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Infectivity of Viable but Non-Cultivable Cells of Pigmented and Nonpigmented *Xanthomonas citri* pv. *anacardii* Strains Demonstrate the Need to Establish Indexing Protocols for Cashew Propagules

Lucas Pontes Lucena [1], Géssyka Rodrigues Albuquerque [1], Elineide Barbosa Souza [2], Ana Maria Benko-Iseppon [3], Rodrigo Dias Oliveira Carvalho [4], Flávia Figueira Aburjaile [5,*,†] and Marco Aurélio Siqueira Gama [1,*,†]

1 Department of Agronomy, Federal Rural University of Pernambuco, Recife 50670-901, PE, Brazil
2 Biology Department, Federal Rural University of Pernambuco, Recife 50670-901, PE, Brazil
3 Genetic Department, Federal Rural University of Pernambuco, Recife 50740-600, PE, Brazil
4 Department of Biochemistry and Biophysics, Institute of Health Sciences, Federal University of Bahia, Salvador 40231-300, BA, Brazil
5 Preventive Veterinary Medicine Departament, Veterinary School, Federal University of Minas Gerais, Belo Horizonte 50670-901, MG, Brazil
* Correspondence: faburjaile@gmail.com (F.F.A.); marco.gama@ufrpe.br (M.A.S.G.)
† These authors contributed equally to this work.

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Abstract: Angular leaf spot of cashew tree in Brazil has been attributed to pigmented and nonpigmented strains of *Xanthomonas citri* pv. *anacardii*. Due to the possibility of dissemination of the disease by propagating material, it is necessary to understand the survival mechanisms of the causal agent. Thus, the present study aimed to characterize the behavior of viable but non-cultivable cells (VBNC) in two pigmented strains (CCRMTAQ13 and CCRMTAQ18) and one nonpigmented strain (IBSBF2579) of *X. citri* pv. *anacardii*, integrating in silico, in vitro, and in vivo studies. Thirteen genes associated with the VBNC phase were identified in the genomes of these strains. The log phase was observed at 24, 48, and 120 h for CCRMTAQ13, CCRMTAQ18, and IBSBF2579 strains, respectively. The death phase was observed at 96 h for both pigmented strains and 168 h for the nonpigmented strain. Using qPCR analyses, it was possible to characterize the occurrence of VBNC for the three strains. When inoculated, the strains showed 100% incidence during the VBNC phase, with the IBSBF2579 strain having the longest incubation period (IP). The strains did not differ concerning final severity (FS) in the VBNC phase. To our knowledge, this is the first report of the occurrence of the VBNC mechanism in *X. citri* pv. *anacardii* strains. Furthermore, it has been demonstrated that *X. citri* pv. *anacardii* in the VBNC state is potentially infective when they meet their host's apoplast, which points to the need to use integrated practices to detect this bacterium in cashew seedlings.

Keywords: qPCR; angular leaf spot; infectivity; *Anacardium occidentale*

1. Introduction

The cashew tree (*Anacardium occidentale* L.) is a tropical plant from Brazil, and it is dispersed throughout almost the entire national territory [1], where it stands out for its domestication and high economic use [2]. The cashew nuts are the main product of this crop, and the Northeast is the main producing region of the country, with more than 137 thousand tons produced in 2019 [3]. Among the several plant pathogens which can infect cashew trees, the bacterium *Xanthomonas citri* pv. *anacardii* stands out due to its capacity to cause angular spots and dark lesions surrounding the veins of the leaves, dieback, branches' death, and water-soaked lesions in fruits [4,5]. From these lesions, pigmented and nonpigmented strains have been obtained, which are equally aggressive to cashew

trees [6]. In addition, *X. citri* pv. *anacardii* appears to be a bacterium spread only within Brazil, where it has spread between states, possibly through contaminated seedlings [4].

So far, there are four genomes of *X. citri* pv. *anacardii* available from Genbank/NCBI [7], which contain important information about genes involved in bacterial metabolism and can help the study of the survival of this bacterium, such as genes related to the mechanism of viable but non-cultivable cells (VBNC) [8,9]. The term VBNC has been used to refer to cells unable to grow in a culture medium, but which remain alive and able to resume metabolic activities under favorable conditions [10], such as nutritionally rich environments [11] or in case of contact with components extracted from the host [12].

