October 2013, Vol. 7, No. 10, pp. 1019-1029
Journal of Life Sciences, ISSN 1934-7391, USA

The Viable but Non-culturable State in *Xanthomonas citri* subsp. *citri* is a Reversible State Induced by Low Nutrient Availability and Copper Stress Conditions

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Received: May 31, 2013 / Accepted: August 09, 2013 / Published: October 30, 2013.

Abstract: *Xcc* (*Xanthomonas citri* subsp. *citri*) causes citrus bacterial canker, a leaf, stem and fruit spotting disease that affects most commercial citrus species and cultivars. Copper compounds, widely used for management of this pathogen, have been reported as inducers of a VBNC (viable but non-culturable state) in plant pathogenic bacteria. VBNC may be considered as a state preceding bacterial death or as a survival mechanism under adverse conditions. Several experiments were performed to characterize the reversibility and persistence of the VBNC state in *Xcc*. VBNC was induced in low nutrient medium or with amendment of copper at concentrations used for field disease control. The VBNC condition was demonstrated to persist up to 150 days after copper treatment and was reversed after the addition of culture media without copper or amendment with citrus leaf extract. *Xcc* viability was evaluated by recovery of colonies on culture media, confirmed by membrane integrity, respiratory activity and by real-time RT-PCR targeting a sequence from the *gumD* gene. Besides, the colonies recovered were pathogenic on citrus leaves. These results confirm that the VBNC state in *Xcc* is inducible and reversible and therefore may occur in the phyllosphere when *Xcc* is under copper stress or starvation.

Key words: VBNC, resuscitation, *Xanthomonas citri* subsp. *citri*.

1. Introduction

CBC (citrus bacterial canker), caused by *Xcc* (*Xanthomonas citri* subsp. *citri*), is one of the major citrus diseases affecting most commercial citrus species and cultivars, and it is a serious leaf, stem and fruit spotting disease [1]. In areas where CBC is present, integrated control of the disease relies on the use of *Xcc*-free plant material, the use of cultivars with low susceptibility, adequate orchard management and the control of CBC epidemics by employing wind breaks and applying foliar sprays of copper formulations [2, 3].

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Although copper is an essential cofactor of many enzymes involved in bacterial metabolism, it is toxic at high concentrations [4], acting as a respiratory poison or by reacting with many bacterial proteins [5, 6]. Copper resistance in Gram negative bacteria, including *Xcc*, is commonly acquired by horizontal transfer of the resistance genes on plasmids as a consequence of repeated exposure to copper sprays [6-8]. On the other hand, bacterial cells cope with environmental stresses by various survival mechanisms [9, 10]. One of these mechanisms in Gram negative bacteria is to enter into the VBNC (viable but non-culturable) state [11], first demonstrated by Colwell and coworkers in *Vibrio cholerae* [12] and later described in several plant

pathogenic bacteria such as *Agrobacterium tumefaciens* [13], *Xanthomonas campestris* [14], *Ralstonia solanacearum* [15], *Erwinia amylovora* [16] and more recently in *Xcc* [17]. VBNC was described as a state which bacteria enter when facing adverse conditions [18]. Bacteria in the VBNC state remain viable but fail to grow on standard culture media [11, 19-21]. In the VBNC state, bacteria exhibit low metabolic activity [22], and are less susceptible to environmental stresses [23]. Many factors induce this dormant condition, as high or low temperatures, osmotic pressure, oxygen concentration, nutrient limitation, radiation or chemical substances [11, 12, 22].

Since the first description of the existence of the VBNC state as an ecologically relevant survival strategy it has been under debate. Some authors considered it as a simple non reversible state previous to cell death and without any epidemiological significance [11]. On the other hand, molecular studies performed in recent years support the claim that bacterial cells adopt the VBNC state as a survival strategy and for example, overexpression of several genes involved in protein synthesis have been detected in VBNC cells in *Vibrio cholerae* [24]. In order to consider the VBNC condition as a survival mechanism, two main requirements must be met: reversibility, ability of cells to resuscitate, and maintenance of the condition for as long as the stress continues, persistence. Resuscitation and persistence of VBNC cells are essential characteristics to discriminate this condition from a step preceding bacterial death that have already been demonstrated in several bacterial models [16, 25].

