*, *fragariae*, *populi*, and *pruni* of *X. arboricola*, as well as 12 strains belonging to other species of *Xanthomonas*. Bacterial strains were cultured in yeast extract glucose carbonate (YGC) medium (yeast extract, 5.0 g;

glucose, 10.0 g; CaCO₃, 30.0 g; agar, 15.0 g; distilled water up to 1.0 liter) at 28°C, except for *X. fragariae*, which was cultured in yeast-peptone-glucose agar (YPGA) medium (yeast extract, 5.0 g; bacto peptone, 5.0 g; glucose, 10.0 g; agar, 15.0 g; distilled water up to 1.0 liter) at 20°C.

DNA was extracted from pure cultures using the EZNA Bacterial DNA Purification kit (Omega Bio-Tek, Norcross, GA), according to the manufacturer's instructions, and quantified using the Qubit 2.0 Fluorometer HS Assay (Invitrogen, Carlsbad, CA).

Isolation of bacteria from walnut leaves and fruits with WBB and BAN symptoms. Leaf and fruit samples of walnut trees (*Juglans regia*) showing WBB and/or BAN symptoms were collected during the spring-summer seasons, from six geographic regions of Portugal (Alcobaça, Azeitão, Beja, Estremoz, Loures, and Seia) (Table 3).

Putative *Xaj* were isolated from infected leaves and fruits by using a sterile scalpel to excise plant tissue adjacent to necrotic areas. Sampled plant fragments were disinfected by immersion in 70% ethanol for 30 s, thoroughly washed with sterile distilled water (SDW), and then macerated with 5 ml of SDW in extraction bags (Bioreba AG, Reinach, Switzerland). The resulting suspensions and the correspondent 10⁻¹ dilutions were streaked on yeast extract dextrose carbonate (YDC) medium (yeast extract, 10.0 g; dextrose, 20.0 g; CaCO₃, 20.0 g; agar, 15.0 g; distilled water up to 1.0 liter) and incubated at 26 ± 2°C for 4 days (Lelliott and Stead 1987). Characteristic mucoid yellow colonies were streaked on fresh nutrient agar medium to ensure purity. All isolates were stored at -80°C at the Portuguese Collection of Phytopathogenic Bacteria (CPBF – Coleção Portuguesa de Bactérias Fitopatogénicas). Bacterial DNA was extracted using EZNA Bacterial DNA Purification kit.

Symptomatic walnut leaves and fruits were additionally used for culture-independent detection of *Xaj* by multiplex PCR. Total DNA was extracted from pelleted cells using 2 ml of the plant suspensions obtained as described above and centrifuged at 16,000 × g for 10 min. DNA extraction was performed using both the QIAGEN DNeasy Plant Mini kit, following manufacturer's instructions (Qiagen, Hilden, Germany) and the CTAB-based DNA extraction method (Doyle 1991; Murray and Thompson 1980). To confirm *Xaj* as the causal agent of infection in these plant samples, bacterial isolates were obtained following the procedure described above.

Molecular analysis of isolates by *gyrB*. The identification of the bacterial isolates was carried out by amplification and sequencing of the *gyrB* gene as previously described by Young et al. (2008), using the primers XgyrB1F and XgyrB1R and a reaction mixture containing 1× DreamTaq Buffer with 2.0 mM MgCl₂ (Fermentas, Ontario, Canada), 0.2 mM of each dNTP (Fermentas), 0.2 μM of each primer, 1U of DreamTaq DNA Polymerase (Fermentas), and 25 ng of DNA template. *gyrB*

Table 1. *Xanthomonas arboricola* pv. *juglandis* specific markers, corresponding primer pair sequences, and expected amplicon sizes. The best BLAST hits (blastn and wgs) are indicated for each marker

