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METHODS AND PROTOCOLS

A reliable qPCR technique for detecting viable *Xanthomonas arboricola* pv. *pruni* cells

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Abstract

Xanthomonas arboricola pv. *pruni* (Xap) is the causal agent of bacterial spot of stone fruits and almond (*Prunus* spp). Detection of Xap is typically carried out using quantitative real-time PCR (qPCR) combined with culture-based isolation. However, qPCR does not differentiate between viable and dead cells, potentially leading to an overestimation of the infective population in a sample. Such overestimation could result in unnecessary phytosanitary measures. The present study aims to develop a specific protocol ideally targeting to detection of only live Xap bacterial cells. To address this challenge, the viable quantitative PCR (v-qPCR) method was evaluated using three nucleic acid-binding dyes: propidium monoazide (PMA), a combination of PMA and ethidium monoazide (EMA), and PMAxx™, an improved version of PMA. PMAxx™ proved to be the most suitable dye for the detection and quantification of living bacterial cells. This methodology was also evaluated in infected plant material over time and can be considered a rapid and reliable alternative to PCR methods for detecting only those putative infective Xap that may pose a risk for *Prunus* crops.

Key points

- *Protocol to detect biofilm and planktonic viable* X. arboricola *pv.* pruni *cells.*
- *Host validated protocol.*
- *Benefits, reduction of chemicals in disease control.*

Keywords Cut-off Ct · LOD · Viable cell detection · *Xanthomonas arboricola* pv. *pruni* · V-qPCR · Intercalating dye

Introduction

Bacterial spot disease of stone fruits and almond, caused by *Xanthomonas arboricola* pv. *pruni* (Xap), affects a wide range of cultivated and ornamental *Prunus* species worldwide (EPPO 2024). Recognized as one of the most important bacterial pathogens affecting *Prunus* spp., it is listed as a regulated pest in the European Union, and it is considered a quarantine pathogen in other several countries around the world (Anonymous 2019). Symptoms can be observed on leaves, twigs, branches, trunks, and young and fully developed fruits; infected fruits of almond either drop prematurely or mummify and remain on the trees after harvest (EPPO 2021). The economic impact of bacterial spot is attributed to the reduction of crop yield, dismissed marketability of fruits, and increased costs of nursery productions. According to Stefani (2010), an epidemic in a commercial plum orchard in northern Italy affecting 30% of the fruits, could result in estimated economic loss as high as 11,200 € per ha for susceptible cultivars. In the USA, 25–75% of the fruits were damaged in neglected peach orchards (Dunegan 1932) and, in recent years, the disease incidence reached 100% on susceptible peach cultivars in some orchards in the middle reach of the Yangtze River in China (Luo et al. 2021). Regarding almonds, losses in production of up to 46% have been reported in Spain (EFSA 2014; Palacio-Bielsa et al. 2014); incidences of about 70–80% of affected fruits have been reported in Italy (Gerin et al. 2019), and around

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90% in Hungary (Kolozsváriné Nagy et al. 2019). In the Netherlands, bacterial spot is a significant issue in nurseries producing ornamental cherry laurel (*Prunus laurocerasus*), which are primarily destined for export (Tjou-Tam-Sin et al. 2012).

Diagnosis and detection of bacterial diseases are crucial tools for their control, as they help prevent their introduction and spread. Quantitative real-time PCR (qPCR) techniques are commonly used due to their rapidity, sensitivity, specificity, and ability to quantitatively detect pathogens. However, a limitation of qPCR is the potential overestimation of viable cells. Since PCR can detect non-viable cells, because intact target nucleic acid sequences persist for an extended period after cell death, positive amplification products do not necessarily indicate the viability of target organisms (Josephson et al. 1993).

The use of DNA-intercalating dyes combined with qPCR protocols potentially enables the quantification of only viable cells (v-qPCR). Generally, the dyes used are ethidium monoazide (EMA), propidium monoazide (PMA), its improved version PMAxx™, or a mix of some of them. These chemicals can penetrate bacterial cells with damaged membranes and covalently bind to double-stranded DNA upon photoactivation. The bounded DNA cannot be amplified in v-qPCR, ideally resulting in the amplification of only viable cells (Nocker et al. 2006; Nogva et al. 2003). EMA and PMA differ with respect to their permeation through cell membranes, with the latter proving to be more selective due to a higher charge of the molecule (Nocker et al. 2006) and enabling more specific penetration into membrane-compromised dead cells. Han et al. (2018) reported that PMAxx™ demonstrates higher sensitivity in discriminating viable and dead cells of *Clavibacter michiganensis* (formerly *Clavibacter michiganensis* subsp. *michiganensis*) with less false binding of the DNA of viable cells compared to PMA. v-qPCR with different nucleic acid-binding dyes have been used for quantification of viable cells for human pathogens (Pedrosa de Macena et al. 2022), biocontrol agents such as *Lactobacillus plantarum* (Daranas et al. 2018) or *Pantoea agglomerans* (Soto-Muñoz et al. 2015) and, in a lesser extent, for plant pathogens, such as the aforementioned *C. michiganensis* (Han et al. 2018), *Xanthomonas fragariae* (Wang and Turechek 2020) and *Xyella fastidiosa* (Baró et al. 2020; Sicard et al. 2019). Moreover, a recent paper presented a strategy for Xap detection (Panth et al. 2024). Despite the success of the v-qPCR technique, some practical limitations have been identified, especially when applied to complex matrix samples that can affect the efficiency of v-qPCR. Such limitations can be partially minimized by selecting experimental parameters and conditions suitable for a particular sample (Fittipaldi et al. 2012).

The aim of the present study is to develop a v-qPCR assay, implemented with a suitable dye agent, for a selective detection of viable Xap cells using the specific primers designed on the virulence-associated *xopE3* gene (Garita-Cambronero et al. 2017) that is regularly used and adopted by organisms such the European and Mediterranean Plant Protection Organization (EPPO) (EPPO 2021). Parameters such as type and concentration of the DNA intercalating dyes and concentration of the microorganism have been carefully optimized and validated in Xap inoculated plants.

