Enumeration of plant-growth promoting bacteria Herbaspirillum seropedicae viable cells by a new propidium monoazide combined with quantitative PCR (PMA-qPCR) assay

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Method Article

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Abstract

The plant growth-promoting bacteria *Herbaspirillum seropedicae* is an endophytic diazotroph found in several economically important crops. Reliable, high-yield, and cost-effective methods are needed to determine bacterial viability in inoculant formulations or in plant. The present study aims to develop a PMA-qPCR assay to evaluate viable cells of *H. seropedicae* in pure culture and maize roots grown in vitro. *H. seropedicae* grown in a culture medium was submitted to heat treatment at 48°C for different periods of time. Maize roots were inoculated, grown in vitro and collected seven days after inoculation. The bacteria viable cells were quantified using qPCR, PMA-qPCR assays, and plate counting. Standard curves were prepared, and the efficiency obtained ranged from 85 to 99%. The limit of detection (LOD) was $10^1$ genome copies, corresponding to 60.3 pg of DNA. Enumeration obtained in pure cultures by qPCR, PMA-qPCR and plate count were $8.85 \pm 0.16$, $6.51 \pm 0.12$ and $2.25 \pm 0.30$ log CFU.mL$^{-1}$ after heat treatment, respectively. These results showed that PMA-qPCR is a powerful approach for quantifying viable and viable but non-culturable cells in inoculants and plants. PMA-qPCR allowed reliable obtained results much faster than culture-dependent methods.

Introduction

*Herbaspirillum seropedicae*, the first described species of the genus *Herbaspirillum*, is an endophytic bacterium known for its beneficial association with several economically important crops. This association has already been well characterized at molecular, physiological, and microscopic levels [1, 2]. The *H. seropedicae* SmR1 is the most studied strain of the genus and has its genome sequenced and annotated [3]. When used as a bioinoculant, the mechanisms by which this strain promotes plant growth include biological nitrogen fixation, phytohormone production, and phosphate solubilization [4].

The delivery of an adequate number of viable cells to the plant and the survival of the inoculum are essentials to achieve a satisfactory colonization rate that results in the desired effect in the field. However, the viability of inoculants is affected by environmental conditions and can be influenced before, during, and after their application in response to biotic and abiotic factors [5, 6]. The lack of adequate formulations that can withstand these influences is one of the challenges of using plant growth promoting rhizobacteria in agricultural inoculants [7].

Evaluating and monitoring bacterial diversity associated with cultivated plants are done using dependent and independent cultivation methods. Counting colony-forming units in the solid medium for a long time was the most used method to monitor the viability of inoculants. However, it is a laborious method and can be time-consuming [5, 8]. Therefore, culture-independent methods, such as quantitative PCR (qPCR), began to effectively monitor the number of cells of rhizosphere inoculants in a fast and accurate way [9].

Regarding the enumeration of *H. seropedicae* DNA in plants, a qPCR assay using the species-specific HERBAS1 primers was developed to quantify bacterial DNA in inoculated maize roots [10]. In addition, the
abundance of *H. seropedicae* and the levels of bacterial transcripts *nifH*, *rpoC*, and *hrcN* were evaluated in maize plants grown under sterile and non-sterile conditions [11].

Although effective in quantifying bacterial DNA in plants, the qPCR technique may have a significant limitation, not being able to differentiate DNA from viable or non-viable cells. Thus, qPCR results may overestimate the number of viable cells. An alternative is the pretreatment of samples with DNA intercalating dyes such as propidium monoazide (PMA). PMA allows only DNA from viable cells to be amplified, including those that can be cultured and those that cannot be cultured. PMA can differentiate cell types due to its ability to penetrate dead cells or cells with compromised membranes and covalently bind to DNA after exposure to halogen light. This DNA-PMA complex is insoluble, and its amplification by qPCR is inhibited [12–14].

PMA-qPCR is a promising approach to assess the cell and storage viability of non-sporulating bacterial inoculants. However, it still needs to be evaluated for different bacterial species and strains [5]. In this sense, the plant-growth promoting bacterium *A. brasilense* as an inoculant was quantified using PMA-qPCR. Regarding the enumeration of *A. brasilense* in inoculant medium and in inoculated maize roots, similar values were obtained by PMA-qPCR and plate counting in selective culture medium. On the other hand, enumeration by qPCR obtained different and higher values, revealing that the PMA-qPCR assay was efficient in quantifying viable cells for this inoculant [15]. This paper is the first report using the PMA-qPCR approach to enumerate viable cells of plant growth-promoting bacteria in inoculants and inoculated plants. Therefore, this work aims to develop an assay to evaluate viable cells of *H. seropedicae* in pure culture and maize roots grown in vitro using the PMA-qPCR method.