VBNC have been reported in several phytobacteria [9,10,13–19]. In *X. campestris* pv. *campestris* [8] and *X. citri* subsp. *citri* [9], the VBNC status has been induced by contacting the bacterial suspension with copper sulfate ($CuSO_4$). In some studies, cell viability has been associated with genes related to motility [20], responsible for the biosynthesis of metabolites [11], involved in protection against oxidative stress [21], biofilm formation [22], adaptation to environmental stresses, energy generation, and cell wall composition [16].

Regarding the ability to reestablish an active metabolism from the state of VBNC, these cells have been characterized as potential inoculum sources for new infections [23]. Thus, given that bacteria in the VBNC state do not produce cultivable colonies, it may be necessary to combine molecular techniques with traditional microbiological isolation methods for more accurate detection of the pathogen [10], which could prevent the introduction of *X. citri* pv. *anacardii* into disease-free areas and avoid disease spread. Therefore, the present study aimed to verify the occurrence of VBNC using quantitative polymerase chain reaction (qPCR) in *X. citri* pv. *anacardii* and assess the capacity of these cells to initiate an infectious process in a leaf of cashew seedlings.

2. Results

2.1. In Vitro Growth Phases

The three strains of *X. citri* pv. *anacardii* showed the apex of the exponential phase at 24, 48, and 120 h, reaching concentrations of 1.15, 0.36, and 0.95 × 10^8 CFU/mL, for the strains CCRMTAQ13, CCRMTAQ18, and IBSBF2579, respectively (Figure 1a). For the death phase, the absence of bacterial growth was observed at 96, 96, and 168 h for strains CCRMTAQ13, CCRMTAQ18, and IBSBF2579, respectively. In turn, the growth curves obtained from the absorbance of the suspension of the three strains showed progressive increases up to 168 h (Figure 1b).

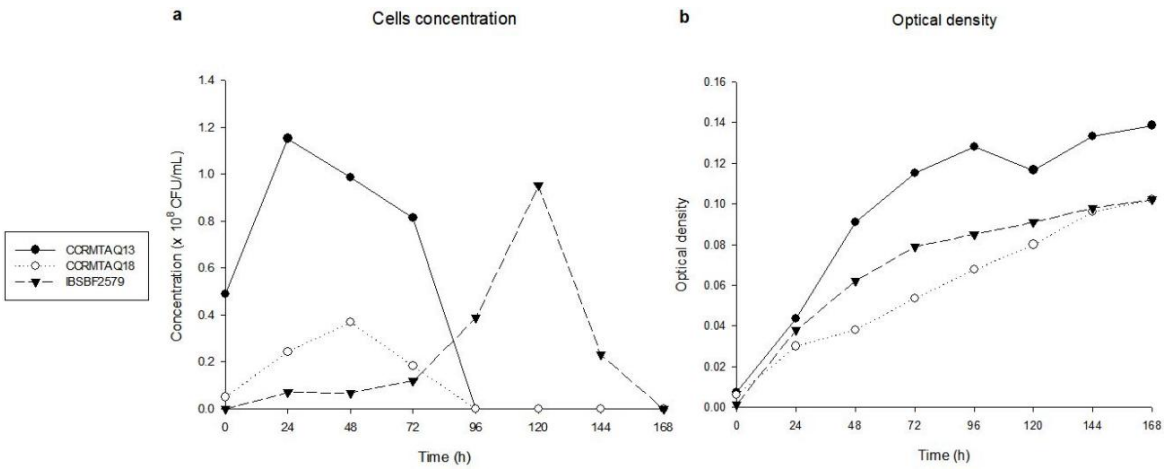

Figure 1. In vitro growth of the pigmented (CCRMTAQ13 and CCRMTAQ18) and nonpigmented (IBSBF2579) strains of *Xanthomonas citri* pv. *anacardii.* (**a**) bacterial cells concentration. (**b**) bacterial optical density over time.

2.2. Genomic Annotation, Comparative Analysis, and Primer Construction for VBNC Status Assessment

Four thousand five hundred ninety-six and 4917 coding sequences were annotated, genomic content of 5,084,712 and 5,183,683 base pairs, 64.4% and 64.1% of GC for CCRMTAQ13 and CCRMTAQ18 strains, respectively.

