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Development of a multi-targeted real-time PCR assay for the detection of the grapevine pathogen *Xylophilus ampelinus*

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Abstract

Background *Xylophilus ampelinus* is a plant pathogenic bacterium that causes bacterial blight in grapevines, which can lead to severe yield losses and economic damage. Owing to its fastidious growth on culture media, detection is primarily based on molecular methods. However, existing tests have produced inconsistent results, particularly when used to detect latent infections and non-validated matrices. There is a risk of false-positive results, with economic consequences such as restrictions on international trade. To enhance the diagnostics of *X. ampelinus*, a genome-informed approach was utilised to identify new potential targets for specific detection. On the basis of these sequences, multiple real-time PCR assays were designed, and their specificity and sensitivity were assessed, as well as their performance validated across three different grapevine tissues, including leaves, roots, and xylem.

Results The newly designed real-time PCR assays were evaluated via high throughput testing for specificity and sensitivity and compared with a reference assay. The most promising assays were selected and validated in different grapevine tissues and included in a test performance study to validate their reproducibility and robustness. Three new assays (Xamp_BA_2, TXmp22.4, and Xamp_BA_7) demonstrated high specificity and sensitivity for *X. ampelinus* detection. The Xamp_BA_2 assay exhibited the best overall performance, offering high diagnostic sensitivity and robustness across diverse plant matrices. Importantly, the assays exhibited no cross-reactivity with non-target bacterial species and maintained high detection accuracy across diverse grapevine tissue types.

Conclusions The newly developed real-time PCR assays provide an enhanced diagnostic framework for the detection of *X. ampelinus* in various plant matrices, significantly improving the applicability of molecular testing. The Xamp_BA_2 assay demonstrates superior performance and is recommended for routine diagnostics, with other validated assays being employed for confirmation of identification. The development of these new assays represents a significant expansion of our toolkit for the precise detection of *X. ampelinus* in grapevines, with the potential to contribute to the mitigation of grapevine bacterial blight, the prevention of yield losses, and the protection of international trade in grapevine material. Further implementation of these assays will support regulatory and phytosanitary efforts to mitigate the spread of *X. ampelinus*.

Keywords *Xylophilus ampelinus*, Grapevine bacterial blight, Molecular diagnostics, *Vitis vinifera*, Real-time PCR, Genome-informed assay development

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Background

Xylophilus ampelinus is a gram-negative betaproteobacterium (order Burkholderiales) that is responsible for the bacterial blight of grapevine (*Vitis vinifera*). Its initial isolation in 1969 in Greece designated it *Xanthomonas ampelina* [1], prior to its subsequent transfer to the newly established genus *Xylophilus* [2]. The only other described member of this genus is *X. rhododendri*, which was isolated from *Rhododendron schlippenbachii*. *X. ampelinus* is widely distributed in the European Mediterranean region, where it has been isolated in Greece, Italy, France, Moldavia, Russia, Slovenia, Spain and Ukraine [3–5]. In addition to Europe, it has also been identified in Jordan [6], South Africa [7] and Japan [8].

X. ampelinus is known to cause systemic infection of the xylem tissue, where the bacterium also overwinters. The bacterium is transmitted both locally, through the use of infected tools and machinery, and over long distances, via infected cuttings used for rooting or grafting. The symptoms include necrotic lesions of the leaves, cracks and cankers along the infected shoots, brown discolouration of the xylem tissue and dead branches [9]. The severity of symptoms exhibited by plants can vary significantly from year to year, contingent on prevailing climatic conditions. Notably, latent infections are prevalent in infected vineyards [3]. The economic impact of *X. ampelinus* is significant, and the disease is listed as a regulated non-quarantine pest (Annex IV, part C of Commission Implementing Regulation (EU) 2019/2072 of 28 November 2019) and is on the A2 list of pests recommended for regulation as quarantine pests by the European and Mediterranean Plant Protection Organisation (EPPO; https://www.eppo.int/ACTIVITIES/plant_quarantine/A2_list; version 2024-09). A significant aspect of the investigations pertains to the international trade in plant material, wherein positive findings can bear substantial economic ramifications, such as the imposition of an import ban.

Owing to the non-specific symptoms of the disease, laboratory tests are required to confirm the infection. Typically, a screening test is performed to detect the pathogen, followed by the isolation of bacteria on culture media if the screening test results are positive or suspicious. However, it should be noted that bacteria may not always be isolated reliably on artificial culture media because of their slow growth rate. Consequently, molecular methods that rely on DNA amplification are predominantly employed for the detection of bacteria in plants. One notable example is the quantitative real-time PCR (real-time PCR) assay developed by Dreo et al. in 2007 [6], which was among the first real-time PCR tests to be incorporated into the EPPO protocol [3]. Other molecular detection methods include PCR [10], which can be combined with an ELISA-based signal amplification system, nested PCR [11] and multiplex PCR [12].

The grapevine samples utilised for detection can be obtained from diverse parts of the plant at varying annual growth stages and can be either symptomatic or asymptomatic. The assays are validated for detection within a limited concentration range in a specific matrix, as plant extracts often contain inhibitors for PCR amplification or may cause cross-reactivity. The use of assays in matrices for which they have not been validated can lead to problematic outcomes. In the case of *X. ampelinus*, contradictory results have been reported from different assays when vine cuttings or root offshoots were analysed for latent infections.

The validation of existing assays on new matrices prior to conducting laboratory tests, as well as the development of new assays capable of detecting *X. ampelinus* in a broader range of matrices, is imperative for reliable detection. The molecular assays currently in use are based on only a few different genetic targets, with real-time PCR by Dreo et al. (2007) and PCR by Manceau et al. (2000) based on the Xamp 1–27 A fragment [6, 13] and nested PCR by Botha et al. (2001) based on the intergenic spacer region of 16–23 S rDNA [11]. This limits the possibility for reliable detection in the event of a false-positive result with one of the currently used tests. The objective of this study was to identify new genetic targets for the specific detection of *X. ampelinus* via a genome-based approach and to utilise these targets for the development of several new real-time PCR assays. These assays were evaluated on the basis of their specificity and sensitivity, with the most promising assays subsequently validated for the detection of *X. ampelinus* in various grapevine samples (leaves, roots, and xylem). To investigate the robustness and applicability of the selected assays, a test performance study (TPS) was conducted in collaboration with other laboratories. The objectives of this study were to validate a series of new real-time PCR assays that should allow reliable detection of *X. ampelinus* in a wide range of grapevine samples and, second, to identify assays suitable for further use in diagnostics (Fig. 1).

Methods

In silico analysis and assay design

The genomes of bacteria of the genus *Xylophilus* used for the in silico analysis were obtained from the publicly accessible GenBank database (Fig. 1: A, Table S1). The average nucleotide identity (ANI) analysis was performed using the online tool enve-omics [14] to calculate the ANI matrix and construct phylogenetic trees using the neighbour-joining method. The program MEGA v11.0.13 was used to visualise the phylogenetic tree. An automated analysis for the determination of unique core sequences (UCS) was performed using a program for the rapid