Materials and methods

Preparation of viable and dead cell suspensions from planktonic and aggregated *bacteria*

Xap strain CITA 33 (deposited at the publicly accessible culture collection International Centre of Microbial Resource – French Collection for Plant-Associated Bacteria. INRAE, CIRM-CFBP, with the reference number CFBP 9272), isolated from almond (*Prunus amygdalus*) (cv. Guara), was used in all the assays (Cuesta-Morrondo et al. 2022; Garita-Cambronero et al. 2014). The strain CITA 33 was routinely grown at 27 °C for 72 h in Luria–Bertani (LB) agar plates (per L: 10 g tryptone, 5 g yeast extract, 5 g sodium chloride and 1.5% agar, pH 7.2 ± 0.2). A bacterial suspension was prepared from a single colony grown in LB broth, incubated overnight at 27 °C in a rotatory shaker, and then adjusted by spectrophotometer to 10^8 colony forming units (cfu)/mL.

Viable planktonic bacteria were obtained from 5 mL of this overnight 10^8 cfu/mL suspension incubated for 24, 48, 72, 96, or 144 h in a rotatory shaker at 27 °C. To produce bacterial biofilms, aliquots of 150 µL of an overnight Xap suspension, prepared as above, were deposited into plastic containers and incubated at 27 °C under static conditions for 72 h. Supernatants were then carefully removed and bacterial pellets were incubated for an additional 24, 48, 72, 96, or 144 h at 27 °C in static and dried conditions. Bacterial cells adhered to the container surface were recovered after adding 2 mL of phosphate-buffered saline (PBS) buffer and 30 min shaking (100 rpm).

Dead cells were obtained by heat treatment from planktonic bacterial suspension or from the bacterial suspension recovered from biofilms. The bacterial suspensions (400 µL) were inactivated on a heat block at 95 °C for 10 and 20 min, or at 100 °C for 15 min.

Assessment of the viability of Xap cells

Cellular viability was confirmed by three methods: (i) plating onto LB agar medium and incubation at 27 °C for 48–72 h; (ii) evaluation of the cellular respiratory activity; and (iii) visualization of cellular membrane integrity. To evaluate respiratory activity, alamarBlue™ Cell Viability Reagent

(Bio-Rad Laboratories Inc., Hercules, CA, USA) was used according to the manufacturer's instructions. Briefly, 10 µL of alamarBlue™ reagent was added to each of a polypropylene 96-well plate containing 100 µL of inactivated or living bacterial suspensions from planktonic and biofilm samples. The samples were incubated in darkness at 27 °C for 48 h after adding the reagent. Samples that turned from blue to pink, indicating respiratory activity, were considered positive. To verify bacterial membrane integrity, LIVE/DEAD *Bac*Light™ Bacterial Viability Kit (Invitrogen, Carlsband, CA, USA) was used following manufacturer's instructions. Images from stained bacteria were visualized with a microscopy (Leica SP5C) (Leica Microsystems, Wetzlar, Germany) with a × 40 objective lens and excitation/emission light of 485/498 nm. Red cells were considered dead while green cells were considered alive, and the double-staining cells were considered an intermediate state of membrane-compromised cells. Three independent repetitions with five technical replicates each were performed for the assays (i) and (ii), whereas two replicates were done for assay (iii).

PMA, PMAxx™, and EMA treatment

Three photoreactive DNA-binding dyes were assayed at different concentrations and incubation conditions: propidium monoazide (PMA), its improved version PMAxx™, and ethidium monoazide bromide (EMA) (Biotium, Fremont, CA, USA). For the assays, 400 µL of live and heat-treated Xap CITA 33 suspensions were supplemented with PMA or PMAxx™ to obtain a final dye concentration of 10, 50, 75, 100, 150, and 200 µM. A mixture of EMA/PMA with final dye concentrations of 10 µM/75 µM was tested according to a previous report (Truchado et al. 2020). In addition, higher concentrations of both agents 35 µM/100 µM were also assayed. After dye addition, samples were gently mixed and shaken at 100 rpm in darkness at room temperature, or at 40 °C for 10, 30, or 40 min. Samples treated and non-treated with DNA-binding dyes were subsequently exposed to blue-light photolysis for 15 min with a PMA Lite™ LED photolysis Device (Biotium, Fremont, CA, USA). Bacterial cells were concentrated by centrifugation (20,000 *g*) for 10 min at 22 °C and pellets were stored at − 20 °C for further DNA extraction and amplification.

The effect of photoactivation process and toxicity of intercalating dyes were checked by growing serial dilution of bacterial suspension from 1 to 10^8 cfu/mL on LB plates.

Bacterial DNA extraction for v-qPCR reactions

Bacterial DNA extraction was performed using QiAmp® DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. Briefly, proteinase K was added to the bacterial pellet suspended in the tissue lysis buffer and incubated at 56 °C for 90 min. For complete cell lysis, samples were incubated with RNase at room temperature for 2 min, followed by incubation with lysis buffer at 70 °C for 10 min, and then mixed with 100% ethanol. Samples were deposited in a QIAamp column and centrifuged (20,000 *g*) for 3 min. Purified DNA was eluted in 200 µL of elution buffer. DNA concentration and purity were measured in a NanoDrop™ ND-2000 UV–Vis spectrophotometer (Thermo Fisher Scientific, Whaltham, MA, USA). Extracted DNA was stored at − 20 °C until used as a template in v-qPCR reactions.

v-qPCR conditions

The effect of photoreactive dyes on the PCR amplification of DNA from Xap CITA 33, and their ability to suppress amplification from dead cells, were assessed through v-qPCR analyses. For PCR, DNA extracted from bacterial culture, obtained as described above, was used as a template. v-qPCR amplifications were performed using primers and probe targeting a sequence within the virulence effector gene *xopE3* (EPPO 2021; Garita-Cambronero et al. 2017) in 25 µL reaction volume containing: 12.5 µL of 2X GoTaqR Probe qPCR master mix (Promega Biotech Ibérica S.L., Madrid, Spain), 0.4 µM final concentrations of each primer, 150 mM TaqMan probe, and 5 µL of template DNA. Amplification reactions were conducted with the following cycling conditions: one cycle of 5 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 1 min, and extension and annealing at 59 °C for 1 min.