In the literature, 60 genes were found upregulated during the state of VBNC in bacteria. From the in silico genomic analyses, 13 different genes were present in a single copy in the bacterial genome of the three strains of *X. citri* pv. *anacardii* (Table 1). Among them, the *relA* gene was detected in silico in the three genomes analyzed, and it was selected for the primer design.

Table 1. Genes differentially expressed during the stage of viable but not cultivable cells (VBNC) reported in bacteria and found in genomes of the strains pigmented (CRMTAQ13 and CCRMTAQ18) and nonpigmented (IBSBF2579) of *Xanthomonas citri* pv. *anacardii*.

Gene	Product	Gene Size (bp)	Reference
fliG	FliG flagellar protein	975	[20]
relA	(P)ppGpp synthase	2163	
omp	Outer membrane protein	1515	[21]
rps	30S Ribossomal protein	789	
dps	DNA-binding ferritin-like protein (Oxidative damage protectant)	540	
mobA	Molybdenum cofactor guanylyltransferase	570	[24]
soxR	Transcriptional regulator soxR family	451	[25]
katG	Catalase-peroxidase	2249	
gltB	Glutamate synthase alfa subunit	4470	[22]
gltD	Glutamate synthase beta subunit	1410	
pilM	Type IV pilus assembly protein	1086	
hfq	RNA Hfq binding protein	282	[26]
murG	UDP-N-acetylglucosamine–N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	1290	[16]

2.3. VBNC State Evaluation

The data obtained through qPCR show that the FXCA and RXCA primers constructed based on the *relA* gene efficiently amplified all the samples used, demonstrating specificity in the melting curve and uniformity of peaks for all samples (Figure 2).

Considering the amplification of the *relA* gene, no significant differences were observed between the exponential and death phases, which presented 1.10 and 1.68, 0.47 and 0.33, 1.17 and 4.32 $\times$ 10^{14} copies/mL for CCRMTAQ13, CCRMTAQ18, and IBSBF2579 strains, respectively (Figure 3a). Considering the bacterial concentration in these phases, significant differences were observed between the exponential and death phases of all strains, with concentrations of 1.15, 0.36, and 0.95 $\times$ 10^{8} CFU/mL for CCRMTAQ13, CCRMTAQ18, and IBSBF2579 (Figure 3b) in the exponential phase, while in the death phase growth was not observed.

The VBNC status was induced by the time of cultivation during 96, 96, and 168 h for the strains CCRMTAQ13, CCRMTAQ18, and IBSBF2579, which presented a high number of copies/mL of the *relA* gene and bacterial concentration equal to zero.