Markers	Primers	Sequences (5'-3')	Length (bp)	Best BLAST hit (E value/Q coverage) ^a	
				blastn	wgs blast
XAJ1	XAJ1F	GTGTCAAGGTGCTACTGGACGC	758	<i>X. campestris</i> 17	<i>X. arboricola</i> pv. <i>celebensis</i> NCPPB 1832
	XAJ1R	CGACAGGTACCGAAAGTGGACG		(2e-08/12%)	(0/100%)
XAJ2	XAJ2F	AGATCGGGCTGGATGAAGAAGAG	753	<i>Desulfomicrobium baculatum</i> DSM 4028	<i>X. perforans</i> TB15
	XAJ2R	CCAGGATCGCAAGTTCTTCAAGTG		(4e-12/17%)	(0/100%)
XAJ3	XAJ3F	CTCGTCTTACCGTTCGTC AAC	785	<i>Pseudomonas suwonensis</i> 11-1	<i>Xanthomonas</i> sp. Leaf148
	XAJ3R	CGCCAAAGGACATTCACACACTC		(6e-16/10%)	(1e-12/21%)
XAJ4	XAJ4F	TCGATCGAATCAGTAGTCACTGGC	748	<i>Bifidobacterium animalis</i> subsp. <i>animalis</i> ATCC 25527	<i>X. hyacinthi</i> DSM 19077
	XAJ4R	CGGATCTCTTCATTCGTCAGGTG		(0.26/5%)	(1e-12/21%)
XAJ5	XAJ5F	AGCCTTGGGGTAGCTTTTGAGG	491	<i>Corynebacterium mustelae</i> DSM 45274	<i>X. axonopodis</i> pv. <i>glycines</i> CFBP 7119
	XAJ5R	CGTTCTTTGACGGCACTCCC		(0.17/6%)	(0/100%)
XAJ6	XAJ6F	AAGTCAGATGCGAAGCGAAAGG	436	<i>Ascaris lumbricoides</i> v1 5 4	<i>X. axonopodis</i> pv. <i>glycines</i> CFBP 7119
	XAJ6R	GCACAGCGGGAAAGTAATAGCAAAC		(0.51/6%)	(0/100%)
XAJ7	XAJ7F	ATAACTACCACCACTTCTCTGGGG	766	<i>Alligator sinensis</i>	<i>X. arboricola</i> pv. <i>celebensis</i> NCPPB 1832
	XAJ7R	TCTGGGCGAGGTAAAGTATTCTAC		(0.93/4%)	(1e-133/35%)
XAJ8	XAJ8F	GGGGTGAATCCAGTACGTGTCAC	588	<i>Monodelphis domestica</i>	<i>X. campestris</i> pv. <i>campestris</i> Xca5
	XAJ8R	CGCAGAACGTACTTTCGGTACTGG		(2.5/4%)	(8e-122/98%)
XAJ9	XAJ9F	CACGGGGATCAGCTTTTCATAC	443	<i>Ralstonia solanacearum</i>	<i>X. hortorum</i> pv. <i>carotae</i> M081
	XAJ9R	GCGTCGGTAACGATGTTTGTGTC		(1e-08/16%)	(2e-166/83%)

^a Q coverage = query coverage.

amplicons were sequenced in both strands and compared with *gyrB* sequences available in GenBank using the BLAST program.

To determine the phylogenetic relatedness of these isolates with other *Xanthomonas* spp., a maximum likelihood tree was built using the Tamura-Nei model in MEGA 6.0 (Tamura et al. 2013). The analysis was generated from the MUSCLE alignment of 145 *gyrB* sequences (34 sequences of *Xaj* Portuguese isolates and 111 sequences representing different species of *Xanthomonas*).

Experimental validation of putative *Xaj*-specific markers by dot blot. Validation of the nine putative *Xaj*-specific markers across a large set of *Xanthomonas* spp. strains and isolates was carried out using a dot blot hybridization platform. DNA probes were prepared from the purified amplicons obtained from PCR amplifications using *Xaj* strain LMG 751 (= NCPPB 1447), from which the fully sequenced genome was used in this study to select the DNA markers. A 20 µl PCR reaction mix consisted of 1× DreamTaq Buffer (Fermentas), 0.2 mM of each deoxynucleotide triphosphate (dNTP) (Fermentas), 0.2 µM of each forward and reverse primers (Table 1), 1U of DreamTaq DNA Polymerase (Fermentas), and 25 ng of DNA template. PCR cycling parameters were carried out with the implementation of a first amplification cycle of 5 min at 95°C, followed by 35 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 30 s, and a final DNA extension at 72°C for 10 min. The identity of each amplicon, used as probe, was

confirmed by sequencing. The obtained PCR products were purified using the illustra GFX GEL Band Purification kit (GE Healthcare, Buckinghamshire, United Kingdom), following the reference protocol available, and sequenced (STAB Vida, Caparica, Portugal).

Probes were labeled with digoxigenin using the DIG-High Prime kit, according to the manufacturer's instructions (Roche Diagnostics GmbH, Basel, Switzerland). For dot blot assays, 100 ng of heat-denatured bacterial DNA were spotted into nylon membranes (Roche Diagnostics GmbH, Basel, Switzerland) using a Bio-Dot apparatus (Bio-Rad, Hercules, CA). Each membrane was hybridized overnight at 68°C to ensure high stringency with a final probe concentration of 100 ng/ml, and stringency washes were performed as described in the DIG application manual. Probe-target hybrids were detected with chemiluminescent alkaline phosphatase substrate (CDP-Star) reagent (Roche Diagnostics, Basel, Switzerland) and the images were acquired using a Molecular Imager ChemiDoc system (Bio-Rad, Hercules, CA).