Cut-off value and limit of detection (LOD)

The cut-off value, i.e., the PCR threshold cycle number (Ct) above which signals were not considered positive, was determined with the receiver operating characteristic (ROC) method that combines both sensitivity and 1-specificity (Nutz et al. 2011). Sensitivity was defined as the probability of detecting low levels of target DNA (true positives). Specificity was defined as the probability to detect true negative rate, then 1-specificity gave the false positive rate. For each cycle of the v-qPCR run, both sensitivity and specificity were calculated and plotted with IBM SPSS Statistics version 22 program (SPSS, Chicago, IL, USA) with a confidence level of 95%. The Ct cut-off value was selected according to the point on the curve where 1 − specificity and sensitivity were the closest to 0 and 1, respectively, and then confirmed by the maximal Youden index J (Youden 1950). DNA sample yielding a Ct value higher than the determined cut-off value was considered negative (dead bacterial cells). The analysis was performed with alive cells, positive status (35 samples), and with dead bacteria (24 samples). The limit of detection (LOD) is defined as the lowest amount of

analyte (measurand) in a sample that can be detected with stated probability, although perhaps not quantified as an exact value (Forootan et al. 2017). The analytical sensitivity of v-qPCR for the effector gene *xopE3* was estimated by determining the concentration at which there is a 95% probability of detection (LOD 95%), considering the established cut-off value. The experiment was repeated at least twice.

Validation of the v-qPCR in planta

Once determined PMAxx™ as the most suitable dye for the discrimination of viable and dead Xap cells, its effect on v-qPCR amplification of viable cells and its ability to suppress amplification of dead cells were evaluated in planta. To prepare the bacterium inoculum, Xap strain CITA 33 was grown on YPGA medium (Ridé 1969) (per L: 5 g yeast extract, 5 g bacteriological peptone, 10 g glucose, and 20 g agar pH 7.0 to 7.2) for 3 to 4 days at 26 °C. As above, bacterial suspensions were prepared from a single colony grown in LB broth and adjusted to 10^8 cfu/mL.

Two-year-old almond (*Prunus dulcis*) plants (cv. Aylés), grown in pots filled with a peat and sand mix and without any fertilization, were used for Xap inoculation. Prior to inoculation, the plants were enclosed for 24 h at 30 °C under transparent plastic to create a humid chamber and facilitate stomatal opening (da Silva Vasconcellos et al. 2014). The bacterial inoculum was then infiltrated into the underside of each young leaf at two sites using a 1-mL syringe without a needle. Two leaves on three almond plants were inoculated with the bacterium, while mock inoculations (PBS) were conducted under the same conditions on two leaves of one plant. The inoculated plants were then incubated at 26 °C during a 16-h daytime period and at 22 °C for an 8-h night period. To evaluate bacterial populations, portions of leaves containing the inoculated area (1 cm wide × 1 cm length) were comminuted in 1 mL PBS amended with 2% polyvinylpyrrolidone (PVP) 40 T (Agdia, Elkhart, IN, USA), vortexed for 30 s and then introduced in 50-mL Falcon tubes containing 1.9 mL of PBS amended with 2% PVP 40. Tenfold serially diluted aliquots of each sample were spread onto YPGA medium and typical yellow colonies were counted and identified by qPCR (Garita-Cambronero et al. 2017). Subsequently, each sample was subdivided into six 400 µL aliquots which were submitted to different treatments: (i) heat-treatment (20 min at 95 °C) and 100 µM of PMAxx™ (two aliquots); (ii) room temperature and 100 µM of PMAxx™ (two aliquots); and (iii) room temperature and no addition of PMAxx™ (two aliquots). All treated samples were processed as described above. Moreover, after heat treatment, and prior to the addition of PMAxx™, aliquots of these samples were plated onto YPGA medium to confirm Xap inactivation (absence of bacterial growth).

DNA extraction of samples was performed with DNeasy® Plant Mini kit (QIAGEN, Hilden Germany) according to the manufacturer's instructions, and its concentration and purity were determined by spectrophotometric measurement as previously described. Extracted DNA was stored at − 20 °C until used as a template in v-qPCR reactions with the cycling conditions detailed above.

To generate calibration curves for bacterial quantification purposes, portions (1 cm wide × 1 cm length) leaves of GF-677 (hybrid rootstock *P. dulcis* x *Prunus persica*) were comminuted and spiked with decreasing concentrations of Xap CITA 33, ranging from 10^7 to 10^2 cfu/mL. Aliquots of these samples were amended with 100 µM PMAxx™ or with PBS. DNA from the serial dilution was extracted (DNeasy® Plant Mini Kit, QIAGEN, Hilden, Germany), and v-qPCR reactions were performed as above. Linear regression curves, plotting the Ct values of each reaction against the logarithmic values of Xap CITA 33 DNA concentrations, were constructed from two independent repetitions with duplicated serial dilutions and three v-qPCR runs for each bacterial concentration. Calibration curves, both with and without the use of PMAxx™, were generated to estimate bacterial abundance in leaves of inoculated almond plants at 7 and 30 days of post-inoculation (dpi). The determination coefficient (R^2) for each curve was calculated from the DNA concentrations extracted from each bacterial dilution series.