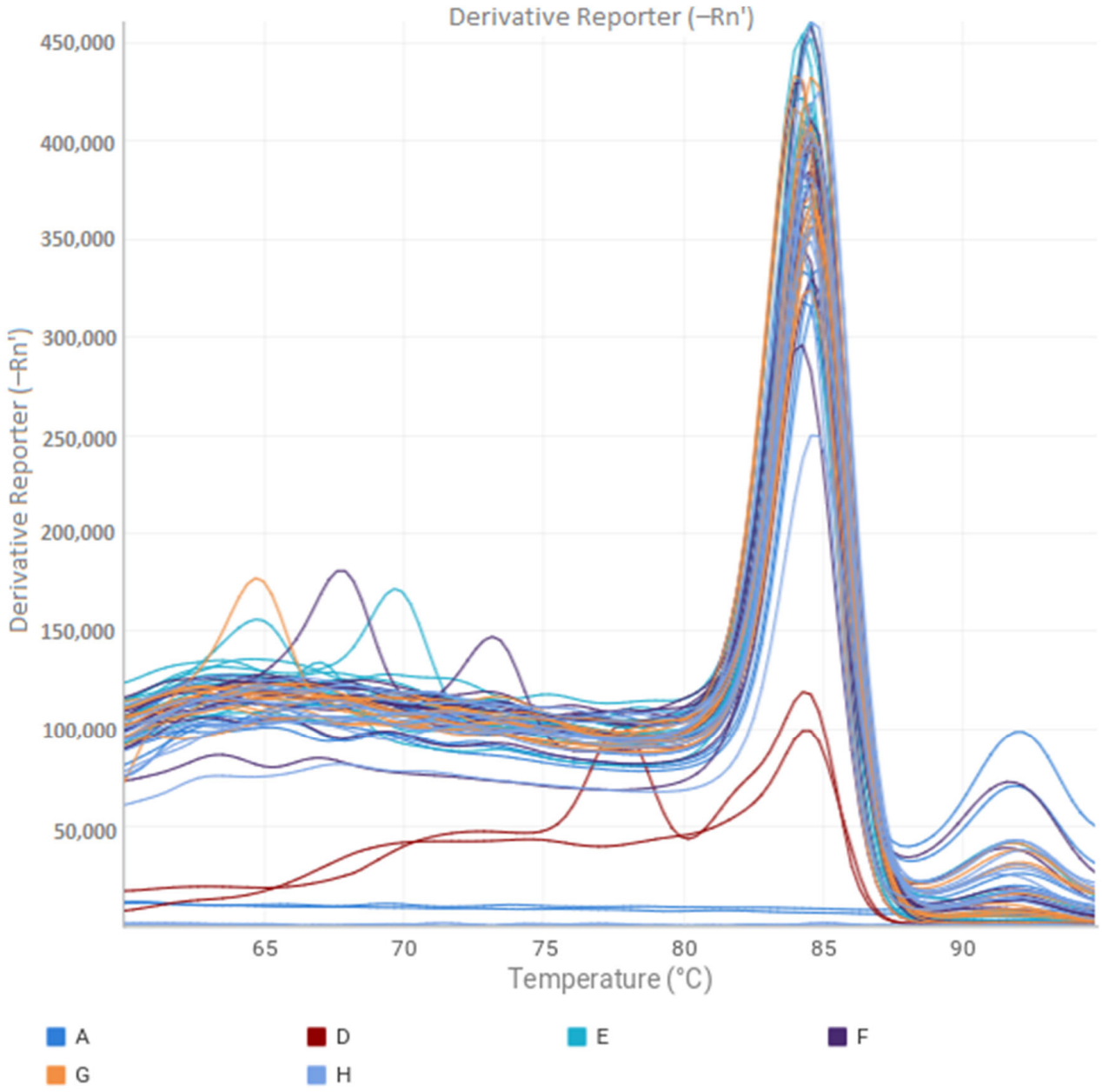

Figure 2. Melting curve of qPCR obtained from samples of strains CCRMTAQ13, CCRMTAQ18, and IBSBF2579 strains of *Xanthomonas citri* pv. *anacardii* for the *relA* gene. A1–A12—serially diluted samples for standard curve. D11–D12—blank test. E11–F4—Diluted samples of isolated CCRMTAQ13. F5–G8—Diluted samples of CCRMTAQ18. G9–H12—Diluted samples of IBSBF 2579.