Multiplex PCR validation. A multiplex PCR targeting the most promising *Xaj*-specific markers was optimized in order to validate a method to rapidly identify *Xaj* isolates and for the direct diagnostics of *Xaj* infection in walnut tree samples. XAJ1, XAJ6, and XAJ8 were the chosen markers since they were shown to hybridize to most of the *Xaj* tested and have distinct amplicon lengths of 758, 436, and 588 bp, respectively. Multiplex PCR assays were performed on DNA extracted from bacterial strains/isolates and from plant tissue extracts. For chromosomal bacterial DNA, the PCR conditions were essentially the same as those used to obtain the DNA dot blot probes with an increase of DreamTaq DNA polymerase to 1.5U per PCR reaction. Concerning DNA extracted from infected plant material, PCR reactions were prepared with 2 µl of DNA extracted from infected walnut leaves and fruits using both the QIAGEN DNeasy Plant Mini kit and the CTAB-based DNA extraction as described above, and 1.5U of GoTAQ G2 Flexi DNA Polymerase (Promega, Madison, WI). For these samples, PCR amplification conditions were 10 initial PCR cycles at an annealing temperature of 55°C, followed by 30 cycles at an annealing temperature of 61°C. To further confirm multiplex PCR results from infected plant material, the same DNA samples were used as a template in PCR reactions using each of the markers individually (XAJ1, XAJ6, and XAJ8). PCR amplification of these three markers was performed as described to prepare the DNA dot blot probes.

Finally, to ensure that multiplex PCR amplification from symptomatic plant material was due to infection by *Xaj*, a multiplex PCR was carried out using the *Xaj* isolates obtained from the same infected plant samples. Partial sequencing of *gyrB* confirmed the *Xaj* identity of these isolates. All the 436 bp amplicons corresponding to the marker XAJ6 and obtained from both the plant samples and *Xaj* isolates were purified using the illustra GFX GEL Band Purification kit and sequenced to confirm their identity.

Detection limit of multiplex PCR. Sensitivity of multiplex PCR was determined with chromosomal DNA, bacterial suspensions, and spiked plant extracts using *Xaj* LMG 747. Chromosomal DNA multiplex PCR conditions were the same as described above for multiplex PCR validation with bacterial DNA template, using 2 µl of each 10-fold DNA dilutions ranging from 100 ng to 10 fg. Multiplex PCR detection limit of cell suspensions was obtained from 10-fold dilutions, prepared in SDW, of *Xaj* LMG 747 cells grown for 48 h at 28 ± 2°C on NA medium, within the range of 10⁸ to 10⁰ CFU/ml. Five microliters of each dilution was used as DNA template for multiplex PCR reactions, which were carried out as described above but increasing the initial denaturation step to 10 min at 95°C to ensure bacterial cell lysis. The *Xaj* LMG 747 suspension of 10⁸ CFU/ml was also used to determine the multiplex PCR detection limit in spiked walnut leaf samples. Walnut leaf fragments (about 0.6 g) were macerated as described previously. The resulting extract was used to prepare 10-fold serial dilutions of spiked walnut leaf samples ranging from 10⁷ down to 10⁰ CFU/ml. From each dilution, DNA was extracted using both CTAB-based method and QIAGEN DNeasy Plant Mini kit as described above. Multiplex PCR was carried out as described for naturally infected plant material.

Table 2. List of reference bacterial strains used for validation of *Xanthomonas arboricola* pv. *juglandis* specific markers

<i>Xanthomonas</i> species and pathovars ^a	Geographic origin	Year of isolation
<i>X. arboricola</i> pv. <i>juglandis</i> CFBP 176	France	1961
<i>X. arboricola</i> pv. <i>juglandis</i> CFBP 877	France	1966
<i>X. arboricola</i> pv. <i>juglandis</i> CFBP 2564	Italy	1985
<i>X. arboricola</i> pv. <i>juglandis</i> CFBP 2632	Spain	1984
<i>X. arboricola</i> pv. <i>juglandis</i> CFBP 5252	France	2000
<i>X. arboricola</i> pv. <i>juglandis</i> CFBP 6556	Italy	1993
<i>X. arboricola</i> pv. <i>juglandis</i> CFBP 6557	Italy	1999
<i>X. arboricola</i> pv. <i>juglandis</i> CFBP 7072	Spain	1993
<i>X. arboricola</i> pv. <i>juglandis</i> CFBP 7179 ^{GSS}	France	2002
<i>X. arboricola</i> pv. <i>juglandis</i> CFBP 7244	France	1978
<i>X. arboricola</i> pv. <i>juglandis</i> LMG 745	Netherlands	1978
<i>X. arboricola</i> pv. <i>juglandis</i> LMG 746	United Kingdom	1955
<i>X. arboricola</i> pv. <i>juglandis</i> LMG 747 ^{TS;GSS}	New Zealand	1956
<i>X. arboricola</i> pv. <i>juglandis</i> LMG 748	New Zealand	1956
<i>X. arboricola</i> pv. <i>juglandis</i> LMG 749	New Zealand	1957
<i>X. arboricola</i> pv. <i>juglandis</i> LMG 751 ^{GSS;*}	Romania	1962
<i>X. arboricola</i> pv. <i>juglandis</i> LMG 752	United Kingdom	1964
<i>X. arboricola</i> pv. <i>juglandis</i> LMG 8047	Netherlands	1979
<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> pv. <i>populi</i> CFBP 3123 ^{PRS}	Netherlands	1979
<i>X. arboricola</i> pv. <i>pruni</i> LMG 852 ^{PRS}	New Zealand	1953
<i>X. axonopodis</i> pv. <i>citri</i> LMG 9322 ^{TS}	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 ^{TS}	United States	1960
<i>X. gardneri</i> LMG 962 ^{GSS;TS}	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 ^{TS}	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;TS}	New Zealand	1955