Statistical data analyses

Statistical analyses for cut-off value and limit of detection study were performed with IBM SPSS software, version 22 (SPSS, Chicago, IL, USA). The normality of the data was verified with the Kolmogorov–Smirnov (Lilliefors correction), Shapiro–Wilk, and Anderson–Darling tests. Group comparisons of means were performed with *t*-test and the Mann–Whitney *U* test for two groups, and analysis of variance (ANOVA) was determined with the Welch, Brown-Forsythe, and Kruskal–Wallis tests for three or more group comparisons. The Levene test was applied to check homoscedasticity. The Tukey and Games-Howell tests were used for multiple comparisons of means. Differences with p-value < 0.05 were considered significant.

To assess differences in bacterial populations determined by various methods in plants inoculated after 7 and 30 dpi, the data were subjected to the Kruskal–Wallis test using SigmaPlot software version 15 (Systat Software Inc., San Jose, CA, USA) (https://systatsoftware.com/products/sigmaplot/).

Results

Verification of bacterial viability

Heat treatments can result in dead bacteria with different levels of cell membrane integrity. To demonstrate the suitability of the v-qPCR assay for specifically detecting viable bacteria, three different tests were used to confirm complete inactivation of Xap strain CITA 33 in both planktonic and biofilm stages (Fig. 1). CITA 33 strain exposed to a temperature of 95 °C for 20 min showed no respiratory activity or cellular growth in LB agar medium (Fig. 1a, b). Furthermore, the membrane integrity of bacterial cell at the different stages assayed was compromised, as evidenced by staining with propidium iodide and SYTO 9 (Fig. 1c). Similar results were observed when the inactivation of bacteria was attempted at 100 °C for 15 min. Membrane integrity and respiratory assay results confirmed cell inactivation, ensuring complete non-viability of the samples.

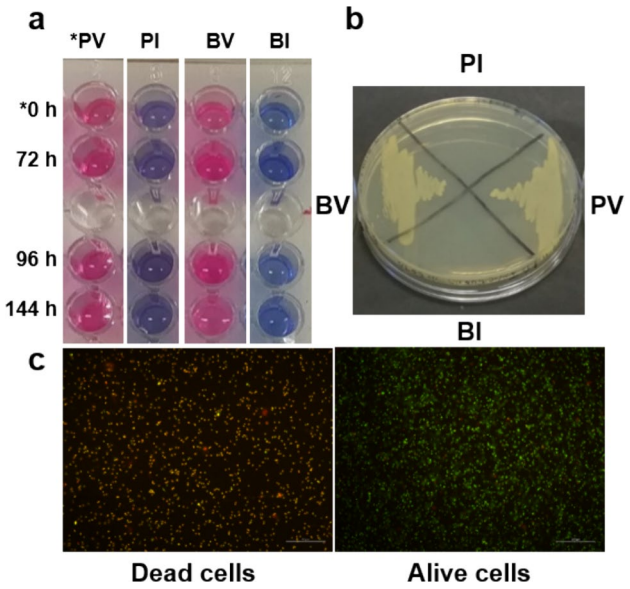

Fig. 1 Viability of the Xap strain CITA 33 in planktonic and biofilm states after 95 °C for 20 min treatment. **a** Evaluation of bacterial respiratory activity by alamarBlue™. Blue, negative result; pink, positive result. **b** Bacterial growth on LB Petri dishes at 72, 96, and 144 h of growth. **c** Visualization of membrane integrity by LIVE/DEAD *Bac*Light™ Bacterial Viability Kit of a Xap CITA 33 suspension at 10^8 cfu/mL. *PV, alive planktonic; PI, inactivated planktonic cells; BV, alive biofilm cells; BI, inactivated biofilm cells

Determination of experimental conditions for the use of different intercalating agents in viability assays

To define the optimal temperature and time of incubation for use with the intercalating agents, planktonic suspensions of Xap CITA 33 at 10^8 and 10^7 cfu/mL, encompassed by dead and alive bacteria, were incubated in the presence of 75 µM PMAxx™ and 10 µM EMA/75 µM PMA at room temperature or at 40 °C for 10, 30, or 40 min. No significant differences were observed among the various combinations; therefore, incubation at room temperature for 10 min was selected for subsequent assays.

Intercalating agents PMA, PMAxx™, and the mixture EMA/PMA were evaluated at different concentrations to select the most effective one for distinguishing between viable and non-viable bacteria (Fig. 2).

When the samples were treated with PMA or PMAxx™ at 100 µM, a difference of 5 to 9 cycles was obtained between those Ct values from alive and heat-inactivated bacteria. The disparity between active and inactive cells was higher with the less concentrated suspension (10^7 cfu/mL) compared to the most concentrated one (10^8 cfu/mL). When the same bacterial suspensions were treated with

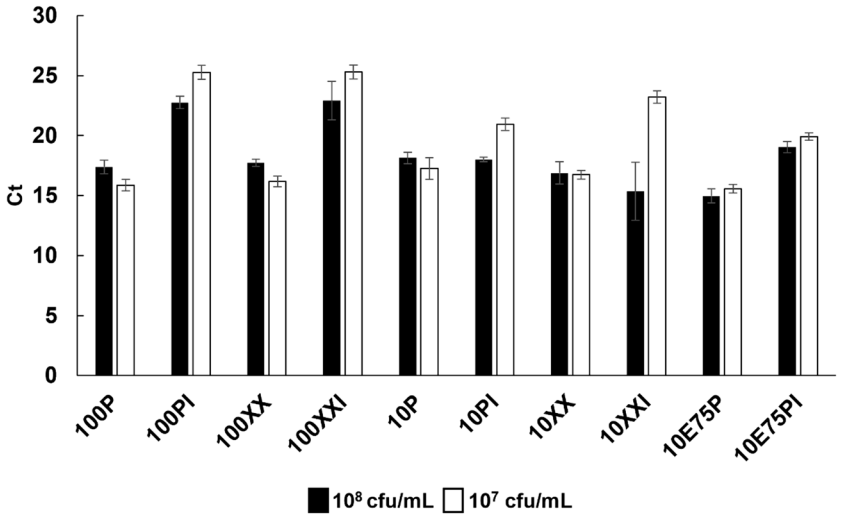

Fig. 2 Ct values obtained from v-qPCR using different intercalating agents from two concentrations of Xap CITA 33 (10^8 and 10^7 cfu/mL). P, samples treated with PMA; I, heat-inactivated cells; XX, samples treated with PMAxx.™; E, samples treated with EMA. Numbers indicate the intercalating agent concentration (µM)