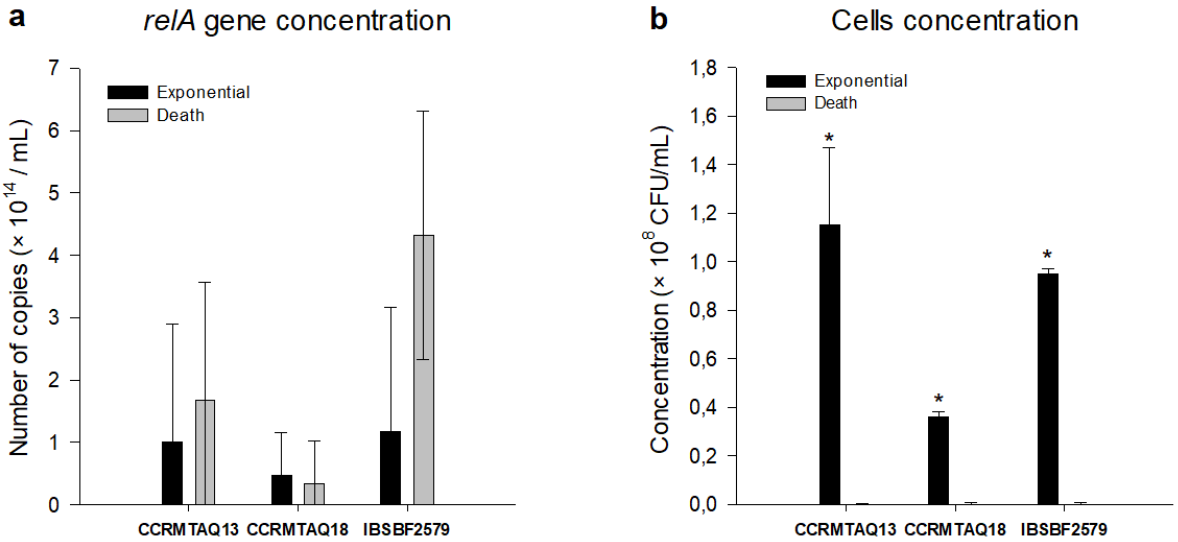

Figure 3. Comparative qPCR amplification and cell formation units of the strains pigmented (CCRMTAQ13 and CCRMTAQ18) and nonpigmented (IBSBF2579) of *Xanthomonas citri* pv. *anacardii* in exponential and death phases. (**a**), Numbers of copy of the *relA* gene. (**b**), cell formation units. Asterisk indicates statistical difference between exponential and death phases.

2.4. VBNC State Cell Infectivity

An incidence of 100% was observed for the three strains evaluated in the exponential and VBNC phases. For IP and FS, a significant interaction ($p \leq 0.05$) was observed between strains and bacterial growth phases (Table 2). The IP of the strains IBSBF2579 and CCRMTAQ13 were lower than the strain CCRMTAQ18 in the exponential phase, while the strains CCRMTAQ18 and CCRMTAQ13 showed an IP lower in the VBNC state. The strains IBSBF2579 and CCRMTAQ18 showed a difference between the IP of the exponential phase and the state of VBNC. Regarding FS, the three strains showed differences among themselves in the exponential phase, while in the state of VBNC no differences were observed among the strains. Higher values of FS were observed in the exponential phase of strains CCRMTAQ13 and IBSBF2579 than in the state of VBNC, while in strain CCRMTAQ18 the values between these phases did not differ.

Table 2. Infectivity of the pigmented (CCRMTAQ13 and CCRMTAQ18) and nonpigmented (IBSBF2579) strains of *Xanthomonas citri* pv. *anacardii* in cashew seedlings inoculated with cells in the exponential growth phase and on VBNC (viable but nonculturable cells) state.

	Incubation Period (Days)		**Final Severity (mm^2)**	
Strain	**Exponential**	**VBNC**	**Exponential**	**VBNC**
CCRMTAQ13	2.44 bA [1]	2.66 bA	6.91 aA	5.43 aB
CCRMTAQ18	3.13 aA	2.69 bA	4.72 cA	5.09 aA
IBSBF2579	2.27 bB	3.41 aA	5.98 bA	5.12 aB
VC [2] (%)	24.9		15.71	

[1] Average of nine replicates. Averages followed by the same lowercase letter in columns and uppercase letter in rows are not significantly different, as measured by LSD test ($p \leq 0.05$); [2] Variation coefficient.

3. Materials and Methods

3.1. Bacterial Strains and Growth Conditions

We used two pigmented strains (CCRMTAQ13 and CRMTAQ18) and one nonpigmented strain (IBSBF2579) of *X. citri* pv. *anacardii*. The genomes of these strains were sequenced in other studies [6,27] and deposited in the database of the GenBank/NCBI (https://www.ncbi.nlm.nih.gov/genome, accessed on 17 May 2021), with the assemblies:

GCA_002898475.1 (CCRMTAQ13), GCA_002898415.1 (CRMTAQ18), and GCA_002837255.1 (IBSBF2579). The strains CCRMTAQ13 and CCRMTAQ18 are deposited in Rosa Mariano Culture Collection from the Phytobacteriology Laboratory (LAFIBAC) of Universidade Federal Rural de Pernambuco (UFRPE), and the strain IBSBF2579 is deposited in the Phytobacteria Culture Collection from the Instituto Biológico of the São Paulo state. In the different assays carried out in this study, the strains were cultivated in Petri dishes containing NYDA medium (20 g L^{-1} agar, 10 g L^{-1} dextrose, 5 g L^{-1} yeast extract, 3 g L^{-1} meat extract, and 5 g L^{-1} peptone) at 29 °C for 36 h.