**Materials And Methods**

**Bacterial growth and experimental conditions**

*H. seropedicae* strain SmR1 (strain Z78 ATCC 35893 SmR) was grown in an orbital shaker (120 rpm) at 30°C in a liquid NFbHPN medium supplemented with 5 g/L malic acid [16]. The optical density (OD) of bacterial cell culture was measured at 600 nm using a Hitachi U2910 Spectrophotometer. A correlation between OD and the number of colonies forming units (CFU) was determined by drop plate counting as the serial dilution (1:10) of known amounts of cells in 0.9% NaCl solution and triplicate dripped in a selective NFbHPN agar medium (Supplementary Figure S1). Bacterial counting was performed after incubation for 48 h at 30°C. Bacterial strains used in qPCR as negative control (*Azospirillum brasilense, Pseudomonas aeruginosa, Bacillus cereus, and Escherichia coli*) were grown as previously described [10].

**Preliminary Temperature Assay In Pure Culture**

To evaluate the applicability of the PMA dye in the differentiation of viable and non-viable cells, a preliminary analysis was performed with samples of pure cultures of *H. seropedicae* SmR1, cultured as described above. Non-viable cells were obtained, and the samples were subjected to heat treatment with
constant temperature and variable time. Four assays were carried out with bacterial cultures of \( H. \) \textit{seropedicae} SmR1 in a thermostatic water bath. In the first test, the temperature of 38°C was tested at times: 0, 15, 30, 60, 90, and 120 minutes. In the second test, the temperature was 45°C; in the third test, 52°C; and in the fourth test, 48°C. In tests 2, 3, and 4, the times used were 0, 30, 60, 90, 120, and 180 minutes. For each time, three microtubes containing 1 ml of pure culture were obtained. The microtubes were placed in a thermostatic water bath at the temperatures and times determined for each assay. At the end of each period of time, the microtubes were placed in cold water for 15 seconds so that the bacterial culture was at room temperature. Afterward, a serial dilution was performed from the dilution of 1 mL of pure culture in 0.9% NaCl solution. Plating was performed by drop on selective NFbHP malate agar medium, and for each dilution point, three drops were pipetted with a volume of 10µL (Supplementary Figure S1). Three replicates were performed per time, each plate being an experimental unit.

**Preparation Of Heat-treated Culture Samples**

\( H. \) \textit{seropedicae} SmR1 was grown until the mid-logarithmic growth phase (~ \( 10^8 \) CFU.mL\(^{-1}\)), and 1.5 mL of culture was placed in microcentrifuge tubes submerged in a thermostatic water bath at 48°C. Tubes were removed each time at 0, 30, 60, 90, 120, and 180 min after incubation. The samples were divided into three groups, the first group remained untreated (samples not treated with PMA), the second group was treated with PMA, and the third group was plated by drop plate in NFbHP medium for colony counting after 48 h of incubation at 30°C. The first and second groups of samples were subjected to centrifugation (6000 \( x \) \( g \), 4°C, 3 min), and the bacterial precipitates (pellets) were washed three times with 0.9% NaCl solution before analysis. Three biological replicates were obtained for each treatment group.

**Germination, Inoculation, And Growth Of Seedlings**

Seeds of \textit{Zea mays} (variety Dekalb390) were surface sterilized in a biosafety cabinet by washing three times with autoclaved ultrapure water, followed by 70% ethanol for 3 min, and shaken in 2% sodium hypochlorite and 2.5% Tween-20 for 30 min. Seeds were washed three times with autoclaved ultrapure water by gentle shaking, transferred to sheets of germination paper (Germitest), and maintained for three days in a growth chamber at a temperature of 25°C, in the dark, for germination. The seedlings were inoculated with the culture of \( H. \) \textit{seropedicae} SmR1 (~ \( 10^8 \) CFU.mL\(^{-1}\)) after dilution to \( 10^7 \) CFU.mL\(^{-1}\) in NFb malate medium (without nitrogen source) and maintained in an orbital shaker for 30 min at 30°C and 80 rpm [10]. Control seedlings were Mock-inoculated under the same conditions. For seedlings grown in vitro, inoculated and control seedlings were washed in autoclaved 0.9% NaCl solution for 1 min and placed in glass tubes containing plant medium solution [17]. Seedlings from each treatment were grown in a controlled-environmental chamber and placed side-by-side for 7 days (16 h photoperiod, 25°C, and 40% humidity). The experiment was conducted in a completely randomized design in three biological replicates. Maize seedlings were randomly collected 7 days after inoculation (DAI). Roots were surface
treated by 2 min immersion at 70% ethanol plus 2 min at 1% sodium hypochlorite solution. Then, roots were washed 3 times in autoclaved distilled water for further analysis.