10 µM PMA or PMAxx™, less differences were recorded from live or inactivated cells but those were higher using PMAxx™. Heat-treated samples with the addition of the mixture 10 µM EMA/75 µM PMA showed lower differences in Ct between active and inactivated cells as compared with either PMA or PMAxx™. Based on these results, PMAxx™ was selected as the more suitable intercalating agent to discriminate between viable and dead bacteria.

Once PMAxx™ was selected, different concentrations of the intercalating agent were tested to ensure total cover of the bacterial population analyzed (Table 1). Although amplification of high concentrations of heat-inactivated bacteria was not fully inhibited with any PMAxx™ concentrations assayed, the Ct average value from dead cells was always significantly higher than this from alive cells. Thus, the average Ct value of 23.6, obtained with 75, 100, 150, and 200 µM of PMAxx™, was 7 cycles higher than the average Ct value of 16.6 for live bacterial cells. Similar Ct values were obtained with alive cells subjected or not to treatment with 100 µM PMAxx™. No influence of photoactivation was observed in non-heat-inactivated bacteria. The potential effect of a significant reduction in bacterial culturability was not elucidated after treatment with 100 µM PMAxx™.

Table 1 Mean Ct of v-qPCR of viable and dead cells with different PMAxx™ concentrations

PMAxx™ concentration (µM)	Cell type	Average Ct	SD
10	V	16.9	0.941018471
10	I	15.4	0.941018471
50	I	20.2	0.642960365
75	V	15.6	0.364329111
75	I	23.4	0.615944063
100	V	17.7	0.283708921
100	I	23.4	0.377425421
150	I	23.7	0.762159264
200	V	15.8	0.275540567
200	I	23.7	0.379812537
0	V	15.8	0.223390054
0	NF	17.0	0.682388095
0	I	18.6	0.398011813

The bacterial concentration assayed was 10^8 cfu/mL

V alive cells photoactivated, *I* heat-inactivated and photoactivated cells, *NF* alive samples no photoactivated, *SD* standard deviation

Selection of a cut-off value for viable quantitative PCR (v-qPCR) on pure bacterial cultures

Based on the statistical analyses on ROC curve, when analyzing concentration of 10^8 cfu/mL, the threshold between alive and dead cells was at Ct of 19.2, with a sensitivity of 0.97, and 1-specificity value of 0.068. These analyses were able to account for 97.8% of the cases. To evaluate the protocol with different bacterial concentrations, serial dilutions (1–10^8 cfu/mL) of alive and dead cells were tested (Fig. 3). Ct values from dead cells treated with PMAxx™ ranged from 26.6, for the highest bacterial concentration, to 33.9, for the lowest bacterial concentrations. Meanwhile, Ct values from serial dilutions of inactivated cells without PMAxx™ treatment and from alive cells with or without intercalating agent showed a similar pattern, with Ct values ranging from 16.9 to 33.4 (Fig. 3).

The cut-off obtained with ROC curve of serial dilutions (10^3–10^8 cfu/mL) of planktonic Xap CITA 33 was a Ct of 26.2, with a sensitivity of 1 and 1-specificity value of 0.1, accounted for 96.9% of the cases.

Xap CITA 33 biofilm time course was tested compared with planktonic bacterial cells (Fig. 4). No differences were observed in the amplification of *xopE3* gene between

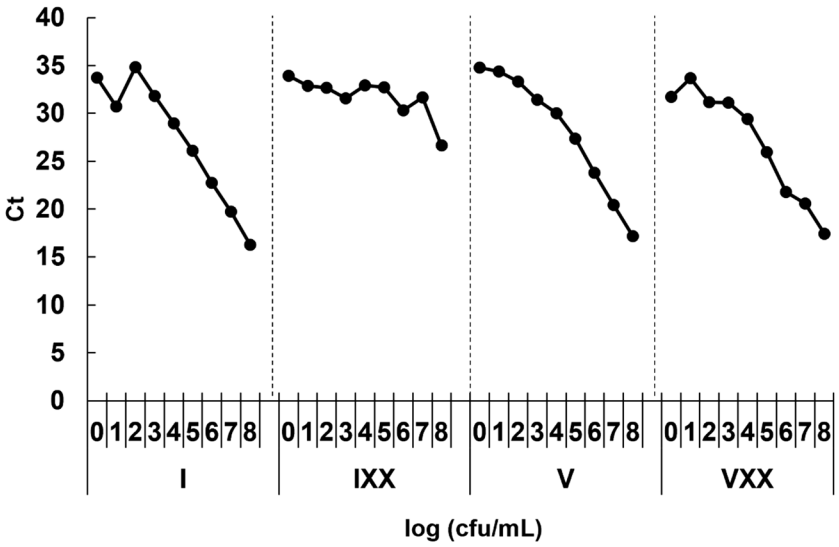

Fig. 3 Ct values obtained after v-qPCR from serial dilutions of DNA from alive Xap strain CITA 33 suspensions ranging from 1 to 10^8 cfu/mL. I, dead cells; XX, samples treated with PMAxx™; V, viable bacteria. The graph shows the means of two different assays, each with two replicates. The "zig-zags" observed at the lowest concentrations are attributable to the non-strict linear correlation between Ct and concentration in these samples (Fig. 5)