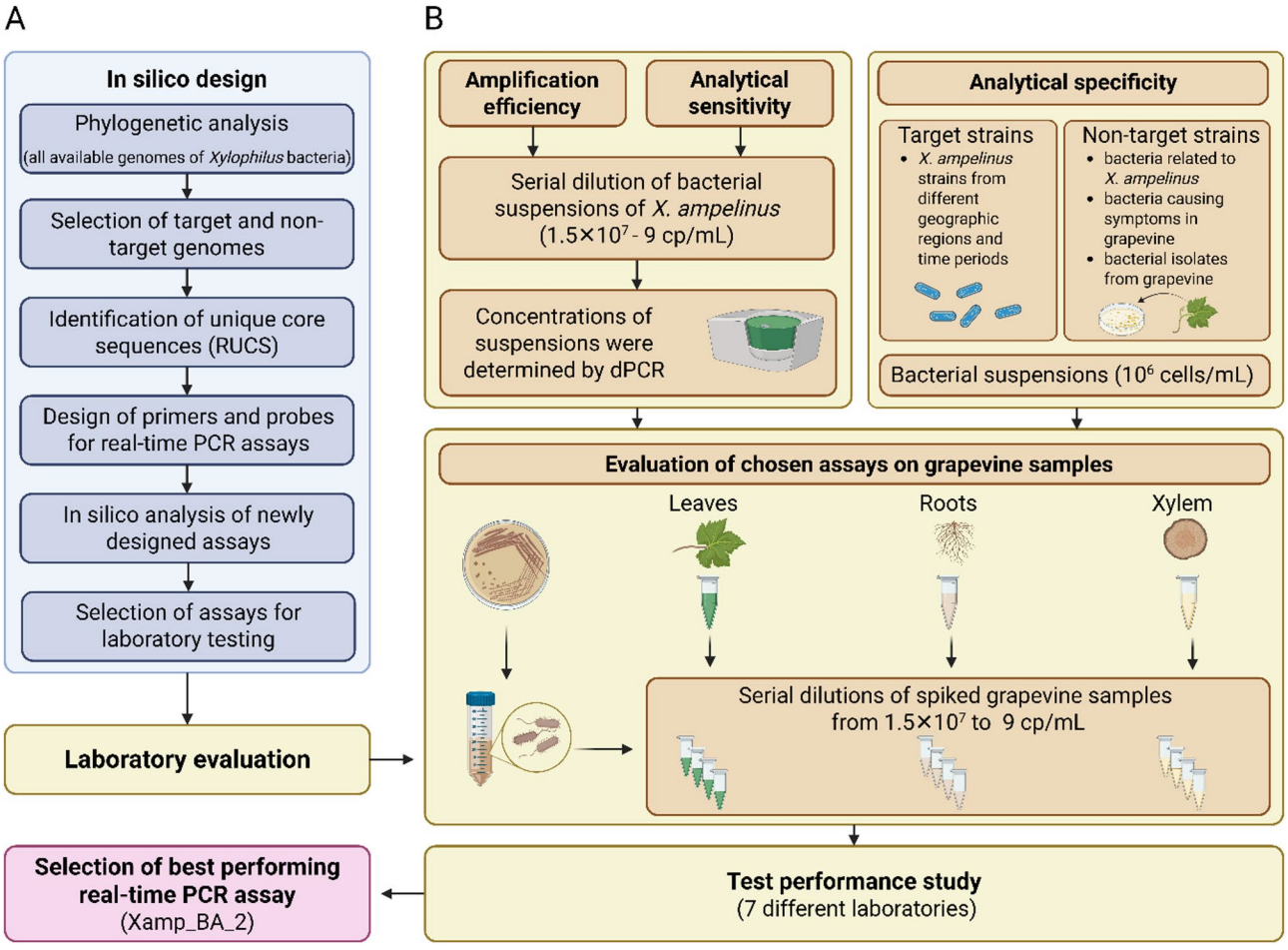

Fig. 1 Schematic overview of the complete real-time PCR design (**A**) and validation (**B**). This involved the in silico design of new assays, which included phylogenetic analysis of all the genomes of the genus *Xylophilus* available at the time. The results of phylogenetic analysis were used to determine target and non-target genomes, which were used in the identification of UCS by RUCS. The primers and probe were designed using Primer Express 2 on UCS. The evaluation of new real-time PCRs included the determination of analytical specificity and sensitivity and amplification efficiency. Five selected assays were validated on grapevine samples from leaves, roots and xylem. Four assays were then used in the TPS, which involved the participation of seven laboratories. The best performing assay was selected on the basis of the TPS and previous in-house validation

identification of PCR primers for unique core sequences: the RUCS [15] web server (https://cge.food.dtu.dk/services/RUCS/). The RUCS programme uses two datasets for the analysis. The first dataset is labelled positive and contains the genomes of all bacteria to be detected. The second dataset is labelled negative and contains the genomes of bacteria that should not be detected. These are usually closely related bacteria that do not belong to the target taxon. Initially, RUCS determines the core genome of the positive genomes, subsequently identifying the sequences present in the core genome but absent in the negative genomes. The application of RUCS was undertaken for the identification of genome sequences specific to *Xylophilus ampelinus*, using a reference genome assembly of the type strain CECT 7646^{T} (GenBank accession number GCA_003217575.1), which was the sole available genome of *X. ampelinus* at the time of analysis. Two separate analyses were performed with two different negative datasets (Table S2). The top ten sequences identified in each RUCS analysis were then used to design the real-time PCR assays. The primers and hydrolysis probes were designed using Primer Express version 2.0 (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA). BLASTN [16] was performed using standard parameters to determine the position of the newly designed assays in the *X. ampelinus* CECT 7646^{T} genome and to assign them to the corresponding genes. To identify similar sequences that might cause cross-reactivity, BLASTN was performed using the Core Nucleotide BLAST database. The calculation of amplicon length, melting temperature, guanine and cytosine percentages, and secondary structures was conducted using the web tool OligoAnalyzer (idtdna.com/calc/analyzer; Integrated DNA Technologies).

Preparation of testing material

Bacterial strains

The bacterial strains used in the study are listed in Table 1. All bacteria were cultivated on nutrient agar media (Bacto Nutrient Agar; Difco), with the exception of *X. fastidiosa*, which was cultivated on BCYE media [17]. The isolates of *X. ampelinus* were cultivated at 25 °C, while the other bacterial strains were cultivated at 28 °C. The new bacterial isolates employed in the study (labelled I in Table 1) were isolated from extracts of grapevine leaves and roots. The isolates were identified by MALDI-TOF mass spectrometry (smartfleX, Bruker, Billerica, US) and 16 S rRNA barcoding using the primers described in EPPO Standard PM 7/129 [2, 18].

Plant material

Asymptomatic leaves, roots and xylem of the grapevine (*Vitis vinifera*) were used for the preparation of the plant extracts (Fig. 1: B). The vine leaves were collected by the Administration for Food Safety, Veterinary Sector and Plant Protection as part of the regular annual monitoring programme coordinated by the Phytosanitary Administration of the Republic of Slovenia in 2023. The plant material used in this study was procured from Trsničarska zadruga Vrhpolje (Vrhopolje, Slovenia). The leaf extracts were prepared by cutting selected leaves (1 g fresh weight per 5 mL buffer) and placing them in sterile phosphate buffer (0.01 M PBS; 1.08 g Na_2HPO_4, 0.4 g $NaH_2PO_4 \times 2H_2O$, 8 g NaCl, 1 L distilled water, pH 7.2) with 10% glycerol (v/v), shaken and incubated for 15 min at room temperature. After incubation, the samples were

Table 1 List of bacterial strains used in validation. The table contains target strains that belong to *X. ampelinus* and non-target strains, which include bacteria related to *X. ampelinus*, bacteria that cause symptoms similar to those of *X. ampelinus*, and bacteria isolated from grapevine. Comments: P-grapevine pathogen, T-type strain, I-strain isolated from grapevine, R-related bacterium

Type	Bacterial species	Strain	Isolated	Comments
Target	*Xylophilus ampelinus*	LMG 5856^T	Greece (1966)	P, T
		LMG 518	Greece (1966)	*P*
		LMG 527	France (1969)	*P*
		LMG 520	South Africa (1972)	*P*
		LMG 510	Spain (1982)	*P*
		CFBP 2875	France (1988)	*P*
		CFBP 4864	South Africa (1994)	*P*
		CFBP 4717	France (1998)	*P*
		CFBP 5787	France (2001)	*P*
		NIB Z 3131	Slovenia (2002)	*P*
		NIB Z 3147	Slovenia (2004)	*P*
		NIB Z 2746	Slovenia (2018)	*P*
Non-target	*Xylophilus rhododendri*	KACC 21,265^T	Korea (2019)	R, T
	Agrobacterium tumefaciens	NIB Z 3648	Slovenia (2023)	I
	Agrobacterium vitis	Av 13 – 2	Slovenia (2002)	*P*
	Ancylobacter novellus	NIB Z 3643	Iran (2017)	I
	Bacillus sp.	NIB Z 3649	Slovenia (2023)	I
	Brevundimonas nasdae	NIB Z 3642	Iran (2017)	I
	Cellulomonas sp.	NIB Z 3645	Iran (2017)	I
	Enterobacter sp.	NIB Z 3656	Slovenia (2023)	I
	Enterobacteriaceae	NIB Z 3654	Slovenia (2023)	I
	Erwinia sp.	NIB Z 3653	Slovenia (2023)	I
	Pantoea agglomerans	NIB Z 2034	Slovenia (2014)	I
	Pantoea agglomerans	NIB Z 3644	Iran (2017)	I
	Pantoea agglomerans	NIB Z 3646	Slovenia (2023)	I
	Pseudomonas syringae	CFBP 2896	Japan (1951)	*P*
	Pseudomonas chlororaphis	NIB Z 3647	Slovenia (2023)	I
	Pseudomonas graminis	NIB Z 2045	Slovenia (2014)	I
	Rahnella aquatilis	NIB Z 3651	Slovenia (2023)	I
	Rouxiella badensis	NIB Z 3652	Slovenia (2023)	I
	Serratia liquefaciens	NIB Z 3655	Slovenia (2023)	I
	Sodalis sp.	NIB Z 3650	Slovenia (2023)	I
	Xylella fastidiosa	CFBP 8075	USA (1997)	*P*
	Xylella fastidiosa	CFBP 8351	USA (1993)	*P*

filtered through a sterile cloth, and the filtrate was stored at -20 °C until further analysis. To produce the root extracts, the grafted vines were grown in sterile water for 3 weeks before the root offshoots were sampled. To prepare the xylem extracts, the bark was first removed from the grafted vines, and then the xylem tissue was cut into small pieces. After the sampling process, the xylem and root extracts were prepared in accordance with a uniform protocol. 1 g of plant tissue was placed in an extraction bag and macerated with a Homex 7 homogeniser (Bioreba AG, Reinach, Switzerland). Next, 5 mL of 0.01 M PBS with 10% glycerol (v/v) was added, and the mixture was incubated for 15 min. The supernatant was then separated from the plant tissue by pipetting and stored at -20 °C until further testing.