^a 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. NCPPB: National Collection of Plant Pathogenic Bacteria, Fera Science Ltd., York, United Kingdom. *: other collection no: NCPPB 1447; TS, type strain; GSS, genome sequenced strain; PRS, pathovar reference strain.

Nucleotide sequences accession numbers. DNA sequences corresponding to the nine DNA markers (XAJ1 to XAJ9) were deposited in the NCBI database with the following accession numbers: KU577313 to KU577316 for XAJ1; KU577317 and KU577318 for XAJ2; KU577319 to KU577322 for XAJ3; KU577323 to KU577326 for XAJ4; KU577327 to KU577330 for XAJ5; KU577331 to KU577334 plus KX530957 for XAJ6; KU577335 to KU577337 for XAJ7; KU577338 to KU577341 for XAJ8; and KU577342 to KU577345 for XAJ9. NCBI accession numbers for *gyrB* of *Xaj* Portuguese isolates are shown in Table 3.

Results

In silico selection of DNA markers specific for *Xaj*. The comprehensive BLAST analysis of all ORFs (>100 bp) inferred from the draft genome of *Xaj* (NCPB 1447 = LMG 751; accession PRJNA84273) resulted in the selection of nine markers, designated as XAJ1 to XAJ9, which showed low identity (E value > 1e-10; query coverage <35%) to sequences of non-xanthomonads species (Table 1). Significant BLAST wgs hits were obtained with *Xanthomonas* species, namely with *X. arboricola* pv. *celebensis*, *X. perforans*, *X. axonopodis* pv. *glycines*, *X. campestris* pv. *campestris*, and *X. hortorum* pv. *carotae* (Table 1). Concerning ORF identity, none of the markers could be unequivocally assigned to a well characterized gene of the reference genome (accession PRJNA84273), being either located in intergenomic regions or in genomic regions coding for hypothetical proteins (Supplementary Table S1).

***gyrB* sequencing analysis of *Xaj* isolates obtained from walnut samples.** A set of 34 isolates was obtained from field samples of leaves and fruits from walnut trees exhibiting characteristic symptoms of WBB or/and BAN (Table 3). The assignment of all isolates

into *X. arboricola* was confirmed by *gyrB* gene sequencing analysis. In fact, all isolates showed higher sequence similarity to *X. arboricola*, with a BLAST E value of 0.0 and identity of 99 to 100%. In addition, the maximum likelihood tree showed that within the genus *Xanthomonas*, all the isolates clustered together with other *X. arboricola* pathovars. Moreover, four isolates (CPBF 1507, CPBF 1508, CPBF 1514, and CPBF 1522) were grouped in a different cluster (Fig. 1).

Experimental validation of the selected markers by dot blot.

The dot blot assays carried out using as probes the nine markers (XAJ1 to XAJ9) obtained from *Xaj* strain LMG 751, confirmed the specificity of the markers to *Xaj* since no hybridization was observed whatever the nontarget *Xanthomonas* spp. (Table 2) tested (data not shown), with exception of markers XAJ1 and XAJ7 that hybridized to *X. arboricola* pv. *celebensis* LMG 677 (Fig. 2), as predicted by the initial BLAST analysis (Table 1). Furthermore, from the 52 *Xaj* strains/isolates tested, 12 distinct hybridization profiles, designated as HP1 to HP12, were observed (Fig. 2). These hybridization patterns varied from *Xaj* hybridizing with the nine markers (HP1), to an *Xaj* strain (CFBP 2632) hybridizing with two markers (HP12). While most of these hybridization patterns were present in two to six *Xaj* strains/isolates (HP1, HP2, HP4, HP6, HP8, HP9, and HP11), one hybridization pattern was dominant and observed in 15 *Xaj* (HP10) and four hybridization patterns were unique to single *Xaj* strains/isolates (HP3, HP5, HP7, and HP12). Concerning the efficacy of the markers, it is evident that XAJ1, XAJ4, XAJ6, and XAJ8 hybridized to most of the *Xaj* strains and isolates, with emphasis for XAJ1, which hybridized with all the *Xaj* tested. Although the remaining markers were unable to hybridize with some *Xaj* strains/isolates, it is important to acknowledge their