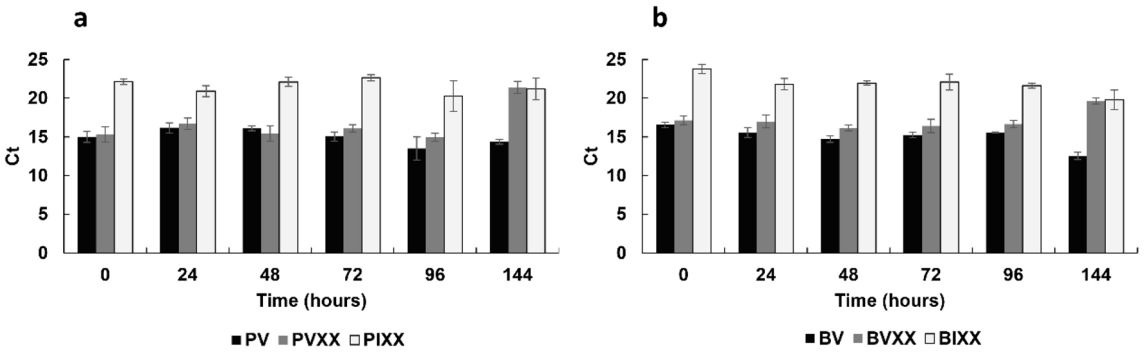

Fig. 4 Ct values of *XopE3* gene amplification of planktonic (**a**) and biofilm cells (**b**) of Xap strain CITA 33 at different cellular growth times. P, planktonic cells; B, biofilm; V, alive cells; I, inactivated cells; XX, samples treated with 100 µM PMAxx™

planktonic or biofilm cells when alive bacterial cells were treated with PMAxx™. Dead cells showed 6–7 cycles of higher amplification when incubated with PMAxx™ compared to those not treated, across all growth times checked. A noticeable reduction in viable cells, indicated by higher Ct values, occurred at 144 h in samples treated with PMAxx™ due to nutrient depletion during the death phase following the stationary phase.

When planktonic and biofilm stage coexist, statistical analyses based on ROC curve give a predicted Ct value of 20.9 (equal to 21) for a sensitivity of 0.91 and 1-especificity value of 0.063, that accounted for 96.8% of cases, considering all days of growth. Cut-off value for Xap CITA 33 at biofilm stage is Ct of 19.3, with a sensitivity of 1 and 0.73 of 1-specificity, that accounted for 96.8% of cases. The analysis of ROC curve for each day tested, 0 h, 24 h, 48 h, 72 h, and 96 h of biofilm maturation resulted in a cut-off of 19–20, with a sensitivity of 1 and 0 of 1-specificity, that explains 100% of cases. The cut-off obtained for 144 h of biofilm formation was 21.9, with a sensitivity of 1 and 0.25 of 1-specificity, that accounted for 93.1% of cases.

Discrimination between viable and dead cells of Xap strain CITA 33 in almond tissue

Calibration curves intended to quantify Xap in plants, obtained from serial dilutions of samples spiked with strain CITA 33 treated and non-treated with PMAxx™, showed slight differences in the slope of the lines and, consequently, in amplification efficiency (Fig. 5). For this reason, in the estimation of bacterial populations by qPCR, the derivatives of qPCR both without PMAxx™ and with PMAxx™ were used in each case.

To evaluate the v-qPCR method in almond plants, Xap strain CITA 33 was inoculated into leaves that exhibited symptoms at 7 dpi, with analyses subsequently performed at both 7 and 30 dpi. At 7 dpi, the estimated bacterial populations by PCR without intercalating dye (qPCR) ($10^{9.4}$ to $10^{10.8}$ cfu/mL) or v-qPCR using 100 µM PMAx™ ($10^{9.2}$

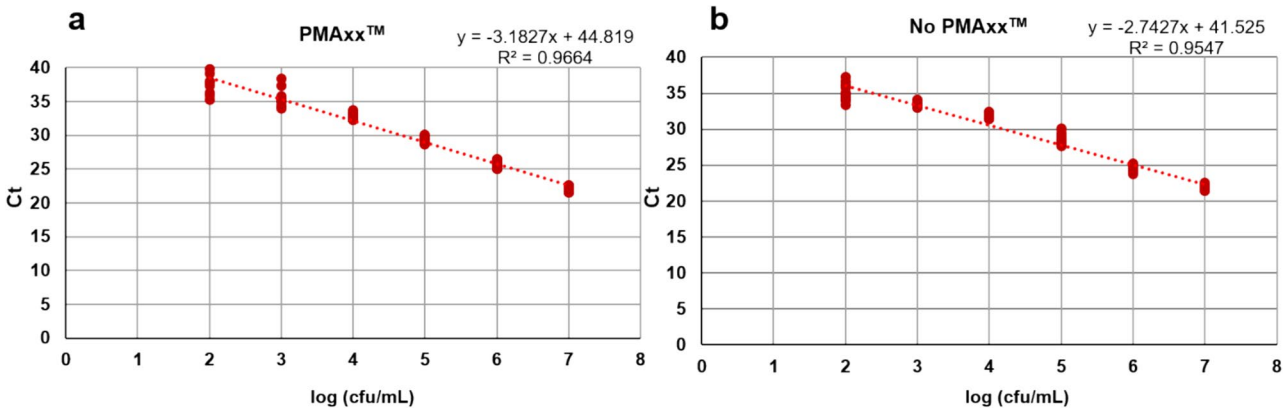

Fig. 5 Calibration curve of log (cfu/mL) versus threshold cycle (Ct) after qPCR using primers and probe targeting *xopE3* gene from serial dilutions of Xap CITA 33 in spiked samples non-treated (**a**) and treated (**b**) with PMAxx™. The corresponding regression equations and coefficients of determination (R^2) are shown on the plot. Data are means of two technical replications

to $10^{10.4}$ cfu/mL) did not show significant differences. At 7 dpi, colony counting indicated a significantly higher concentration, likely due to an overestimation of the inoculated bacteria resulting from the use of a non-specific culture medium for isolation. However, these differences became evident at 30 dpi, with a higher population estimated by qPCR ($10^{10.1}$ to $10^{10.9}$ cfu/mL) compared to v-qPCR ($10^{7.5}$ to $10^{8.9}$ cfu/mL) with the intercalating agent. Moreover, the population estimated with qPCR and the intercalating agent was not significantly different from that determined by plate colony counting ($10^{8.2}$ to $10^{9.6}$ cfu/mL). In both cases, the population estimated from heat-inactivated samples was significantly lower (Fig. 6).