Preparation of the samples

The bacterial suspensions were prepared in sterile 0.01 M PBS with 10% glycerol (v/v). The concentrations of the bacterial suspensions were determined turbidimetrically using a densitometer (DEN-1B; BioSan, Riga, Latvia). The bacterial suspensions of the strains listed in Table 1, which were used to determine the analytical specificity, were diluted to a concentration of 10^6 cells/mL. To determine the analytical sensitivity, 10-fold serial dilutions ranging from 10^8 to 10 cells/mL of a bacterial suspension of *X. ampelinus* (strain LMG 5856^T) in 0.01 M PBS with 10% glycerol (v/v) were prepared. The effects of the plant extracts on sensitivity were evaluated, and the diagnostic sensitivity of the selected real-time PCR assays was determined. Plant extracts from leaves, roots and xylem were spiked with 10-fold serial dilutions of a bacterial suspension of *X. ampelinus* (strain LMG 5856^T). The target concentrations of *X. ampelinus* in the spiked samples were 10^8, 10^7, 10^6, 10^5, 5×10^4, 10^4, 5×10^3, 10^3, 10^2 and 10 cells/mL. The concentrations of *X. ampelinus* in the serial dilution samples and the spiked samples were additionally determined by dPCR (QX200, Bio-Rad, Pleasanton, USA) using primers and probes from Dreo et al. (2007) [9] (Fig. 1: B, Table S3).

DNA extraction

DNA was extracted from 100 µL aliquots of bacterial suspensions, plant extracts and spiked plant extracts via the magnetic bead-based QuickPick SML Plant DNA Kit (Bio-Nobile, Turku, Finland) on an automated KingFisher mL system (Thermo Fisher Scientific). Molecular grade water (100 µL) was used as a negative control for DNA extraction. Extraction was performed as previously described by Pirc et al. (2009) [19] with slight modifications (415 µL of lysate for purification) [20]. The DNA was stored at -20 °C until analysis.

Digital PCR

The DNA of *X. ampelinus* extracted from 10-fold serial dilutions of bacterial suspensions in PBS and samples used in the TPS was quantified using the QX200™ Droplet Digital™ PCR system (Bio-Rad) and primers and probe from Dreo et al. (2007). PCR reactions consisted of 10 µL of ddPCR Supermix for probes (Bio-Rad) and 4 µL of sample DNA, with the same primer and probe concentrations used for real-time PCR. After droplet generation, 40 µL of the generated droplet emulsion was transferred to a 96-well PCR plate and amplified in a thermal cycler. The amplification conditions were 10 min of DNA polymerase activation at 95 °C, followed by 40 cycles of a two-step thermal profile of 30 s at 94 °C for denaturation and 60 s at 60 °C for annealing and extension, followed by a final hold of 10 min at 98 °C for droplet stabilisation and cooling to 4 °C. The temperature ramp was set to 2.5 °C/s, and the lid was heated to 105 °C. After the thermal cycle, the plates were transferred to a droplet reader. The software package supplied with the dPCR system (QuantaSoft 1.7.4.0917, Bio-Rad) was used for data acquisition.

High-throughput real-time PCR

High-throughput real-time PCR was performed to determine the analytical specificity and sensitivity of the newly designed real-time PCR assays. This was done using a microfluidics-based platform (Standard BioTools - formerly known as Fluidigm, South San Francisco, USA) and a 48.48 Dynamic Array IFC (Integrated Fluidic Circuit) chip (Standard BioTools). Both the assays and the samples were run in two replicates, resulting in four technical replicates. The analysis included DNA extracted from bacterial suspensions (10^6 cells/mL) of the strains in Table 1 and DNA extracted from the samples in Table S3 at concentrations of 2.8×10^4, 2.8×10^5, 2.4×10^6 and 1.5×10^7 cp/mL. The assay mixtures were prepared with the following components per sample: 3 µL 2× Assay Loading Reagent (Standard BioTools, PN 85000736) and 3 µL 20× mixture of primers and probe. The sample premix was prepared with the following components: 3 µL of TaqMan Universal PCR Master Mix (Applied Biosystems), 0.3 µL of 20× GE Sample Loading Reagent (Standard BioTools) and 2.7 µL of bacterial DNA. The 48.48DA was primed with the IFC Controller MX (Standard BioTools) prior to loading the samples and assays. 5 µL of the sample mixture and 5 µL of the assay mixture were pipetted into the inlets on the chip. The chip was then inserted into the IFC controller, where it undertook the loading and mixing of the samples and assays. After loading and mixing, thermal cycling was performed using the BioMark HD (Standard BioTools) under the following conditions: 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 60 s at 60 °C. Two non-template controls were included to control for

non-specific amplification and sample contamination. The data collected with the BioMark HD system were analysed with Fluidigm Real-Time PCR Analysis Software version 3.1.3 (Standard BioTools).

Quantitative real-time PCR

Real-time PCR was performed using newly designed real-time PCR assays and the reference assay from Dreo et al. (2007) (Table S4). All reactions were performed in three technical replicates on a ViiA 7 real-time PCR system (Applied Biosystems) under the same universal cycling conditions: 2 min at 50 °C and 10 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 60 s at 60 °C in standard temperature ramp mode (1.6 °C/s). The reaction volumes of 10 µL contained the following final concentrations: 900 nM primers (Integrated DNA Technologies, Inc.), 250 nM 6-carboxyfluorescein (FAM) and Black Hole Quencher (BHQ)-1 labelled probe (Integrated DNA Technologies, Inc.), TaqMan Universal PCR Master Mix (Applied Biosystems) and 2 µL of DNA sample. QuantStudio software v1.6 (Applied Biosystems) was utilised for the acquisition of fluorescence and the calculation of the quantification cycle (C_q) value. The baseline was set automatically, and the fluorescence threshold was manually adjusted to overlap with the linear portion of the amplification curves of all real-time PCR assays, which yielded the final C_q value for each well.

Performance characteristics of real-time PCR assays

Analytical specificity was assessed using DNA extracted from bacterial suspensions (10^6 cells/mL) of target and non-target bacterial strains (Table 1). Analytical specificity was also determined using 10 µL real-time PCR reactions, with the results interpreted as positive if at least one replicate was positive. The analytical sensitivity of the designed real-time PCR assays was determined on the basis of DNA extracted from 10-fold serial dilutions of a bacterial suspension of *X. ampelinus* (strain LMG 5856^T) in 0.01 M PBS with 10% glycerol (v/v). All samples and controls were tested in three technical replicates using the ViiA 7 real-time PCR system (Applied Biosystems). The effects of the plant matrices, leaves, roots and xylem on amplification efficiency and sensitivity were analysed via five new real-time PCR assays and the reference assay Dreo et al. (2007). The diagnostic sensitivity was evaluated on DNA extracted from samples of plant extracts of leaves, roots and xylem spiked with 10-fold serial dilutions of a bacterial suspension of *X. ampelinus* (strain LMG 5856^T). The assigned concentrations of *X. ampelinus* determined by dPCR in the spiked samples were 1.5×10^7, 2.4×10^6, 2.8×10^5, 2.8×10^4, 1.8×10^4, 6.2×10^3, 2.3×10^3, 3.7×10^2, 71 and 9 cp/mL (Table S3). To determine the cross-reactivity of the DNA extracted from the plant extracts, real-time PCR assays were used. Molecular grade water was used as a no-template control, whereas DNA extracted from *X. ampelinus* (strain LMG 5856^T) was used as a positive control.

Test performance study (TPS)

The performance of the assays selected in a previous validation (Dreo et al. (2007), TXmp22.4, Xamp_BA_2, Xamp_BA_6 and Xamp_BA_7) was evaluated in a TPS. The TPS sample panel consisted of 30 test items, including positive samples containing DNA extracted from a pure culture of *X. ampelinus* (strain LMG 5856^T); positive samples containing plant extracts from leaves, roots and xylem spiked with *X. ampelinus*; negative samples containing DNA from other non-target bacteria; and negative samples containing plant extracts without *X. ampelinus*, along with the corresponding control samples (Table S5). The quantitative reference values (target concentrations) were determined turbidimetrically and assigned to the concentrations by dPCR Dreo et al. (2007) for the test samples and controls. The stability of the test samples and controls was tested by real-time PCR by Dreo et al. (2007). The stability tests were performed under two conditions: first, at 25 °C for a period of one week, and second, at the standard storage temperature of -20 °C for the duration of the TPS. Each participant analysed all the samples in three separate reactions. A sample was designated as positive if it elicited at least one positive reaction. The collected TPS results were then analysed on the basis of their agreement with the assigned reference values (Table S5). Test records were considered valid if the results of the controls were concordant and if the overall percentage of non-concordant results did not exceed 15%. Participating laboratories reported which instruments, reagents and protocols were used to process the samples. The data for each participating laboratory were analysed on the basis of the number of positive matches (positive versus expected positive), negative matches (negative versus expected negative), positive deviations (positive versus expected negative) and negative deviations (negative versus expected positive).