**Preparation Of Maize Roots Samples**

For maize grown in vitro, the root samples were divided into three groups, the first group remained untreated (samples not treated with PMA), the second group was treated with PMA, and the third group was plated by drop plate in NFbHP medium for colony counting after 48 h of incubation at 30ºC. For PMA treated samples, 200 mg of roots were crushed using a mortar and pestle, in 1mL of 0.9% saline solution and transferred to microcentrifuge tubes. The aliquots were centrifuged (9000 x g, 10 min) and stored on ice until PMA treatment [15].

**Pma Treatment For Viable Cell Quantification**

PMA (Biotium Inc., Hayward, CA, USA) was dissolved (1 mg) in ultrapure water according to manufacturer instructions to obtain a 20 mM stock solution (stored at −20°C in the dark). Heat-treated bacteria culture and maize root pellets were resuspended in ultra-pure water at 500 µL and 1000 µL, respectively, and treated with PMA to obtain a final concentration of 50 µM PMA [18]. The tubes were incubated for 5 min in the dark (with intermittent agitation) and then exposed for 15 min to a 500 W halogen light source. The samples were placed 20 cm from the light source on ice to prevent heating. Then, the tubes were submitted to centrifugation (6000 x g, 10 min), and pellets were stored at −80°C until DNA extraction.

**Dna Extraction**

Genomic DNA was extracted from bacterial cultures of *H. seropedicae* SmR1 using Wizard® Genomic DNA Purification Kit (Promega™, Madison, WI, USA) with modifications [10]. Total DNA from maize roots was isolated using hot CTAB protocol [19] with modifications. Approximately 200 mg of tissue was used for extraction. Roots untreated with PMA were frozen in nitrogen liquid and ground to a fine powder right before being transferred to microtubes. Roots pretreated with PMA were macerated with saline solution as described previously. Initially, it was added 1.5% PVP directly in the falcon containing 100 mL of CTAB buffer (2% CTAB, 0.1 M Tris-HCl, 1.4 M NaCl, 0.2 M EDTA disodium), followed by pre-heating at 65 ºC for 5 min. Then, 4.5 µL of the 2-mercaptoethanol and 4 µL of RNAse were added directly to the sample tube. After, the solution was incubated in a thermostatic water bath at 65 ºC for 60 min. Each extract corresponded to a pool of four seedling roots. The concentration and quality of the DNA were measured in a Thermo Scientific Nanodrop™ 2000 spectrophotometer (Wilmington, DE, USA) by measuring O.D. of DNA sample at 260 and 280 nm.

**Real-time Pcr Quantification And Construction Of Standard Curves**
HERBAS1 primer pair was used to specifically amplify a fragment of 76 bp in a noncoding region of the *H. seropedicae* SmR1 genome [10], forward and reverse primers were 5’- TTTCGCGGTAGGCGATCA − 3’ and 5’- GAGCAATTGACCGGCAAGAC − 3’, respectively. Quantitative real-time PCR was performed in ABI PRISM 7500 Detection System (Applied Biosystems, Foster City, CA, USA). The amplification reactions contained 12.5 µL of 2 x SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 100 nM of HERBAS1 forward and reverse primers, water and 10 ng of template DNA in a final volume of 25 µL. qPCR reactions were carried out in triplicate following the cycling conditions: an initial incubation step at 50°C for 2 min, 95°C incubation for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting curve analysis was performed automatically by continuous heating from 60°C to 95°C. All real-time PCR runs were analyzed using automatic software settings. To quantify the genomic DNA amount in a pure culture of *H. seropedicae* SmR1, standard curves were prepared by serial dilution. Genomic DNA was 10-fold serially diluted in ultra-pure water to final concentrations ranging from 10^6 to 10^0 DNA copies, equivalent to concentrations of 6.03 ng to 6.03 fg. The number of genome copies (m) was calculated based on *H. seropedicae* SmR1 genome size (5.51 Mbp) [3] as described previously [10]. Amplification efficiency values were calculated from the equation $E = (10^{-1/s} - 1) \times 100$, where $E$ is the efficiency (percent) and $s$ is the slope obtained from the standard curve. Limit of detection (LOD), limit of quantification (LOQ), and other qPCR parameters were determined as previously described [20].