Table 3. List of bacterial isolates collected in Portugal used in this study and the correspondent GenBank accession number of partial *gyrB* sequences (835 bp)

Isolates ^a	Host plant	Isolation sources ^b	Location, year	Accession number
CPBF 1271	<i>Juglans regia</i>	n/a	Azeitão, 2009	KU058325
CPBF 1479	<i>J. regia</i>	Leaves	Azeitão, 2014	KU058326
CPBF 1480	<i>J. regia</i>	Fruits	Azeitão, 2014	KU058327
CPBF 1484	<i>J. regia</i> cv. Hartley	Leaves	Alcobaça, 2014	KU058329
CPBF 1485	<i>J. regia</i> cv. Hartley	Fruits	Alcobaça, 2014	KU058330
CPBF 1486	<i>J. regia</i> cv. Lara	Leaves	Alcobaça, 2014	KU058331
CPBF 1487	<i>J. regia</i> cv. Franquette	Leaves	Alcobaça, 2014	KU058332
CPBF 1489	<i>J. regia</i> cv. Rego	Leaves	Alcobaça, 2014	KU058333
CPBF 1490	<i>J. regia</i> cv. Rego	Fruits	Alcobaça, 2014	KU058334
CPBF 1491	<i>J. regia</i> cv. Come	Fruits	Alcobaça, 2014	KU058335
CPBF 1492	<i>J. regia</i> cv. Amigo	Leaves	Alcobaça, 2014	KU058336
CPBF 1496	<i>J. regia</i> cv. Franquette	Fruits	Alcobaça, 2014	KU058337
CPBF 1497	<i>J. regia</i> cv. Come	Leaves	Alcobaça, 2014	KU058338
CPBF 1502	<i>J. regia</i> cv. Hartley	Leaves	Alcobaça, 2014	KU058339
CPBF 1503	<i>J. regia</i>	Leaves	Seia, 2014	KU058340
CPBF 1504	<i>J. regia</i>	Leaves	Seia, 2014	KU058341
CPBF 1505	<i>J. regia</i>	Fruits	Seia, 2014	KU058342
CPBF 1506	<i>J. regia</i>	Fruits	Seia, 2014	KU058343
CPBF 1507	<i>J. regia</i>	Leaves	Beja, 2014	KU058344
CPBF 1508	<i>J. regia</i>	Leaves	Beja, 2014	KU058345
CPBF 1509	<i>J. regia</i>	Fruits	Azeitão, 2014	KU058346
CPBF 1510	<i>J. regia</i>	Fruits	Azeitão, 2014	KU058347
CPBF 1511	<i>J. regia</i>	Fruits	Azeitão, 2014	KU058348
CPBF 1512	<i>J. regia</i>	Leaves	Seia, 2014	KU058349
CPBF 1513	<i>J. regia</i> cv. Hartley	Leaves	Estremoz, 2014	KU058350
CPBF 1514	<i>J. regia</i> cv. Hartley	Leaves	Estremoz, 2014	KU058351
CPBF 1518	<i>J. regia</i> cv. Lara	Leaves	Estremoz, 2014	KU058354
CPBF 1519	<i>J. regia</i> cv. Tulana	Leaves	Estremoz, 2014	KU058355
CPBF 1520	<i>J. regia</i> cv. Tulana	Leaves	Estremoz, 2014	KU058356
CPBF 1521	<i>J. regia</i>	Leaves	Loures, 2014	KU058357
CPBF 1522	<i>J. regia</i> cv. Howard	Leaves	Estremoz, 2014	KU058358
CPBF 1525	<i>J. regia</i> cv. Lara	Leaves	Estremoz, 2014	KU058359
CPBF 1526	<i>J. regia</i> cv. Lara	Leaves	Estremoz, 2014	KU058360
CPBF 1527	<i>J. regia</i> cv. Lara	Leaves	Estremoz, 2014	KU058361

^a CPBF: Coleção Portuguesa de Bactérias Fitopatogénicas, Instituto Nacional de Investigação Agrária e Veterinária, Oeiras, Portugal.

^b n/a: not available.

utility to obtain distinct hybridization patterns. For instance, the *Xaj* broad spectrum markers XAJ4, XAJ6, and XAJ8 did not hybridize with the group of *Xaj* strains and isolates sharing exactly the same hybridization pattern, i.e., HP9 and HP12 for marker XAJ4, HP11 for marker XAJ6, and HP8 and HP11 for marker XAJ8 (Fig. 2).