Statistical data analysis

Using the programming language R for statistical computing [21] and the integrated development environment RStudio [22] in combination with the *drc* package [23], non-linear modelling of the target detection probability was calculated and the target concentration detected with 95% probability (LOD95) was determined from the real-time PCR results of the samples used to determine analytical and diagnostic sensitivity. The dichotomous positive and negative results of the real-time PCR assays were analysed via a 2-by-2 contingency table. Kruskal–Wallis tests were performed using the *vegan* package [24]

to determine whether there was a statistically significant difference between the datasets analysed.

Results

Phylogenetic analysis

Prior to the in silico design of the primers, a phylogenetic analysis was performed to decide which genomes should be included in the positive and negative datasets (Fig. 1: A). All published genomes assigned to the genus *Xylophilus* were included in the calculation of the ANI matrix and the construction of the phylogenetic tree (Fig. 2, Table S1). The analysis revealed that only the genome of the type strain CECT 7646^T belonged to the species *X. ampelinus*. Strains BgEED09 and CCH5-B3, which were assigned to *X. ampelinus* at the time of analysis, did not belong to this species, as their ANI with the genome of the type strain was 80% for both strains. These two genomes were therefore excluded from further assay design. The strain most closely related to *X. ampelinus* was identified as Leaf220, with an ANI of 93%. The remaining genomes analysed presented ANIs ranging from 78 to 83%, indicating their affiliation with the genus *Xylophilus* (Fig. 2, Figure S1).

Identification of unique core sequences and design of real-time PCR assays

New real-time PCR assays were designed to amplify segments of unique core sequences (UCS) that are present in *X. ampelinus* and absent in other related bacteria. At the time of the study, there were thirteen published genomes of the genus *Xylophilus*, only one of which belonged to the species *X. ampelinus*. A further challenge was the fact that ten of these genomes were derived from metagenomic data, which generally had lower coverage and quality (Table S1). For this reason, two separate RUCS analyses were performed. The first included only two genomes of high quality, the genome of *X. ampelinus* (CECT 7646^T) as a positive dataset and the genome of *X. rhododendri* as a negative dataset. The UCSs identified in this analysis were then used for the design of eight real-time PCR assays (TXmp22.1-7, TXmp22.10). In contrast to the first analysis, the second analysis incorporated all available genomes with the exception of BgEED09 and

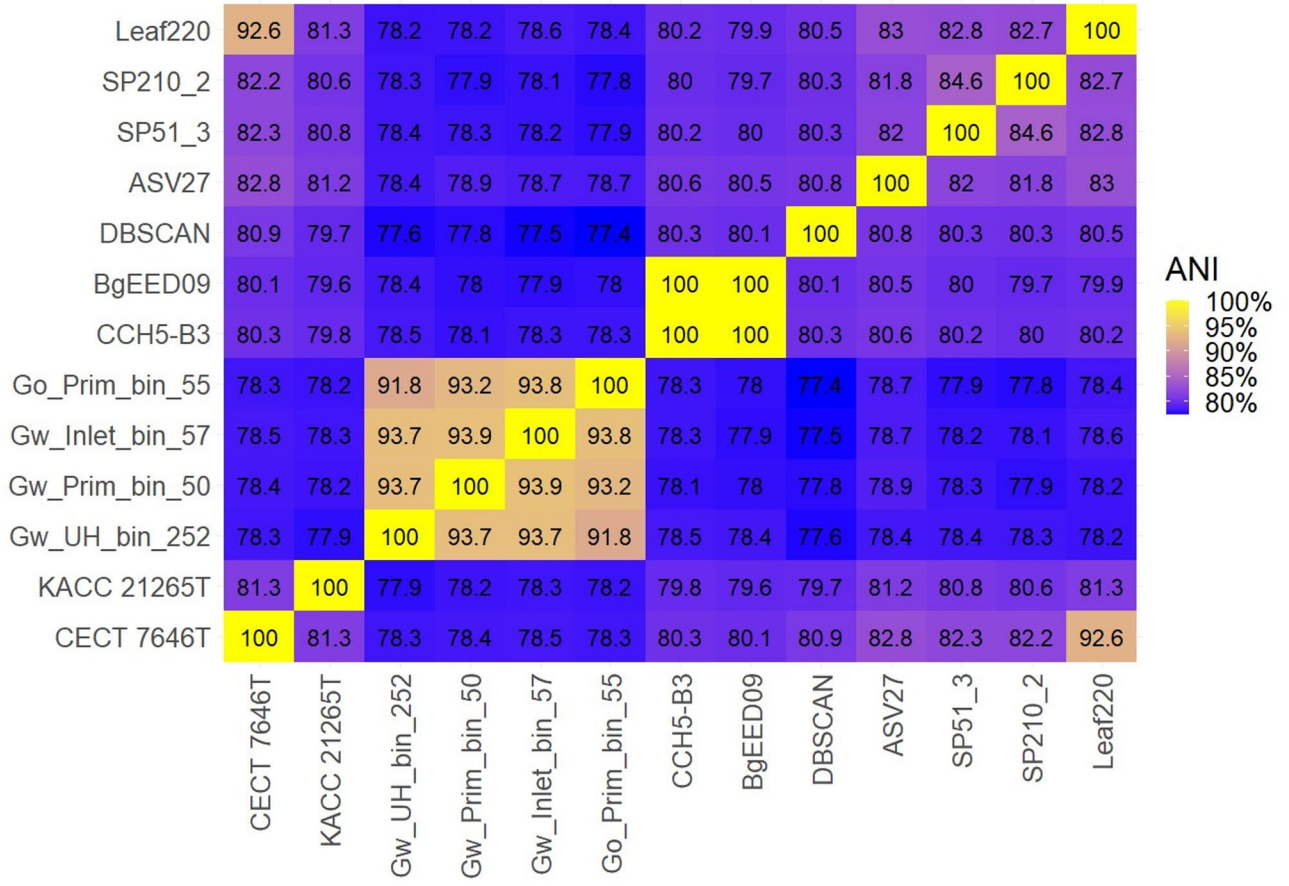

Fig. 2 Heatmap of the average nucleotide identity values matrix in percentages for he genomes of the genus *Xylophilus* available in the GenBank database at the time of this study and listed in Table S1. CECT7646^T is a genome from a type strain of *X. ampelinus*, and KACC21265^T is a genome from a type strain of *X. rhododendri*. Other genomes belong to different *Xylophilus* strains that have not yet been assigned to a specific species

CCH5-B3, with in the *X. ampelinus* genome (CECT 7646^T) again being the sole genome in the positive dataset, whereas all other genomes were allocated to the negative dataset. The UCSs identified in this analysis were then used to develop 10 real-time PCR assays (Xamp_BA_1–10) (Tables S2, S4). Each set of primers and probe was designed for different genomic sequences using Primer Express 2 and TaqMan hydrolysis probes. The melting temperatures of the primers ranged from 63 to 68 °C, whereas those of the probes ranged from 68 to 72 °C. The GC content of the primers varied from 31 to 73%, and that of the probes ranged from 50 to 67%. The amplicons ranged in size from 58 to 108 base pairs (bp), with melting temperatures ranging from 75 to 85 °C (Table S4). BLASTN analyses revealed that the new real-time PCR assays amplified non-coding sequences as well as sequences coding for known or hypothetical proteins (Table S4). It was also shown that four assays (TXmp22.1, TXmp22.2, TXmp22.6 and Xamp_BA_3) amplified prophage sequences and were therefore excluded from further analysis.

Performance and selection of real-time PCR assays

Analytical specificity

To determine the analytical specificity, the newly designed real-time PCR assays were tested using the target and non-target bacterial strains listed in Table 1. The target bacteria included various *X. ampelinus* strains isolated from different geographical regions and time periods, whereas the non-target bacteria included the related bacterium *X. rhododendri*, bacteria that cause similar symptoms in grapevines, and bacterial isolates from grapevine leaves and roots. The analytical specificity was determined using DNA extracted from a bacterial suspension (concentration of 10^6 cells/mL) (Fig. 1: B). A microfluidics-based high-throughput real-time PCR system was used to test multiple assays simultaneously. Eight newly designed real-time PCR assays were able to detect all *X. ampelinus* strains used in the study, while the others showed significantly lower specificity. No instances of false positive results were identified (Tables S6, S7). The analytical specificity determined by high-throughput real-time PCR was confirmed using 10 µL real-time PCR reactions. This confirmed the ability of eight new assays to detect *X. ampelinus* with high specificity, inclusivity and accuracy (Fig. 3, Tables S6, S7).