3.2. Determination of Exponential and Death Phases In Vitro

From the growth of each strain, a suspension in sterilized distilled water (SDW) was prepared to adjust the concentration to A_{570} = 0.06, corresponding to 10^8 colony-forming units (CFU) mL^{-1}. Subsequently, 1.0 mL of bacterial suspension was added to test tubes containing 9.0 mL of NYD liquid medium (NYDA, without agar), with subsequent incubation at 29 °C. Every 24 h, for seven days, the suspension concentration in the tubes was determined by removing 5 mL aliquots of the suspension to read the absorbance using a spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) at 570 nm. Simultaneously, serial dilutions were performed by removing 1.0 mL aliquots of the suspension, diluting in SDW up to 10^{-7}, and plating 0.1 mL of the last three dilutions (10^{-5}, 10^{-6}, and 10^{-7}) in Petri dishes containing NYDA medium, spreading the suspension with the aid of a Drigalski loop. The colonies formed were counted 48 h after plating to determine the concentration of the bacterial suspension using the formula CFU mL^{-1} = average number of colonies x sample dilution factor x correction factor, where the dilution factor corresponds to the potency of base 10 in which the dilution was plated, and the correction factor corresponds to 10, which refers to the fact that only 0.1 mL of suspension was plated. After counting the colonies, concentrations were standardized to 10^8 CFU mL^{-1} to make easy comparisons among the curves built for the three strains.

The experiment was performed in a completely randomized design, with eight replicates per strain, and each replicate was characterized by a tube. For each day of absorbance reading, a tube has been randomly selected. From each test tube, bacterial concentration quantifications were performed by plating on six Petri dishes for each quantified dilution. The data were used to build growth curves to determine the exponential and death phases. The experiment was carried out twice.

3.3. Genomic Annotation and Design of Primers for Evaluation of VBNC State

The automatic annotation was performed using the Rapid Annotation using Subsystem Technology (RAST) Server [28]. Then, there was a manual curation of each of these genes of the VBNC state in the Artemis program [26].

A bibliographic survey was carried out in scientific articles contained in the database of the PubMed, Web of Science, and SCOPUS platforms, aiming to identify candidate genes previously reported as expressed during the state of VBNC in bacteria. The genomes of *X. citri* pv. *anacardii* were analyzed for the presence of these genes in a single copy using the Artemis program [29], and the detected genes were selected to design primers.

The alignment of genes expressed during the VBNC state was performed using the BLASTn database [30,31]. The primers FXCA F' GATCGAAATCCAGATCCGTACC and RXCA R' TGCCGCCTTCCTTGTATTT were designed based on the *relA* using the Primer-BLAST platform [32]. These primers were verified using the Geneious Prime 2021.1.1 program (https://www.geneoius.com, accessed on 25 June 2021). Subsequently, the primers were synthesized by IDT (Integrated DNA Technologies Inc., San Diego, CA, USA) for evaluation using qPCR.

3.4. Assessment of VBNC Status via Quantitative PCR (qPCR)

DNA extraction from the strains used in this study was performed using the MiniPrep kit for bacterial genomic DNA extraction (Axygen Biosciences, Union City, CA, USA)

following the manufacturer's recommendations. The genomic DNA was quantified as previously described [4]. Six samples were extracted from bacterial growth cultivated in NYD medium at 29 °C until the exponential and death phases, which corresponded to 24 and 96 h, 24 and 96 h, and 24 and 168 h, for the strains CCRMTAQ13, CCRMTAQ18, and IBSBF2579, respectively. Collections were carried out in triplicate.