**H. seropedicae SmR1 enumeration by qPCR and plate counting**

Bacterial counting estimation (log CFU.mL⁻¹) of *H. seropedicae* SmR1 pure culture and maize root samples by qPCR were calculated as described previously [15] with modifications. qPCR run replicates were performed in different days. Plate counting of *H. seropedicae* strain SmR1 pure culture were determined by the drop plate method on selective agar media. Data were expressed as the number of log CFU per mL (culture). Seedlings grown in vitro were randomly collected after 7 DAI to determine bacterial root colonization. Roots were washed in autoclaved distilled water, and 200 mg were crushed in mortar and pestle in 1 mL of saline solution 0.9%. Homogenates were serially diluted and plated on selective agar media to determine CFU per gram of fresh root after 48 h of incubation at 30°C [21].

**Results**

**Preliminary viability assay in pure culture**

Four assays were developed to determine the ideal temperature to obtain non-viable cells after heat treatment. The temperatures tested were defined based on the work developed by Baldani et al. [22]. In this study, the authors determined that the ideal temperature range for the growth of the species *H. seropedicae* is from 20° to 38° C. The first temperature tested was 38° C, which had little effect on the cell viability of *H. seropedicae* SmR1 (Supplementary Figure S2A). The second temperature tested was 45° C, which caused a slight decline in the growth of *H. seropedicae* SmR1 (Supplementary Figure S2B). This temperature, although higher than the range tolerated by the bacteria, has not yet been able to cause marked damage to bacterial cells. At the temperature of 52° C, there was a sudden drop in growth
(Supplementary Figure S2B). No bacterial counting was observed in the last evaluated times. For this reason, the temperature of 48°C was tested (Supplementary Figure S2B). There was a decrease in bacterial growth, but less markedly, so the temperature of 48°C was used in the heat treatment.

**qPCR parameters for** *H. seropedicae* **strain SmR1 quantification**

qPCR standard curves were prepared to obtain qPCR parameters such as efficiency, slope, and correlation coefficient ($R^2$) (Supplementary Figure S3). Efficiency values varied from 86 to 99%, with slopes ranging from −3.33 to −3.69 (Table 1). All standard curves presented linear correlation coefficients between 0.97 and 0.99. LOD and LOQ of qPCR using pure culture samples were established as log 1 genome copy number (mean Cq = 33.46), corresponding to 60.3 fg of DNA. The repeatability standard deviation (%RSDr) of the experimental DNA copy numbers obtained in eighteen qPCR reactions presented values below 1.19%, and accuracy was evaluated as the bias (%Bias), with values ranging from −4 to 90% (Table 2).

<table>
<thead>
<tr>
<th>Pure <em>H. seropedicae</em> culture</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>qPCR run</td>
<td>Efficiency (%)</td>
<td>Slope</td>
<td>$R^2$</td>
</tr>
<tr>
<td>1</td>
<td>99</td>
<td>-3.336</td>
<td>0.996</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>-3.470</td>
<td>0.984</td>
</tr>
<tr>
<td>3</td>
<td>98</td>
<td>-3.372</td>
<td>0.977</td>
</tr>
<tr>
<td>4</td>
<td>86</td>
<td>-3.696</td>
<td>0.999</td>
</tr>
<tr>
<td>5</td>
<td>88</td>
<td>-3.654</td>
<td>0.985</td>
</tr>
<tr>
<td>6</td>
<td>96</td>
<td>-3.432</td>
<td>0.997</td>
</tr>
<tr>
<td>Mean</td>
<td>93.5</td>
<td>-3.493</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>5.4</td>
<td>0.149</td>
<td></td>
</tr>
<tr>
<td>CV (%)</td>
<td>5.7</td>
<td>4.253</td>
<td></td>
</tr>
</tbody>
</table>
Table 2
Repeatability of species-specific HERBAS1 qPCR assay using serial dilutions of DNA extracted from *H. seropedicae*.