The cut-off determined at 7 dpi by ROC curve analysis to discriminate between viable and dead bacterial cells in almond leaves was 27.63 (equal to 28), with a sensitivity of 1 and a value of 1-specificity of 0.2. This cut-off accounted for 92% of the cases. At 30 dpi, the cut-off value was 29.20 (equal to 29), with a sensitivity of 0.96 and a value of 1-specificity of 0.16. This cut-off accounted for 90% of the cases. Moreover, at 7 dpi, Ct values from heat-inactivated samples ranged from 28.14 to 33.61 and, at 30 dpi, from 29.55 to 32.24. The bacterial populations estimated in heat-inactivated samples by v-qPCR using PMAxx™ were also significantly lower than those from non-inactivated cells assessed by qPCR, v-qPCR with PMAxx™, or colony counting. Moreover, they were not considered positive according to the calculated cut-off.

Analytical sensitivity

The cut-off for determining negative results in plants was set at Ct > 28–29, which was used to transform the quantitative data into reliable qualitative results. The LOD 95% was determined using the Gompertz mathematical model, which was identified as the best-fitting model to calculate an estimated LOD 95% with bacterial dilutions in almond leaves. The R^2 of the model was strongly influenced by the data points at 0 and 1, with a value of 0.989. The probable LOD estimated was 10^3 cfu/mL, corresponding to 64.41 bacteria per reaction.

Discussion

Determining the population of an infectious pathogen accurately and with high sensitivity is crucial for implementing effective control measures against any plant disease. However, the absence of false-positive results is also of utmost importance, especially when detecting regulated or quarantine pathogens. A false positive can be defined as any sample that does not represent a true epidemiological threat. This may occur either because a pathogen is detected when it is not present, due to a detection strategy that lacks specificity, or because, even if present, it lacks infectious capacity due to viability deficiencies. Therefore, false positives can lead to unjustified and unacceptable economic losses, and potentially harmful effects on the environment or human health due to unnecessary phytosanitary treatments. Moreover, epidemiological studies also require reliable data to model the

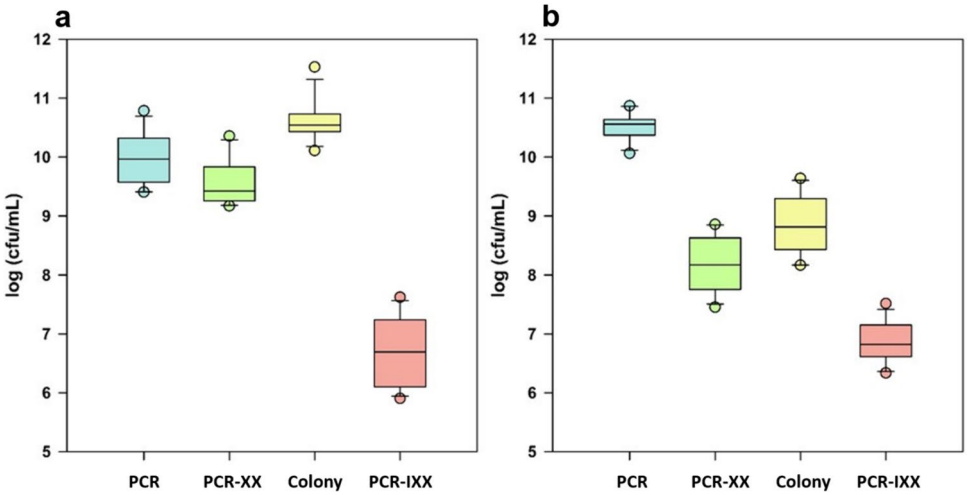

Fig. 6 Detection of Xap strain CITA 33 in inoculated *Prunus* at 7 (**a**) and 30 (**b**) dpi, by PCR compared to plate colony counting. PCR without PMAxx™ (PCR), combined with PMAxx™ (PCR-XX), colony counting (Colony), and PCR combined with PMAxx™ after heat inactivation (PCR-IXX). Statistical analysis was performed using the Kruskal–Wallis one-way analysis of variance to evaluate the significance of observed differences ($p < 0.05$). Boxes depict the upper and lower quartiles. The horizontal line indicates the median, while whiskers indicate boundaries of maximum/minimum points within the interquartile range. Outliers are indicated by points

spatial–temporal dispersal of truly pathogenic organisms, and this means that the pathogens detected are viable and capable of growing and producing new infection foci.

qPCR techniques are routinely used for the detection and identification of various pests, including especially regulated or quarantined plant pathogenic bacteria. However, qPCR's inability to discriminate between viable and dead microbial populations necessitates validation to determine the actual biological risk of the detected pathogen. Several studies have demonstrated the utility of v-qPCR for discriminating and quantifying live and dead cells present in different types of bacterial samples (Baró et al. 2020; Daranas et al. 2018; Fittipaldi et al. 2012; Guo et al. 2024; Han et al. 2018; Kralik et al. 2010; Louzada et al. 2022; Sicard et al. 2019; Soto-Muñoz et al. 2015; Wang and Turechek 2020). For bacterial spot of stone fruits, several qPCR methods have been described (Ballard et al 2011; Bergsma-Vlami et al. 2012; Garita-Cambronero et al. 2017; Palacio-Bielsa et al. 2011). Additionally, a very recent report introduced a method that combines PCR with DNA intercalating agents to potentially discriminate between living and dead cells of Xap (Panth et al. 2024). However, this last study did not fully validate the methodology and relied on previously untested primers. In response, we developed a v-qPCR protocol based on a previous protocol that is commonly used and adopted by phytosanitary organisms like EPPO (EPPO 2021). This v-qPCR was validated to detect and distinguish viable bacteria, which may be infectious, from those that are not, using strict statistical parameters to determine the optimal Ct value for discriminating between viable and dead Xap cells. Our work also ensures that the intercalating agents used do not interfere with the viability of the detected bacteria considering both their planktonic and biofilm stages as previously characterized (Sabuquillo and Cubero 2021), and it was also assessed to evaluate the bacterial progress in inoculated almond leaves plants.