Analytical sensitivity

To determine the analytical sensitivity, linear range and amplification efficiency of the newly designed assays, 10-fold dilutions (10^8 – 10 cells/mL) of bacterial suspensions of the *X. ampelinus* type strain were used. The copy number of target DNA extracted from these suspensions was determined by dPCR and adjusted for losses during DNA extraction, resulting in slightly lower concentrations ranging from 1.5×10^7 – 9 cp/mL (Fig. 1: B, Table S3). For assays utilising high-throughput real-time PCR, the lowest concentrations that still yielded positive results ranged from 2.8×10^4 to 2.8×10^5 cp/mL for distinct assays (Table S8, Figure S2). For assays in 10 µL real-time PCR reactions, the lowest concentrations that yielded positive results ranged from 71 to 6.2×10^3 cp/mL (Table S9). The linear range for the majority of the assays was observed to be between 3.7×10^2 and a minimum of 1.5×10^7 cp/mL (Table 2). The LOD95, defined as the lowest target concentration that led to positive results in 95% of the reactions, was between 1.2×10^2 cp/mL (BA_Xamp_2) and 1.8×10^3 (TXmp22.4 and Xamp_BA_7) (Table 2). The amplification efficiencies ranged from 0.94 (TXmp22.3) to 1.07 (Xamp_BA_5) and were within the acceptable range for all the assays tested (Table 2). The real-time PCR assays demonstrated high sensitivity, a wide linear range, and good amplification efficiency, confirming their ability to detect *X. ampelinus* over a wide concentration range.

Testing of selected assays for the effects of different matrices

To determine which assays are suitable for testing different grapevine samples, five newly designed real-time PCR assays (TXmp22.4, TXmp22.5, BA_Xamp_2, BA_Xamp_6 and BA_Xamp_7) were selected for further evaluation (Fig. 1: B, Table 3). The selection of these assays was based on the results of analytical specificity and the approach used to identify the target sequences. Initially, assays with an inclusivity of less than 100% were excluded on the basis that they were unable to detect all strains of *X. ampelinus*. Eight assays remained, of which five were selected, taking care to include assays designed on the basis of the two different negative datasets for the RUCS analyses. The selected assays were additionally evaluated using DNA from grapevine extracts of asymptomatic leaves, roots and xylem spiked with a bacterial suspension of the *X. ampelinus* type strain (LMG 5856^T) at different concentrations (Fig. 1: B, Table S3). Grapevine extracts not spiked with bacteria were also tested, and no cross-reactivity with the extracts or the corresponding microflora was observed. A reference assay Dreo et al. (2007), which had not previously been validated for use on matrices other than leaves, was also included in additional tests. The results of the new assays and the reference assay were then compared to determine any differences between the assays and the possible effects of the plant matrices on the performance of the assays. Despite the presence of some differences, the matrices had no significant detrimental effects on amplification efficiency, diagnostic sensitivity, analytical sensitivity and overall performance of the real-time PCR assays. The exception was TXmp22.5, which demonstrated inhibition

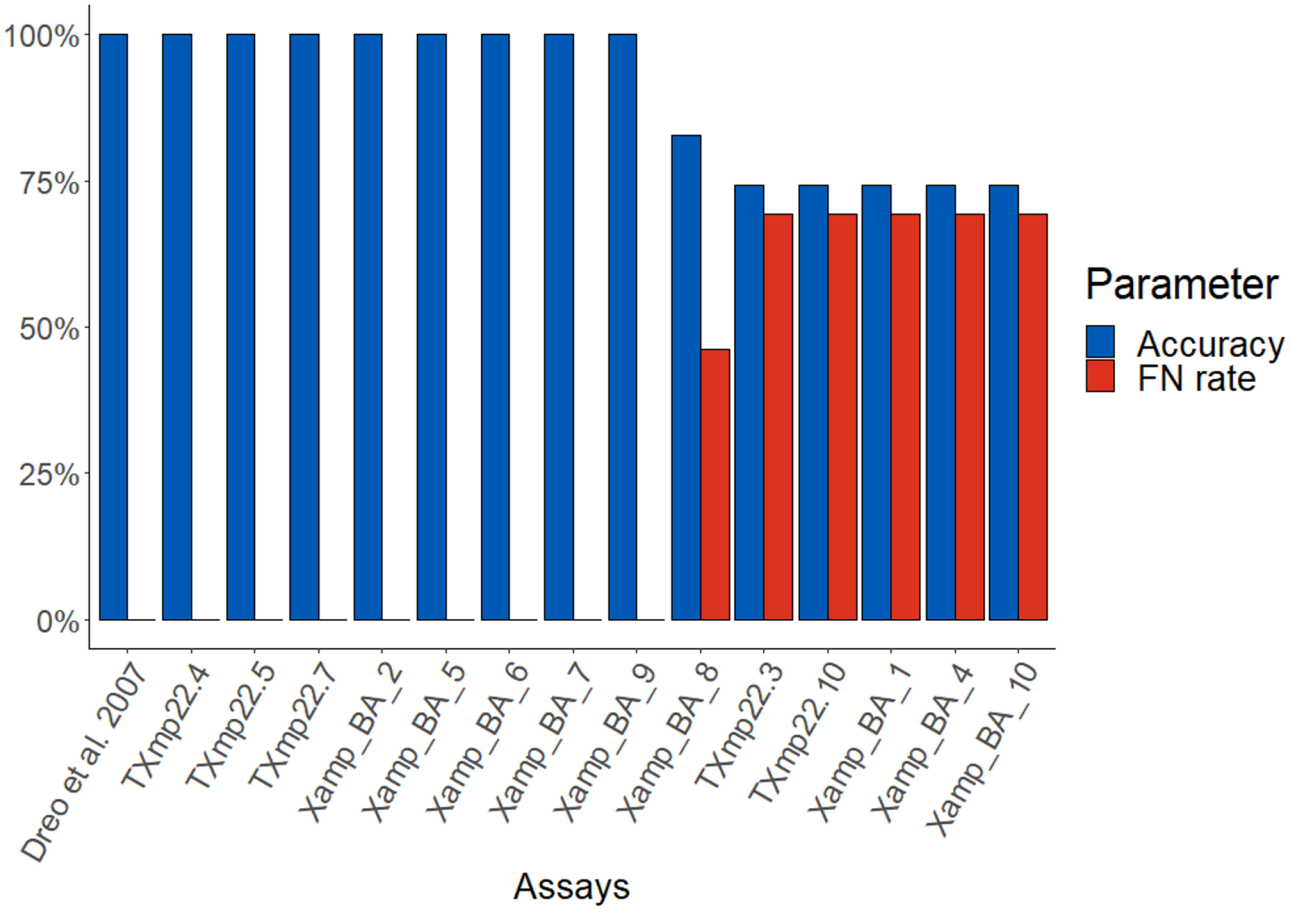

Fig. 3 Bar plot showing the accuracy (dark green bars) and false negative (FN) rate (dark red bars) expressed as percentages of different real-time PCR assays for which analytical specificity parameters were determined during validation

Table 2 Performance characteristics of real-time PCR assays with DNA extracted from *X. ampelinus* suspensions in PBS

Assays	C_q(min–max)[a]	Linear range (cp/mL)[b]	Linear regression [c]			LOD95 [cp/mL]	C_q (1 cp/rxn)
			k	R^2	E		
Dreo	18.8–36.7	3.7×10^2-1.5×10^7	-3.66	0.996	0.88	1.28×10^2	36.72
TXmp22.2	18.9–37.7	3.7×10^2-1.5×10^7	-3.75	0.997	0.85	1.28×10^2	36.54
TXmp22.4	18.9–36.1	3.7×10^2-1.5×10^7	-3.56	0.992	0.91	1.77×10^3	36.27
TXmp22.5	20.2–38.1	3.7×10^2-1.5×10^7	-3.66	0.995	0.88	7.94×10^2	38.13
TXmp22.7	19.5–37.0	3.7×10^2-1.5×10^7	-3.65	0.997	0.88	1.28×10^2	37.37
TXmp22.10	20.9–34.2	6.2×10^3-1.5×10^7	-3.74	0.997	0.85	9.91×10^2	38.45
Xamp_BA_1	20.3–37.7	3.7×10^2-1.5×10^7	-3.61	0.996	0.89	1.28×10^2	38.09
Xamp_BA_2	20.9–39.1	3.7×10^2-1.5×10^7	-3.67	0.996	0.87	1.15×10^2	38.76
Xamp_BA_4	20.8–37.7	6.2×10^3-1.5×10^7	-4.76	0.998	0.62	9.91×10^2	38.22
Xamp_BA_5	19.2–32.2	6.2×10^3-1.5×10^7	-3.68	0.997	0.87	1.53×10^3	36.48
Xamp_BA_6	22.0–41.0	6.2×10^3-1.5×10^7	-3.64	0.992	0.88	9.91×10^2	40.24
Xamp_BA_7	19.0-36.6	3.7×10^2-1.5×10^7	-3.90	0.997	0.81	1.77×10^3	36.23
Xamp_BA_8	18.0-35.4	3.7×10^2-1.5×10^7	-3.53	0.996	0.92	1.28×10^2	35.66
Xamp_BA_9	18.9–31.6	6.2×10^3-1.5×10^7	-3.59	0.997	0.90	1.53×10^3	36.28
Xamp_BA_10	18.7–35.6	3.7×10^2-1.5×10^7	-3.70	0.997	0.86	1.28×10^2	35.88