Multiplex PCR for identification of *Xaj* isolates and direct detection in infected walnut leaves and fruits. Reliable identification of putative *Xaj* isolates by multiplex PCR was optimized for markers XAJ1, XAJ6, and XAJ8. The multiplex PCR was validated with DNA extracted from all the strains and isolates listed in Tables 2 and 3, respectively. The results showed three distinct amplification profiles: amplification of the three markers for most *Xaj* strains/isolates (41 out of 52); amplification of two markers (XAJ1 and XAJ6) for one *Xaj* strain (CFBP 2632) and five *Xaj* isolates (CPBF 1484, CPBF 1485, CPBF 1486, CPBF 1487, and CPBF 1496); and amplification of a single marker (XAJ1) for four isolates (CPBF

1507, CPBF 1508, CPBF 1514, and CPBF 1522). As expected, whatever the marker, no specific amplification was obtained with any representative of other *Xanthomonas* species and with any other *X. arboricola* pathovars (pv. *corylina*, pv. *fragariae*, pv. *populi*, and pv. *pruni*), with exception of pv. *celebensis* (LMG 677) for which marker XAJ1 was amplified (Supplementary Fig. S1). Interestingly, the larger amplicon (with 1,669 bp) observed for isolate CPBF 1497 was confirmed to correspond to marker XAJ6. Sequencing analysis of this amplicon (accession no. KX530957) revealed the insertion of a gene coding for a transposase within marker XAJ6. Altogether, these multiplex PCR results corroborated the dot blot patterns obtained with these three markers. In fact, matching results were observed for dot blot patterns and multiplex PCR for all the *Xaj* strains/isolates tested, including the *X. arboricola* pv. *celebensis* (LMG 677) the only non-*juglandis* pathovar strain for which amplification/hybridization of marker XAJ1 was observed.

Fig. 1. Phylogenetic analysis based on 145 *gyrB* sequences of *Xanthomonas* species, including *Xanthomonas arboricola* isolates studied in this work. The tree was constructed according the maximum likelihood method and Tamura-nei model using MEGA 6.06. Bootstrap values higher than 50 are shown. *X. arboricola* strains and Portuguese isolates are highlighted in bold.

Multiplex PCR was also able to detect *Xaj* in DNA extracted from naturally infected walnut leaves and fruits (Fig. 3). Indeed, marker XAJ6 was efficiently amplified for the 11 plant samples assayed, regardless the DNA extraction procedure and plant organ, i.e., infected leaves (L1 to L5) or fruits (F1 to F6). Marker XAJ1 was amplified for all samples, with the exception of F6, and its amplification efficiency was higher for infected fruit samples and using CTAB-based DNA extraction. Marker XAJ8 was clearly less efficient, with positive amplification for five out of six fruit samples, and no amplification observed for F6. Regarding the leaf samples, this marker showed low efficiency in multiplex PCR with no amplification for most of the samples and poor amplification in sample L1. Faint nonspecific PCR bands were also observed for some samples. The efficiency of each of these three markers was higher in single PCR reactions for each of the 11 plant samples assayed, particularly for the samples where no amplification was obtained with the multiplex PCR, emphasizing the utility of single PCR reactions to resolve the lower efficiency of multiplex PCR particularly observed with leaf samples and marker XAJ8 (Fig. 3).

In parallel, to demonstrate that plant samples were infected by *Xaj*, at least one putative *Xaj* isolate obtained from each of these infected leaves samples (L1 to L5) was screened for the presence of the XAJ6 marker and further identified by *gyrB* sequencing (Supplementary

Fig. S2). With exception for the *Xaj* putative isolate obtained from L1 sample, for which no XAJ6 and *gyrB* amplification was obtained, all the other isolates were positive for marker XAJ6 and *gyrB*. Sequence analysis of both XAJ6 and *gyrB* for each of these isolates (L2 to L5) further confirmed their identity as *X. arboricola*. Taken together, these results underline the robustness of multiplex PCR for the direct detection of *Xaj* in symptomatic walnut leaves.

Multiplex PCR sensitivity. The detection limit of multiplex PCR targeting the specific *Xaj* markers XAJ1, XAJ6, and XAJ8, determined by serial dilution of chromosomal DNA, bacterial cell suspensions, and spiked plant extracts, was 20 pg DNA (10 pg/μl), 10⁴ CFU/ml, and 10³ CFU/ml, respectively (Supplementary Fig. S3).

Discussion

Aiming to develop robust and culture-independent DNA-based approaches for identification of *Xaj* isolates and in planta detection of *Xaj*, several specific DNA loci were selected based on a thorough BLAST analysis of the first *Xaj* draft genome made available (accession PRJNA84273). The putative *Xaj*-specific loci were then filtered to infer their suitability as DNA markers considering their genomic stability and detection by PCR or dot blot hybridization, as previously described (Albuquerque et al. 2012).