Various intercalating agents under different conditions were shown not to interfere with the biological systems and, among them, no effect was confirmed when 100 µM PMAxx™ was used. In such condition, amplification occurred around 7 cycles later in dead bacterial cells treated with PMAxx™ compared to live cells. However, amplification from inactivated planktonic cells occurred and, therefore, may generate putative false positives. As described by numerous authors, detection of the proper amplicon from dead cells using v-qPCR and DNA intercalating agents could be attributable to the fact that some inactivated cells with no metabolic activity maintained their intact membranes, and then, intercalating agent could not bind DNA (Àlvarez et al. 2013; Codony et al. 2015; Song and Wood 2021). In the tests conducted during our method's development, damaged bacteria were confirmed by checking membrane integrity, using fluorescence microscopy, and assessing viability through the detection of cellular respiration. Besides, these false positives may be a consequence of the use of intercalating agents at concentrations insufficient to cover all non-intact cells (Immanuel et al. 2020). In our work, we have addressed this issue by demonstrating that the effectiveness of nucleic acid intercalating dyes is not compromised by high concentrations of dead cells. This was shown through a titration of PMAxx™, which yielded similar Ct values even when concentrations were increased. Other authors have stated that amplicon lengths are associated with either a reduction or complete suppression of amplification in dead cells (Immanuel et al. 2020; Martin et al. 2013; Panth et al. 2024; Wang and Turechek 2020). However, the use of large target sizes results in lower sensitivity of the v-qPCR detection technique and decreased diagnostic efficiency; this is an essential issue under conditions where sensitivity is most needed, such as in asymptomatic samples where, for example, the concentration of plant pathogenic bacteria is usually low (Van Holm et al. 2021).

Herein, we demonstrate that despite the amplification from inactivated cells, the statistical analyses enabled differentiation between v-qPCR positives that correspond to viable cells and those that do not. This was first clear when the evolution of the Ct values from the v-qPCRs of both planktonic bacteria and biofilm stages were analyzed. When the majority of the population would be dead due to the absence of nutrients at 144 h, the difference in Ct values between samples treated with the intercalating agent and those not treated was over 7 cycles, representing a two order of magnitude difference. This indicates amplification of less than 1% of the population when comparing samples treated and untreated with PMAxx™. Moreover, when the bacterial population in plants was estimated by qPCR in samples containing both dead and live bacterial cells, with and without PMAxx™, a clear correlation emerged between v-qPCR combined with PMAxx™ and colony counting. In contrast, qPCR without the intercalating agent overestimated the population, as it amplified DNA from both live bacteria and the DNA that had accumulated since the onset of bacterial progression, which included DNA from both live and dead cells. It is important to highlight that when the target organisms are detected by v-qPCR, reliable results are underpinned by a preliminary determination of reliable cut-off values. In turn, the determination of this false-positive rate was made possible by calculating a reliable cut-off value. Late Ct values can be reliable providing that a veracious cut-off value is determined using a statistical approach such as the ROC curve as used here (Grosdidier et al. 2017). The ROC-based method seems to be flexible and not influenced by the specificity of the test of DNA extraction but was limited by the sensitivity of the v-qPCR equipment (Grosdidier et al. 2017). In the experiments with *Prunus* inoculated leaves, the cut-off at 7 and 30 dpi was concluded to be Ct values of

28 and 29, respectively. High levels of tannins, polyphenols, and polysaccharides may interfere in the v-qPCR process (Nunes et al. 2011). Complex matrices, as those found in environmental samples, could negatively influence the efficiency of the v-qPCR technique due to chemical adsorption and organic and inorganic compounds interfering with photo-activation (Fittipaldi et al. 2012). Probably, residues of host compounds not removed in the DNA purification may explain the different cut-off values with or without the host. However, the most relevant cut-off is the one obtained with Xap strain CITA 33 and its host, since this situation is the most similar to reality to determinate the health status of the plant and apply the most suitable measures to control the disease.

Our study demonstrates the usefulness and accuracy of v-qPCR with PMAxx™ intercalating agent methodology for discriminating between live and dead Xap cells. From this perspective, this methodology can resolve issues associated with overestimating bacterial counts caused by the presence of non-viable bacteria or extracellular DNA when using qPCR alone. Treating with intercalating agents to inhibit or restrict the amplification of dead cells is simple to carry out and does not notably prolong the time needed to obtain results. However, while this approach is applicable to various systems, it requires specific adjustments of v-qPCR protocols for each individual system. Finally, it is important to emphasize that while this methodology represents an advancement in diagnostic techniques, further research is necessary to fully prevent amplification from dead cells, thereby simplifying the analysis process.

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Author contribution PS, JC, and APB conceived and designed the study. PS, APB, and IMB conducted the laboratory experiments. PS, APB, IMB, and JC analyzed the data. PS, APB, and JC supervised the final version of the text. All authors read and approved the final manuscript.

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Data availability All data supporting the findings of this study are available within the paper. Additional details can be provided by the corresponding authors upon reasonable request.

Declarations

Ethics approval and consent to participate Not applicable.

Competing interests The authors declare no competing interests.

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