[a] Minimal and maximal C_q values that still enable detection

[b] The range of concentrations for which C_q values were in linear relationship with logarithms of concentrations (determined by exploring slope values across sections of C_q values × log number of the cells)

[c] Linear regression of all positive samples in a plot of C_q values against the logarithmic number of *X. ampelinus* cells: k = slope of the linear regression line; R^2 = average square regression coefficient; E = efficiency of amplification

Table 3 List of real-time PCR assays that were selected for further evaluation. For each assay, the target inside the genome, forward primer, reverse primer, probe with modifications, amplicon length in base pairs (bp), melting temperature (Tm), and percentage of guanine and cytosine bases (%GC) are listed

Assay (target gene)	Length [bp]	T_m [°C]	%GC
TXmp22.4 (Non-coding sequence)	65	80.8	60
CCCGCGAAGGCCATTC			
GGAGAGACGGCGGGAAAT			
56-FAM/CATCCCTTCACACGGAGTTCTTCCATGAC/3BHQ_1			
TXmp22.5 (ABC transporter permease)	61	81.6	61
AACCCCCTGCGGAAGTG			
GCGTCGCCGCGAAA			
56-FAM/AGGGCTGAGATGGAATCCTACGCACTG/3BHQ_1			
Xamp_BA_2 (alanine racemase)	64	81.1	61
GGTGGCCTCCCTGTTCAGTA			
CGGCATCCTGTTGCAGAAAT			
56-FAM/TCGATCCCGAGCAATGGCGC/3BHQ_1			
Xamp_BA_6 (Non-coding sequence)	88	79.6	49
CCCTCCAAAACTGTGATTGCTAT			
CACCCGGATTTGAATGGAAA			
56-FAM/AGCTATAAGCCGGCACTCGAAGCCG/3BHQ_1			
Xamp_BA_7 (hypothetical protein)	75	79.2	48
GATTCGGCTTCTATCCTTCACAA			
TTCGGAGTTTCCAGGAGATGA			
56-FAM/ACGGCTTCTGGCAGGTTTCACCC/3BHQ_1			

in root and xylem extracts (Table 4; Fig. 4, Table S10). The amplification efficiencies of the assays ranged from 0.45 (TXmp22.5; xylem) to 0.92 (Xamp_BA_7; PBS). The leaf and root extracts showed on average, the lowest and highest amplification efficiencies, respectively. However, the differences were not significant and did not affect the overall performance of the assays (Table 4; Fig. 4: A). No significant differences in analytical sensitivity were detected between the bacterial suspension samples and the samples of the plant extracts spiked with *X. ampelinus.* The limits of detection at 95% confidence (LOD95) values were determined via nonlinear modelling and ranged from 1.15×10^2 cp/mL (BA_Xamp_2; PBS) to 2.67×10^4 cp/mL (TXmp22.5; xylem). The lowest concentration at which we could reliably detect *X. ampelinus* in all the matrices was 3.7×10^2 cp/mL (Fig. 5). The diagnostic sensitivity of the assays ranged from 73 to 82%, with no statistically significant differences observed between the matrices (Table 4; Fig. 4: B). Overall, the new assays demonstrated high sensitivity and amplification efficiency, with the exception of TXmp22.5, which was excluded from further analysis. In comparison, Xamp_BA_2 and Xamp_BA_7 exhibited slightly superior results in terms of sensitivity across different matrices compared with the other evaluated assays.

Test performance study

The reproducibility and robustness of the newly designed assays and the reference assay were investigated through a test performance study (TPS). In this study, seven participants were tasked with testing samples provided by the study organiser. The TPS sample panel consisted of DNA from *X. ampelinus* or non-target bacteria and grapevine extracts of leaves, roots and xylem with and without the addition of *X. ampelinus* (Table S5).

The analysis incorporated only valid experimental recordings, accounting for 86% of the total results submitted by the participants. The TPS demonstrated high reproducibility for all the assays, with 96.1% of the total results being concordant. All the assays exhibited a diagnostic specificity of 100% for all the matrices, with no false positive results observed. The overall diagnostic sensitivity of the assays included in the TPS was greater than 90%, with Xamp_BA_2 having the highest (97%) and Xamp_BA_6 the lowest (90%) diagnostic sensitivity (Fig. 6, Table S11, S12). The performance of the assays was not affected by the different matrices, except for the leaves, which showed inhibition in all assays. The Xamp_BA_6 assay exhibited the highest level of inhibition in the leaf samples (19.0% of non-concordant results), and it also had the highest percentage of non-concordant results in the xylem samples (7.1%). Considering these findings, the use of the Xamp_BA_6 assay is not recommended, particularly for samples from leaves.

To assess the robustness of the assays, the participating laboratories used either the reagents and equipment recommended by the organiser or their own. They used different combinations of DNA extraction methods, PCR mastermixes and thermal cyclers. When the recommended DNA extraction method (QuickPick SML Plant

Table 4 Performance characteristics of real-time PCRs with DNA extracted from samples of plant extracts spiked with *X. ampelinus* and from suspensions of *X. ampelinus* in PBS

Assays	Matrix	Linear range (cp/mL)[a]	Linear regression[b]			LoD95 (cp/mL)[c]	Diagnostic sensitivity
			k	R^2	E		
Dreo	PBS	3.7×10^2-1.5×10^7	-3.66	0.994	0.88	1.8×10^3	75%
	leaf	6.2×10^3-1.5×10^7	-4.16	0.988	0.74	6.2×10^2	73%
	root	3.7×10^2-1.5×10^7	-3.66	0.993	0.88	6.2×10^2	73%
	xylem	3.7×10^2-1.5×10^7	-3.87	0.992	0.81	1.3×10^3	73%
TXmp22.4	PBS	3.7×10^2-1.5×10^7	-3.56	0.992	0.91	7.9×10^2	88%
	leaf	2.3×10^3-1.5×10^7	-3.91	0.991	0.8	7.1×10^2	73%
	root	3.7×10^2-1.5×10^7	-3.55	0.989	0.91	6.2×10^2	73%
	xylem	2.3×10^3-1.5×10^7	-3.92	0.988	0.8	2.7×10^4	73%
TXmp22.5	PBS	3.7×10^2-1.5×10^7	-3.66	0.995	0.88	1.2×10^2	75%
	leaf	2.3×10^3-1.5×10^7	-3.74	0.992	0.85	6.2×10^2	73%
	root	non-linear	NA	NA	NA	1.4×10^2	73%
	xylem	2.8×10^4-1.5×10^7	-6.23	0.995	0.45	7.1×10^2	45%
Xamp_BA_2	PBS	3.7×10^2-1.5×10^7	-3.67	0.996	0.87	9.9×10^2	88%
	leaf	3.7×10^2-1.5×10^7	-3.75	0.989	0.85	6.2×10^2	73%
	root	3.7×10^2-1.5×10^7	-3.63	0.988	0.89	6.2×10^2	82%
	xylem	2.3×10^3-1.5×10^7	-3.65	0.989	0.88	7.1×10^2	73%
Xamp_BA_6	PBS	6.2×10^3-1.5×10^7	-3.90	0.997	0.81	1.8×10^3	75%
	leaf	3.7×10^2-1.5×10^7	-3.87	0.988	0.81	1.3×10^3	73%
	root	3.7×10^2-1.5×10^7	-3.59	0.981	0.9	6.2×10^2	73%
	xylem	6.2×10^3-1.5×10^7	-4.02	0.993	0.77	2.2×10^3	73%
Xamp_BA_7	PBS	3.7×10^2-1.5×10^7	-3.53	0.996	0.92	1.3×10^2	88%
	leaf	3.7×10^2-1.5×10^7	-3.73	0.987	0.85	6.2×10^2	82%
	root	3.7×10^2-1.5×10^7	-3.54	0.991	0.92	1.3×10^2	73%
	xylem	3.7×10^2-1.5×10^7	-3.77	0.994	0.84	6.2×10^2	82%

[a]The range of concentrations for which C_q values were in linear relationship with logarithms of concentrations (determined by exploring slope values across sections of C_q values × log number of the cells)

[b]Linear regression of all positive samples in a plot of C_q values against the logarithmic number of *X. ampelinus* cells: k = slope of the linear regression line; R^2 = average square regression coefficient; E = efficiency of amplification

[c]LOD = limit of detection; for the purpose of this study, LOD95 was defined as the concentration at which 95% of the samples were positive

DNA Kit) was used, the results were 100% concordant regardless of other variables (Figure S3). The volume of plant extract used for DNA extraction and PCR mastermix had no effect on the results. The results of the TPS confirmed the high sensitivity of the assays and demonstrated the reproducibility and robustness of the newly designed assays in several laboratories.