Each qPCR reaction was composed of 1× GoTaq qPCR Master Mix (2×), 2.5 nM of each primer and 3 µL of DNA. For the qPCR reaction, QuantStudio 5 Real-Time PCR System from Thermo Fischer Scientific (Waltham, MA, USA) was used, starting the amplification with 2 min at 95 °C, and 45 cycles of 15 min at 98 °C, 1 min at 60 °C. For DNA quantification, a standard curve was developed based on a 10× serial dilution from 1000 ng/µL. Aiming to quantify the number of cells in the exponential and death phases in the samples, the calculation of the total number of copies/mL was determined from the ratio of the number of base pairs of the *relA* gene x number of base pairs in the genome and the DNA concentration in ng/µL per sample. The cycle limit was determined after manually adjusting the limit number of the linear part of the qPCR logarithmic amplification curves [33]. Procedures were performed in triplicate for all samples. Samples without the presence of DNA were used to verify the reliability of the data obtained. To determine the VBNC state, the strains were cultured in NYD medium at 29 °C until the exponential and death phases, and the concentrations in CFU/mL were estimated as described above. The VBNC status was determined by comparing the number of copies/mL of the *relA* gene in the exponential and death phases with the concentration of total cells in CFU/mL in the suspensions of the strains in the respective phases [33], and the values were standardized to 10^{14} to make possible comparisons among the concentrations obtained. The VBNC status was considered active when the number of copies of the *relA* gene/mL remained high, and the concentration of CFU/mL was equal to zero.

Student's *t* test ($p < 0.05$) was used to assess significant differences between the exponential and death phases for the number of copies/mL of the *relA* gene and the bacterial concentration in CFU/mL using the STATISTIX software 9 (Tallahassee, FL, USA).

3.5. VBNC Cell Infectivity in Cashew Seedlings

The strains CCRMTAQ13, CCRMTAQ18, and IBSBF2579 were cultivated in 10 mL of NYD medium at 29 °C until reaching the exponential and death phases. Bacterial suspensions were obtained by centrifugation of NYD medium at 12,000 rpm, followed by removal of the supernatant. The concentration of the bacterial growth precipitated was adjusted in SDW to 10^8 CFU/mL. The strains were inoculated on the four apical leaves of CCP 76 cashew tree seedlings grown in a greenhouse using the infiltration method of 100 µL of suspension on the abaxial surface of the leaf blade with the aid of a needleless syringe. Leaves similarly treated with SDW were used as a control. After inoculations, the plants were incubated in a greenhouse (28 ± 2 °C, 65% RH) for 23 days, evaluating the disease incidence, the incubation period (IP), and the final disease severity (FS), as described by Gama et al. [4].

The experiment was carried out twice, using five and four repetitions per treatment in the first and second experiment, respectively. Each repetition consisted of a leaf containing four inoculation points along the leaf surface. The experiment was carried out in a completely randomized design in a factorial arrangement (3 strains × 2 growth phases). The means were submitted to analysis of variance (ANOVA) and compared by the LSD test ($p < 0.05$) using the STATISTIX 9 software (Analytical Software, Tallahassee, FL, USA).

4. Discussion

The in vitro growth curve performed with pigmented (CCRMTAQ13 and CCRMTAQ18) and nonpigmented (IBSBF2579) strains of *X. citri* pv. *anacardii* satisfactorily allowed the determination of the exponential and death phases (Figure 1a), which were used to assess the state of VBNC together with the values obtained by qPCR. The growth behavior of all strains was variable until reaching the apex of the exponential phase.

Regarding the duration of the stationary phase and, consequently, the beginning of the death phase, it was observed that the pigmented strains (CCRMTAQ13 and CCRMTAQ18) presented similar behavior to other *X. citri* strains [34], with no growth being observed at 96 h, while the nonpigmented strain (IBSBF2579) showed no growth only at 168 h. Although the bacterial concentration decreased and reached zero for all strains, the optical density curve of the bacterial suspensions kept growing until 168 h (Figure 1b), when the experience was completed. Thus, the VBNC was determined when the concentration of the suspension reached zero, and the optical density value continued to be high. These data indicated for the first time the occurrence of VBNC status in pigmented and nonpigmented strains of *X. citri* pv. *anacardii* and the ability of this bacterium to enter this state stimulated by the time of cultivation of its cells. A similar result was observed for *X. campestris* pv. *campestris*, which reached the VBNC status through liquid microcosms and sterile soil [8] and *X. citri* subsp. *citri*, with induction through low nutrient availability and copper stress [12].

Although they have been sequenced in another study [6], the genomes of pigmented strains of *X. citri* pv. *anacardii* (CCRMTAQ13 and CCRMTAQ18) had not yet been annotated. The annotations performed in this study showed a smaller number of base pairs (5,084,712 and 5,183,683 bp) and N_{50} (109,779 and 128,886 bp) and a higher number of coding sequences (4,761 and 4,917) and GC content (64.4 and 64.1%) for the strains CCRMTAQ13 and CCRMTAQ18, when compared to the number of base pairs (5,348,596 bp), N_{50} (418,068 bp), coding sequences (4,427) and GC content (63.84%) observed in the strain IBSBF2579 [27].