<table>
<thead>
<tr>
<th>True Copy number</th>
<th>DNA quantity (ng)</th>
<th>%RSDr(^a)</th>
<th>Experimental copy number</th>
<th>Bias %(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10(^6)</td>
<td>6.03</td>
<td>0.99</td>
<td>1284237</td>
<td>28</td>
</tr>
<tr>
<td>10(^5)</td>
<td>0.603</td>
<td>0.92</td>
<td>110734</td>
<td>11</td>
</tr>
<tr>
<td>10(^4)</td>
<td>0.0603</td>
<td>0.49</td>
<td>10059</td>
<td>1</td>
</tr>
<tr>
<td>10(^3)</td>
<td>0.00603</td>
<td>0.88</td>
<td>960</td>
<td>-4</td>
</tr>
<tr>
<td>10(^2)</td>
<td>0.000603</td>
<td>0.71</td>
<td>90</td>
<td>-10</td>
</tr>
<tr>
<td>10(^1)</td>
<td>0.0000603</td>
<td>1.19</td>
<td>19</td>
<td>90</td>
</tr>
</tbody>
</table>

\(^a\) Relative repeatability standard deviation

\(^b\) Bias standard deviation

**PMA-treatment efficiency for *H. seropedicae* SmR1 viable cell quantification**

Intending to evaluate PMA treatment efficiency to inhibit DNA amplification of dead cells, aliquots of pure culture *H. seropedicae* SmR1 were submitted to heat treatment at 48°C for zero to 180 minutes to obtain different amounts of viable and non-viable cells. Aliquots were treated or not with PMA, DNA was extracted and submitted to qPCR and PMA-qPCR. The DNA copy number of each sample was calculated and compared among qPCR runs (Fig. 1). As expected, DNA copy number was lower for PMA-treated samples compared to samples without PMA. All samples with and without PMA presented similar DNA copy number, regardless of the heat treatment (30–180 min). As expected, the number of viable cells determined by PMA-qPCR was lower for heat treated samples compared to control samples (not heat-treated samples, time zero).

**Enumeration of *H. seropedicae* viable cells in heat-treated culture and maize roots samples**

Viable cells of *H. seropedicae* SmR1 in heat-treated pure culture and (control and inoculated) roots of maize grown in vitro were enumerated by plate count, qPCR, and qPCR coupled with PMA (PMA-qPCR). DNA was extracted in triplicate for each heat-treated culture sample and submitted to qPCR assay (Table 3). Standard curves Cq versus log CFU were plotted using mean CFU by plate counting of *H. seropedicae* (Supplementary Figure S3A). The same was carried out for in vitro assay root samples (Supplementary Figure S3B). Then, using the obtained Cq values (Table S1) and the equation described previously [20], the *H. seropedicae* count was calculated by qPCR and PMA-qPCR. The experimental values (log CFU.mL\(^{-1}\)) obtained by PMA-qPCR were lower than those obtained by qPCR (PMA-untreated samples) for all heat treatment samples (Table 3). The same was observed for bacterial count (log CFU.g\(^{-1}\)) of maize root samples by PMA-qPCR and by qPCR (Fig. 2), so higher Cq values, and
consequently lower CFU count, were obtained for PMA-treated samples compared to PMA-untreated samples (Supplementary Tables S1 and S2).

### Table 3
Comparison of *H. seropedicae* count (log CFU.mL\(^{-1}\)) obtained by qPCR, PMA-qPCR and plate count of heat-treated culture samples (48°C).

<table>
<thead>
<tr>
<th>Sample</th>
<th>qPCR count(^a) (log CFU.mL(^{-1}))</th>
<th>PMA-qPCR count(^a) (log CFU.mL(^{-1}))</th>
<th>Plate count(^b) (log CFU.mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0'</td>
<td>8.99 ± 0.12</td>
<td>7.83 ± 0.47</td>
<td>8.83 ± 0.03</td>
</tr>
<tr>
<td>30'</td>
<td>8.89 ± 0.21</td>
<td>6.02 ± 0.73</td>
<td>5.75 ± 0.35</td>
</tr>
<tr>
<td>60'</td>
<td>8.94 ± 0.16</td>
<td>6.30 ± 0.57</td>
<td>4.19 ± 0.26</td>
</tr>
<tr>
<td>90'</td>
<td>8.85 ± 0.35</td>
<td>6.50 ± 0.51</td>
<td>3.81 ± 0.23</td>
</tr>
<tr>
<td>120'</td>
<td>8.83 ± 0.09</td>
<td>6.59 ± 0.23</td>
<td>n.d.</td>
</tr>
<tr>
<td>180'</td>
<td>8.85 ± 0.16</td>
<td>6.51 ± 0.12</td>
<td>2.25 ± 0.30</td>
</tr>
</tbody>
</table>

\(^a\) Values are mean ± SD (n = 12), qPCR triplicate four independent runs.