Discussion

In this study, an innovative genome-informed approach was utilised to identify novel targets, which were then employed to develop a panel of real-time PCR assays capable of detecting *X. ampelinus* in various plant matrices with high sensitivity and selectivity. The sequences unique to *X. ampelinus* were identified using a bioinformatics pipeline based on RUCS, a freely available program that has already been shown to be useful for the design of specific real-time PCR assays [25]. A total of 18 primer/hydrolysis probe sets were designed, eight of which could detect all *X. ampelinus* strains without generating false positive results. Despite the fact that *X. ampelinus* is regarded as a highly homogeneous species [26], a significant proportion of assays yielded false negative results, suggesting the potential for intraspecies diversity. This could be because only one genome of *X. ampelinus* was used for the design of the primers. Consequently, the core genome could not be determined, and some shell and cloud genes were probably also included in the UCSs, which were used to design new assays. Despite the limited number of genomes utilised in the analysis, the successful design of numerous specific assays underscores the robustness of the RUCS-based pipeline.

To limit the scope of validation, five out of eight promising assays were selected for further evaluation. These were selected to include assays designed on UCS identified in both RUCS analyses. The five selected assays and the reference assay of Dreo et al. (2007) were then subjected to further evaluation on matrices, including leaves, roots and xylem. It was established that all assays demonstrated acceptable amplification efficiency and diagnostic sensitivity across all the matrices, with the exception of

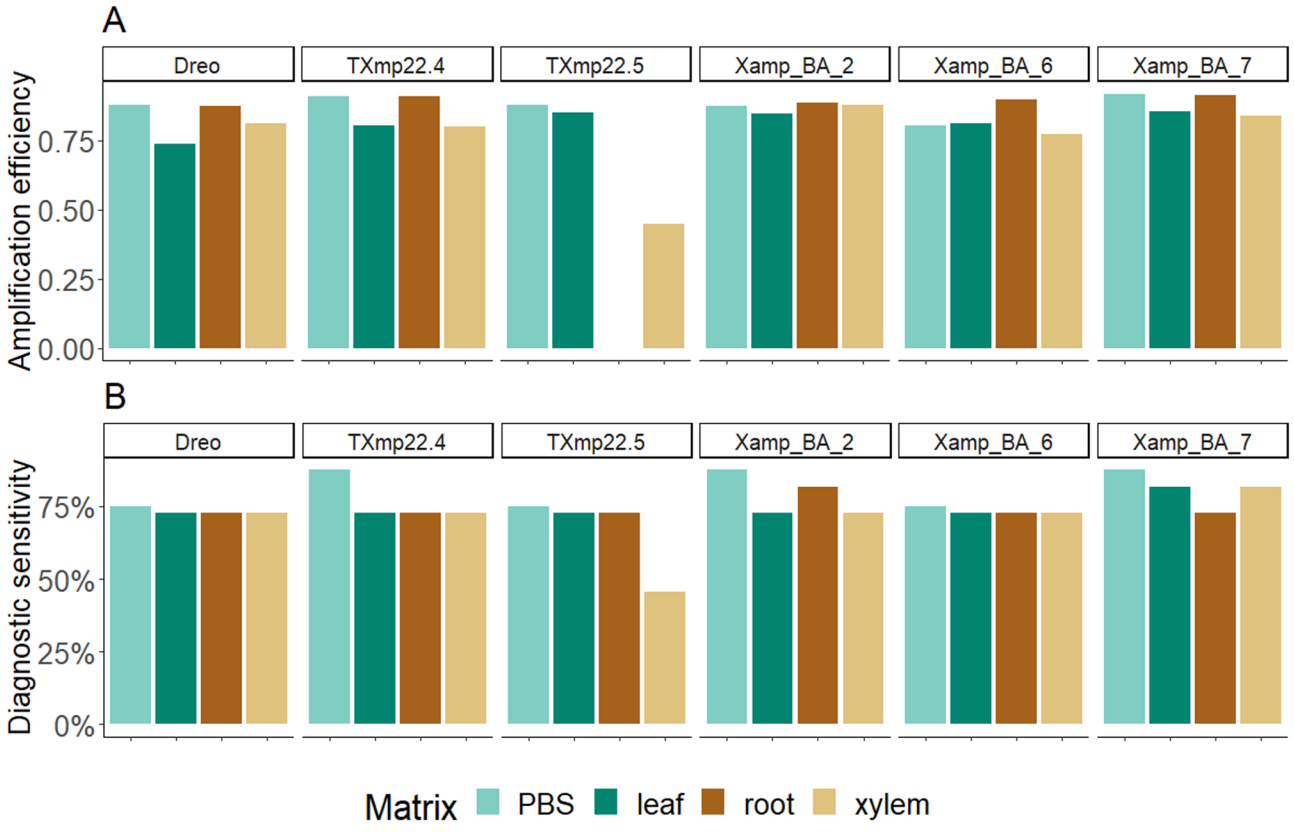

Fig. 4 Comparison of the performance expressed as amplification efficiency (**A**) and diagnostic sensitivity (**B**) of the selected real-time PCR assays TXmp22.4, TXmp22.5, BA_Xamp_2, BA_Xamp_6 and BA_Xamp_7 and the reference Dreo et al. (2007). The DNA used was isolated from samples of plant extracts of leaves, roots and xylem fortified with *X. ampelinus* and from a bacterial suspension of *X. ampelinus* in phosphate-buffered saline (PBS). For TXmp22.5, the amplification efficiency in the root extract could not be calculated because of the lack of a linear range

TXmp22.5. Further evaluation of this assay revealed inhibition in spiked xylem and root samples, resulting in its exclusion from the study. Nevertheless, some differences in assay performance were observed in different matrices, with samples from leaves showing slightly lower amplification efficiency. The remaining assays (TXmp22.4, BA_Xamp_2, BA_Xamp_6 and BA_Xamp_7) were found to be suitable for the detection of *X. ampelinus* in different plant samples and at concentrations as low as 10^2 cp/mL. The validation of the assays for the detection of *X. ampelinus* in different matrices is of great importance, as it enables the analysis of a broad spectrum of samples ranging from green plant parts to woody tissue from grafted plant material. The assays demonstrate a high level of sensitivity, enabling the detection of pathogenic bacteria at low concentrations. This capacity for detection extends to latent infections, a significant consideration given the prolonged incubation periods associated with slow-growing bacteria such as *X. ampelinus*.

The TPS in which new assays were evaluated identified the leaf extract as the most problematic matrix, with the highest percentage of non-concordant results. The Xamp_BA_6 assay demonstrated the lowest diagnostic sensitivity, particularly in the leaf and xylem matrices, and was consequently also excluded. Among the assays evaluated, Xamp_BA_2 exhibited the highest diagnostic sensitivity and is recommended for further diagnosis of *X. ampelinus* infection. The assays TXmp22.4, BA_Xamp_7 and Dreo et al. (2007) also demonstrated high sensitivity and specificity, making them suitable for detection. In instances where a positive result is obtained with Xamp_BA_2, these assays should be employed to ascertain the identity of *X. ampelinus*. To assess the robustness of the novel assays, the participating laboratories were permitted to use their own equipment and reagents to perform TPS. It was found that there were some discrepancies between the participating laboratories. For example, the leaf samples that presented the most challenges in both the initial evaluation and the TPS were found to be 100% concordant when specific DNA extraction methods were employed. This highlights the importance of the DNA extraction method, which should be carefully selected for each matrix in which target bacteria are to be detected. Nevertheless, owing to the limited number of participants, it is challenging to ascertain that these discrepancies are attributable to differing protocols. Importantly,