Fig. 2. Dot blot validation of *Xanthomonas arboricola* pv. *juglandis* (*Xaj*) specific markers (XAJ1 to XAJ9). Markers were tested across 18 *Xaj* reference strains, 34 *Xaj* isolates, and five non-*juglandis* pathovars of *X. arboricola* (Tables 1 and 2). Twelve different hybridization patterns (HP1 to HP12) were identified. The strain LMG 751 was used as positive hybridization control, and 13 non-*arboricola* *Xanthomonas* species (Table 2) were used as negative hybridization control (data not shown). DNA probes for the nine markers were prepared from the purified amplicons obtained from PCR amplifications using *Xaj* strain LMG 751 (= NCPPB 1447).

Fig. 3. Multiplex and single PCR reactions for detection of *Xanthomonas arboricola* pv. *juglandis* in naturally infected walnut leaves (L1 to L5) and fruits (F1 to F6) using three markers, XAJ1 (758 bp), XAJ6 (436 bp), and XAJ8 (588 bp). Each number represents a different walnut (*Juglans regia*) tree. For each sample, two PCR reactions were carried out, one using DNA extracted with the QIAGEN DNeasy Plant Mini kit (a), and the other with a CTAB-based DNA extraction procedure (b). For all the samples the identity of XAJ6 marker was confirmed by sequencing. Strain LMG 751 was used as positive control (C+).

The BLAST analysis revealed that the selected markers (XAJ1 to XAJ9), despite the low query coverage for markers XAJ4 and XAJ7, shared similarity with other *Xanthomonas* species (Table 1, wgs blast). Significant BLAST hits were obtained with other plant pathogens never identified from walnut, such as *X. arboricola* pv. *celebensis* (a banana plant pathogen), *X. perforans* (associated with tomato bacterial spot disease), *X. axonopodis* pv. *glycines* (causal agent of bacterial pustule of soybeans), *X. campestris* pv. *campestris* (attacks *Brassica* spp., responsible for the black rot of crucifers), and *X. hortorum* pv. *carotae* (a bacterial pathogen of carrot). The fact that these *Xanthomonas* spp. do not share the same ecological niche with *Xaj* (Hayward 1993; Leyns et al. 1984) supports the adequacy of these markers to establish new molecular methodologies of detection and identification. Meanwhile, a complementary BLAST search revealed the presence of these markers, with exception for marker XAJ2, in the recently released *Xaj* genomes (Cesbron et al. 2015; Higuera et al. 2015; Pereira et al. 2015).

In addition to the BLAST analysis, a validation by dot blot hybridization was carried out in order to confirm the consistency and specificity of the nine markers across a broad range of xanthomonads (Fig. 2). All the 18 *Xaj* strains and 34 *Xaj* isolates clustered into 12 different hybridization profiles, varying from hybridization with all the nine markers corresponding to HP1 (strains LMG 751 and CFBP 2564; Portuguese isolates CPBF 1479 and 1480), to hybridization with only two markers corresponding to HP12 (strain CFBP 2632). No evident relatedness was observed between these hybridization patterns and the geographic origin from which the isolates were obtained. Indeed, isolates from the same location displayed different hybridization profiles (e.g., HP4 for CPBF 1489 and HP10 for CPBF 1502 from Alcobaça), and inversely, identical hybridization patterns were found for isolates from distinct locations (e.g., HP10 for the isolates CPBF 1510 from Azeitão and CPBF 1513 from Estremoz). Taken together, these results demonstrate the utility of this dot blot platform to disclose the high genomic diversity of *Xaj*, as reported by several genotyping studies (Essakhi et al. 2015; Giovanardi et al. 2016; Hajri et al. 2010, 2012; Ivanović et al. 2015; Kałużna et al. 2014; Loreti et al. 2001; Marcelletti et al. 2010; Scortichini et al. 2001). Most importantly, the distinct hybridization patterns can be informative to characterize different *Xaj* lineages, which can be particularly convenient before carrying out laborious fine tune methods of genotyping such as multilocus sequence analysis (MLSA) and multilocus sequence typing (MLST), or full genome sequencing.

When looking at the dot blot efficacy, it is evident that markers XAJ1, XAJ4, XAJ6, and XAJ8 hybridized with most of the *Xaj* strains tested (Fig. 2) and should be primary choices for detection and identification of *Xaj*. Interestingly, the *Xaj* isolates that did not hybridize to markers XAJ4, XAJ6, and XAJ8 shared identical hybridization profiles, suggesting that the genotype of these markers might be determined by either their presence or absence. In this regard, it is worth mentioning that the four *Xaj* isolates (CPBF 1507, 1508, 1514, and 1522) corresponding to HP11 were PCR positive exclusively to marker XAJ1 in the multiplex assay as expected, and clustered together in the *gyrB* tree (Fig. 1).