\(^b\) Values are mean ± SD (n = 6) plate count.

n.d. not detected

Regarding pure culture samples (Table 3), *H. seropedicae* count (log CFU.mL\(^{-1}\)) by PMA-qPCR ranged from 7.8 at time zero to 6.0 after heat treatment. On the other hand, for qPCR, the count remained above 8.8. Different values were observed by plate counting, count decreased from 8.8 to 2.3 while heat treatment increases until 90 min. No bacterial counting was observed at 120 min. However, at 180 minutes, bacterial growth resumed with a count of 2.3 log CFU.mL\(^{-1}\). Although no bacterial counting was observed at 120 min of heat treatment by plate count enumeration, 6.6 log CFU.mL\(^{-1}\) was detected by PMA-qPCR.

Regarding maize root samples (Fig. 2), *H. seropedicae* enumeration in inoculated maize roots was 6.1 log CFU.g\(^{-1}\) by qPCR and 3.7 log CFU.g\(^{-1}\) by PMA-qPCR and 4.9 log CFU.g\(^{-1}\) by plate count. Bacterial enumeration in control maize roots was 2.7 log CFU.g\(^{-1}\) by qPCR, 2.2 log CFU.g\(^{-1}\) by PMA-qPCR and <1 log CFU.g\(^{-1}\) by plate count.

**Discussion**

The presence of a large number of viable cells in the inoculant is a prerequisite for satisfactory colonization to occur and for beneficial effects on the plant to be observed [5]. Regarding bacterial detection and monitoring in inoculants, molecular methods have been used because they are fast and
specific. The use of PMA associated with qPCR is an efficient technique that has been used to differentiate viable and non-viable cells [15]. This approach has been extensively used to quantify a wide range of microorganisms, in different matrices [23–26]. Methods developed to quantify microorganisms in cultivated plants used viability dyes to quantify phytopathogenic microorganisms and beneficial microorganisms associated with plants of economic interest [15, 27–30]. In the present study, we evaluated the applicability of PMA-qPCR method to enumerate *Herbaspirillum seropedicae* viable cells in heat-treated culture medium and inoculated maize roots sand compared it with qPCR and plate counting.

To establish a reliable DNA quantification of *H. seropedicae*, qPCR parameters for SYBR Green assays were defined using standard curves. Using a substantial standard curve based on a known DNA concentration makes it theoretically possible to quantify DNA from any source [31]. Considering that a consistent standard curve should have a $R^2$ value of more than 0.95 and a slope between −3.0 and −3.9 (corresponding to PCR efficiencies of 80 and 115%) [20, 32], all curves presented reliable qPCR parameters (Table 1 and Supplementary Figure S3). Similar results were already observed in qPCR assay using HERBAS1 primers for *H. seropedicae* quantification [10, 11].

Our results showed that the PCR runs (Table 2) presented low variation, indicating a suitable degree of assay accuracy. The repeatability of qPCR assays is expressed as the standard deviation of relative repeatability (RSDr). According to Kralik; Richi. [20], in clinical, veterinary and food microbial detection, there are no specific recommendations for the RSDr value in terms of its proportion to the mean value. Accuracy was also evaluated as the BIAS of the experimental mean value from the theoretical value (true copy number) for each DNA sample. The Limit of Detection (LOD) provides the sensitivity of qPCR quantification experiments, being defined as the lowest concentration of DNA in a sample that can be reliably detected. In qPCR assays, it is accepted that the LOD is the amount of DNA in which 95% of positive samples are detected [33]. The qPCR assay developed in this study ensured the reliable detection of amounts of *H. seropedicae* DNA ranging from log 6 genome copies (6.03 ng) to log 1 genome copies (60.3 fg). Similar results were obtained in previous studies about quantification of *H. seropedicae* by qPCR [10, 11].