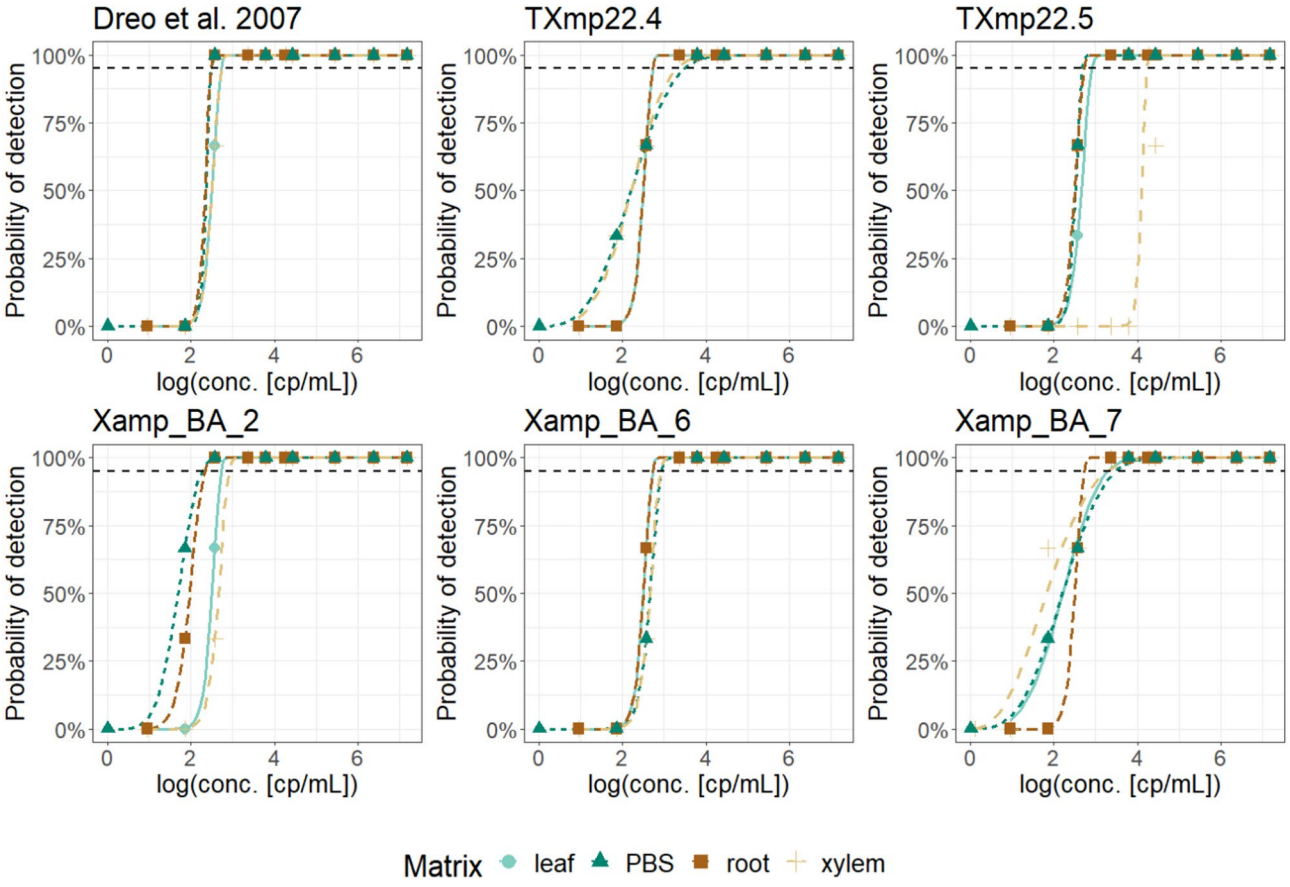

Fig. 5 Plots showing the detection probabilities for selected real-time PCR assays, TXmp22.4, BA_Xamp_2, BA_Xamp_6 and BA_Xamp_7, and reference Dreo et al. (2007), which were calculated using non-linear modelling and the Weibull type 2 model. The dashed lines indicate the 95% probability (LOD95) of detection. Probabilities were calculated for four different matrices (leaf, root, xylem and PBS)

the discrepancies between protocols were small showcasing the high robustness of the three new and reference assays.

In previous studies, molecular tests were found to be more reliable than serological tests and had a lower sensitivity and a higher proportion of false positives [27]. Although other molecular methods have shown results similar to those of real-time PCR, it offers certain advantages, as it is a rapid and straightforward method with high sensitivity that could also be adapted for use as dPCR. The primary benefit of dPCR is its capacity for absolute quantification, a particularly advantageous feature when dealing with complex samples or in scenarios where reference materials are not available, a common occurrence in many research contexts. Compared with other molecular methods, such as nested or conventional PCR, real-time PCR has the advantages of the absence of an electrophoresis step, which reduces the possibility of contamination, and lower sensitivity to inhibitors present in plant tissues.

Conclusions

In this study, five new real-time PCR assays were validated, three of which (TXmp22.4, Xamp_BA_2 and BA_Xamp_7) were found to be suitable for testing different grapevine samples for the presence of *X. ampelinus*. Additionally, the application of the reference assay of Dreo et al. (2007) was expanded to include root and xylem matrices. These assays have been demonstrated to be valuable tools for the detection of *X. ampelinus*, which remains an important pathogen in viticulture, as it can cause yield losses and restrictions in the international trade of grafted plants. Furthermore, these tests are instrumental in preventing the spread of the disease in areas where it has not yet been detected. It is recommended that the new Xamp_BA_2 assay be used in conjunction with other assays for the screening of infected plants to reduce the possibility of false negative results.

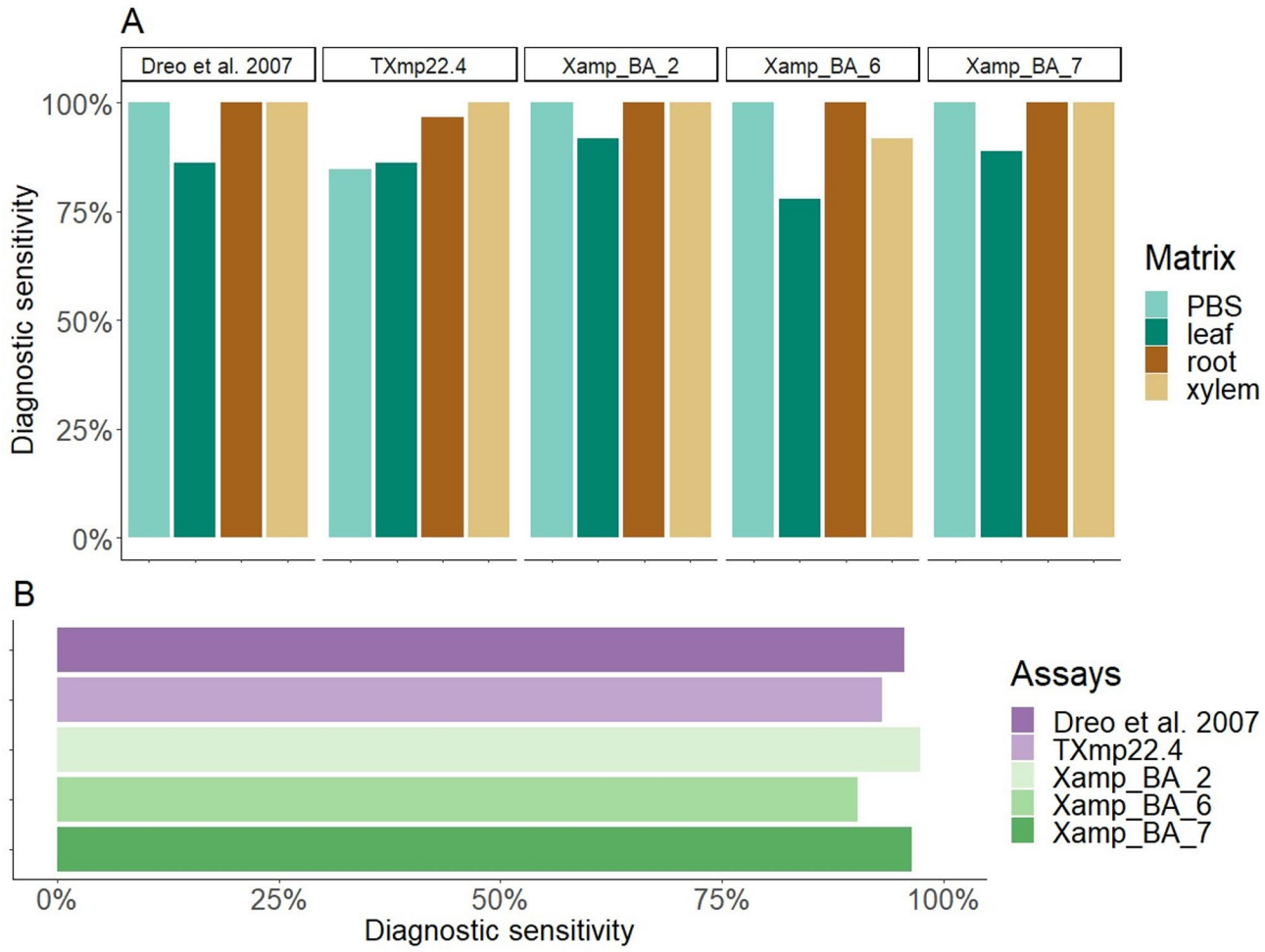

Fig. 6 Results of the test performance study (TPS) showing the diagnostic sensitivity of real-time PCR assays (TXmp22.4, TXmp22.5, BA_Xamp_2, BA_Xamp_6, BA_Xamp_7 and reference from Dreo et al. (2007)) in different matrices (**A**) and over all different matrices

Abbreviations

ANI	Average nucleotide identity
BHQ	Black hole quencher
dPCR	Digital PCR
ELISA	Enzyme-linked immunosorbent assay
EPPO	European and Mediterranean Plant Protection Organization
FAM	6-carboxyfluorescein
LOD95	Limit of detection at 95% confidence
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
NA	Not applicable
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
rDNA	Ribosomal DNA
RUCS	Rapid identification of PCR primers for unique core sequences
TPS	Test performance study
UCS	Unique core sequences

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions
A.B. contributed in aquisition and interpreatation of data, wrote the main manuscript text and prepared figures. A.B.K. contributed in aqusition and interpretation of data. J.M. contributed in data aquisition. M.P. contributed in aqusition and interpretation of data. N.T. contributed in data aquisition. T.D. contributed in design of work and interpretation of data. All authors reviewed the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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