The specificity of the selected markers to *Xaj* was shown by the fact that none of the nine markers hybridized with the 12 non-*arboricola* strains of *Xanthomonas* spp., or to any of the other five *X. arboricola* pathovars studied, with exception for *X. arboricola* pv. *celebensis* LMG 677, which hybridized to markers XAJ1 and XAJ7, confirming the blast wgs analysis (Table 1).

Currently, only a PCR method has been reported for identification of *Xaj* isolates (Gironde et al. 2009). Although this PCR approach has been used in recent studies (Burokiene and Pulawska 2012; Ivanović et al. 2015; Kałużna et al. 2014), the method is based on a single DNA marker and the sequence of the primers has not been published, undermining the full assessment of this method, namely in infected plant material. In this work, we propose a triplex PCR using markers XAJ1, XAJ6, and XAJ8, optimized to ensure an efficient, reliable, and rapid method to identify *Xaj* isolates, as well as for culture-independent detection of *Xaj* in plant samples. These three markers were chosen due to their different amplicon sizes and their consistency across the different *Xaj* strains (Fig. 2). The validation of the multiplex

PCR was carried out with DNA extracted from all the *Xaj* strains and isolates, other pathovars of *X. arboricola*, and non-*arboricola* species of *Xanthomonas* included in this work (Tables 2 and 3). The PCR multiplex results fully confirmed the dot blot data, i.e., amplification was observed for any of the three markers, whenever the dot blot was positive for the corresponding marker, and whatever the *Xaj* strain or isolate tested. It should be noted that a simplex PCR using marker XAJ1 would also be satisfactory for routine detection of *Xaj* in infected plant samples, since this marker is highly specific and present throughout all tested *Xaj* strains. However, the higher detection confidence provided by the use of three independent markers cannot be underestimated, especially when considering *Xaj* diversity.

PCR based methods can be a valuable approach for direct detection of *Xaj* in plant tissues, allowing a rapid and culture-independent detection of *Xaj* in walnuts, relevant for an early diagnosis of WBB or BAN and to determine the bacteria life cycle overwinter. In this study, we were able to amplify the markers XAJ1, XAJ6, and XAJ8 in DNA extracted from naturally infected walnut leaves and fruits indicating the presence of *Xaj* (Fig. 3). It has been acknowledged that the quality, purity, and quantity of DNA can impair the detection in plant material (Demeke and Jenkins 2010; López et al. 2003). The multiplex efficiency, particularly low for the larger markers (XAJ1 and XAJ8), could be related with the presence of plant-derived compounds or even treatment derived chemicals that may interfere with the amplification (Schrader et al. 2012). The higher efficiency observed for DNA extracted using the CTAB procedure in comparison with DNeasy, and for fruit samples rather than for leaves, seems to support this hypothesis. Furthermore, when the three markers were amplified in independent PCR reactions, markers' efficiency increased considerably, whatever the plant material and the DNA extraction procedure (Fig. 3). The faint unspecific amplifications observed in the multiplex PCR were likely favored by the lower annealing temperature, since most of these unspecific bands disappeared in the single PCR amplification of each marker (Fig. 3). The detection threshold of multiplex PCR for chromosomal DNA (10 pg/μl) and bacterial cells (10⁴ CFU/ml corresponding to 50 cells per reaction) are within the sensitivity range of multiplex PCR for other *Xanthomonas* spp. (Araújo et al. 2012; Pothier et al. 2011; Toth et al. 1998). In addition, the multiplex PCR detection limit of 10³ CFU/ml *Xaj* in spiked walnut samples (i.e., two cells per reaction) demonstrate that the load of *Xaj* cells in field infected walnut leaves and fruits (Fig. 3) are well above this detection limit, which strengthens the effectiveness of this method for routine detection and to access *Xaj* in latently infected plant material (Hayward 1974).

In conclusion, this work proposes nine novel molecular markers for identification of *Xaj* isolates and its detection in infected plant samples using a dot blot hybridization assay or multiplex PCR. While four markers (XAJ1, XAJ4, XAJ6, XAJ8) were shown to be particularly effective, since they were detected in most of the *Xaj* tested, regardless of their genetic diversity, the other five markers, although clearly specific to *Xaj*, displayed a narrower, but complementary detection range. Beyond the usefulness of these markers to identify and discriminate different *Xaj* lineages, we demonstrated that the broad range markers XAJ1, XAJ6, and XAJ8 might be used in culture-independent approaches. Attending that direct detection of *Xaj* in plant material has never been described, these results are a promising contribution for the early and reliable diagnostics of walnut diseases caused by *Xaj*.

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