This work evaluated PMA dye performance using DNA extracted from heat-treated pure culture of *H. seropedicae* SmR1 collected in different periods of time. To verify the efficiency of PMA dye to inhibit DNA amplification, cells were submitted to heat treatment, and mixtures of viable and non-viable cells were treated with PMA before qPCR [34]. A similar methodology was employed to quantify viable cells of *A. brasilense* and to determine the number of dead cells obtained by heat-treatment [15]. In that study, PMA dye could differentiate between viable and non-viable cells since differences were observed between CFU counts obtained by qPCR and PMA-qPCR. Observed differences show that qPCR assay detected total DNA, while PMA-qPCR detected DNA from intact membrane cells. In the quantification of *H. seropedicae*, the CFU count values by qPCR were higher than by plate count and by PMA- qPCR (Table 3). This difference can be explained because qPCR quantifies total DNA, including dead cells, whereas PMA-qPCR and plate counting quantify only viable cell DNA.
In heat-treated samples, *H. seropedicae* count values by culture medium plating reduced from 8 to 3 log CFU.mL\(^{-1}\) according to increase heat treatment until 90 min, no bacterial counting was observed at 120 min. At 180 minutes, bacterial growth was summarized with a count of 2 log CFU.mL\(^{-1}\) (Table 3).

Although the plate count decreased with the time of exposure to heat treatment, the count by PMA-qPCR ranged from 7 to 6 log CFU.mL\(^{-1}\). Similar behavior was observed in quantifying viable cells of *Pantoea agglomerans* CPA-2. After spray-drying, cell numbers obtained by PMA-qPCR (assessing intact cells) were two orders of magnitude higher than those obtained by plate-counting [27]. This indicates that a loss of culturability does not necessarily coincide with membrane damage. This cellular comportment can be explained due to the induction of the viable but not cultivable (VBNC) state, the adaptive stress response of cells against environmental conditions. Cells in the VBNC state cannot form colonies or maintain their growth but continue with the membrane intact [35, 36]. The condition of non-culturability is a reversible process. Cellular functioning resumes when the environment returns to favorable conditions or if cells can adapt to stressful situations [37, 38].

To evaluate the efficiency of PMA treatment in the presence of matrix, *H. seropedicae* was quantified by PMA-qPCR in roots of inoculated maize seedlings grown in vitro and harvested 7 DAI. Comparing the bacterial enumeration of viable cells, it was possible to observe that the quantification by PMA-qPCR was approximately twice lower than by qPCR, otherwise bacterial enumeration in inoculated maize roots by plate count was higher than estimated by PMA-qPCR (Fig. 2). Other bacterial species could grow in selective culture medium used for *H. seropedicae* plate count, so bacterial enumeration by plate count is not so specific to *H. seropedicae* as PMA-qPCR assay using *H. seropedicae* species-specific primer pair.

Reliable, high-throughput and cost-efficient methods for determining bacterial viability in inoculants are needed to facilitate the screening of an extensive range of processing parameters. PMA coupled with qPCR is a powerful approach compared with culture-dependent methods for quantifying viable cells [39]. The main advantages of PMA-qPCR are that it allows easy, simple, and more precise quantification. PMA-qPCR assay has already been proposed to quantify plant growth-promoting bacteria in maize roots and pure culture, but to our knowledge, a PMA-qPCR assay for *H. seropedicae* viable cells quantification has not been described. In conclusion, our results showed that this molecular approach allowed estimation for *H. seropedicae* SmR1 viable cell quantification in pure culture and maize root samples.

**Declarations**

**Competing interests**

The authors declare that they have no competing interests in this paper.

**Author Contributions**

A.M.P., conceptualization, writing, methodology, and investigation. E. T. C. and N. G., methodology and investigation. A. C. M. A. conceptualization, supervision, and writing – review, and editing.
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References


**Figures**

![Figure 1](image)

Figure 1

Log DNA copy number of *H. seropedicae* strain SmR1 estimated by qPCR (square) and PMA-qPCR (triangles) in four independent runs. Pure cultures were submitted to heat treatment at 48 °C for different periods of time. Values are mean ± SD of three DNA extracts (n = 3).
Figure 2

Comparison of *H. seropedicae* SmR1 count (log CFU.g\(^{-1}\)) obtained by qPCR and PMA-qPCR of maize roots samples collected at 7 days. Each bar represents the mean ± SD (n = 3) values

**Supplementary Files**

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