To prove the occurrence of the VBNC state in *X. citri* pv. *anacardii*, the genomes of the three strains used in the present study were analyzed for genes reported to be expressed during this state. Thirteen genes were detected in the genomes of *X. citri* pv. *anacardii* as upregulated in the VBNC state, which showed functions related to motility (*fliG*), hydrogen peroxide metabolism (*katG*), glutamate metabolism (*gltB* and *gltD*), bacterial conjugation pili (*pilM*), ribosomal metabolism (*rps*), transcriptional (*soxR*) and translational regulation (*hfq*), protection against oxidative stress (*dps*), peptidoglycan metabolism (*murG*), outer membrane composition (*omp*), molybdenum metabolism (*mobA*), and protection against amino acid deprivation (*relA*) (Table 1). This last gene was selected for the design of the primers because it is a gene previously successfully used to detect the VBNC phase in *Vibrio cholerae* strains [35], being responsible for both the synthesis and degradation of guanosine pentaphosphate synthetase ((p)ppGpp), as well as the regulation of this molecule, which is related to adaptation to environmental changes, such as nutritional deficit and low temperatures [36]. In addition, (p)ppGpp also promotes resistance to adverse conditions such as nutritional stress, antibiotics, and metabolic inhibitors [20,37].

All samples from the exponential and death phases were amplified with the FXCA and RXCA primers, used to assess the amplification of the *relA* gene (Figure 2). Analyses via qPCR showed a high number of copies/mL in the exponential and death phases, with no significant differences ($p < 0.05$) between these phases being observed for the three strains, demonstrating an adaptation in the stationary phase [38] concerning other phases of bacterial growth. Furthermore, the concentration of cells in the exponential phase ranged from 0.36 to 1.15×10^8 CFU/mL, while in the death phase the concentration was equal to zero. These results indicate an active cell multiplication even after the estimated time for cell death, demonstrating for the first time the occurrence of this mechanism in *X. citri* pv. *anacardii*. However, in the genus *Xanthomonas*, the occurrence of VBNC has been previously reported in *X. citri* subsp. *citri* [9,12] and in *X. campestris* pv. *campestris* [8], indicating that this survival mechanism may be consistently present in this genus.

Artificial inoculations in cashew seedlings with strains CCRMTAQ13, CCRMTAQ18, and IBSBF2579, both in the exponential phase and in the VBNC state, indicated a high capacity for infection, as demonstrated by the incidence of disease in 100% of inoculations. The significant interaction ($p \leq 0.05$) among strains and growth states (exponential and VBNC) revealed that depending on the strain, lower IP values and higher FS values can be induced in each phase (Table 2). These results demonstrate a high pathogenic capacity of

X. citri pv. *anacardii* in the VBNC state. The pathogenicity of phytopathogenic bacteria in the VBNC state has also been shown in *X. citri* subsp. *citri*, which causes symptoms typical of a citrus canker at this stage, similarly to the inoculation of exponential stage strains [9]. In this context, survival in the VBNC state and its high capacity to cause disease to suggest that infected organs can fully function as a source of inoculum [23]. Therefore, the application of conventional and molecular techniques for detection of *X. citri* pv. *anacardii* in propagation material is essential since detection accuracy is a highly relevant factor for the integrated management of diseases caused by *Xanthomonas* species and pathovars [39].

The high number of copies/mL of the *relA* gene in the death phase of the bacterial growth curve demonstrated the occurrence of the VBNC state in pigmented and non-pigmented strains of *X. citri* pv. *anacardii*. In this phase, the strains CCRMTAQ13, CCRMTAQ18, and IBSBF2579 showed high infectiousness in cashew leaves, indicating the possibility of dissemination of the pathogen in propagation materials. These results indicate that when used in isolation, traditional methods of diagnosis may underestimate the viability of the bacteria to be detected or even not detect the presence of the pathogen. Therefore, the results obtained in this study point to the need to integrate conventional and molecular approaches for more accurate detection of the presence of *X. citri* pv. *anacardii* in cashew seedlings.

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