Although the VBNC condition has recently been reported in *Xcc* by del Campo et al. [17], recovery of culturability was not demonstrated, leaving the biological significance of this bacterial state unanswered. In addition, the non culturable state described by these authors was induced by short term exposure using a copper concentration higher than occurs as a result of copper spray applications in orchards [17].

Consequently, to assess the reversibility and persistence of VBNC and to provide evidence for metabolic and gene activity for *Xcc* cells in the VBNC state, the aim of this work was to study the effect of nutrient starvation and copper as inducers of VBNC, at concentrations that are encountered in the phyllosphere of citrus trees.

2. Materials and Methods

2.1 Bacterial Strains and Growth Conditions

Strain 306 of *Xcc* from the Collection of Plant Pathogenic Bacteria of IAPAR (Instituto Agronômico do Paraná, Brazil), for which the complete genome sequence is available [26], and strain CFBP 2911, from the Collection Nationale des Bactéries Phytopathogènes (France), were used in all the assays. Both 306 and CFBP 2911 are Asiatic type (A) canker strains, and were isolated from different geographic areas (Brazil and Pakistan, respectively). Bacteria were maintained on Yeast Peptone Glucose Agar (YPGA) medium [27] and grown for inoculum preparation in Luria Bertani (LB) broth. LB cultures were incubated under shaking at 26 ºC for 48 h and concentrations were monitored by optical density at 600 nm.

2.2 Copper and Nutrient Limitation Assay

Batch cultures were prepared in 250 mL flasks with sterilized AB (1 g/L NH_4Cl, 0.3 g/L $MgSO_4$, 0.15 g/L KCl, 0.01 g/L $CaCl_2$, 2.5 mg/L $FeSO_4$, 3 g/L K_2HPO_4 and 1 g/L NaH_2PO_4) and supplemented with $CuSO_4$ at 0.5, 5, 10 and 50 μM. Medium not supplemented with copper was used to determine the starvation effect on the VBNC condition. AB medium was used because of its minimal ability to bind copper ions [16]. An exponentially growing suspension of 10^7 CFU/mL of *Xcc* strain, 306 or CFBP 2911, was added to each batch culture and kept at 26 ± 2 °C for 5 months in the dark. Two replicates were performed for each strain.

2.3 Culturability of Xcc Cells in Batch Cultures

Culturability of *Xcc* in batch cultures was determined by plating and recovery of colonies on YPGA medium. An aliquot of 1 mL of each bacterial suspension was regularly pelleted from each treatment during a five-month period, resuspended in 1 mL of PBS at pH 7 and 100 µL plated in triplicate on the medium. CFU (colony forming units) were counted from 2 to 7 days after incubation at 26 °C.

2.4 Membrane Integrity and Respiratory Activity of Xcc in Batch Cultures

To determine membrane integrity, bacterial cells were harvested and stained with BacLight Live/Dead kit (Molecular Probes, Inc., Eugene, OR, USA) as follows: 500 µL of *Xcc* from the batches were mixed with 500 µL of a mixture of 300 µL of SYTO-9 and 200 µL propidium iodide, and incubated in the dark for 15 min. The suspension was then filtered through a 0.22 µm pore filter, and green and red cells were counted and classified as live or dead using an epifluorescence microscope (Nikon ECLIPSE E800, Tokyo, Japan) under 100× magnification [28]. Twenty microscopic fields were evaluated for each sample and the average of viable and non-viable cells calculated for comparative purposes.

The alamarblue fluorometric cell viability assay (Invitrogen, Rockeville, MD), was applied for evaluation of respiratory activity as previously described [29]. Microtiter plates were amended with 100 µL alamarblue reagent, inoculated with 100 µL of bacterial suspension from the batches and incubated overnight at room temperature in the dark before color evaluation. Bacterial suspensions with active or viable cells turned pink while those with non active or non viable cells turned blue. Ten replicates per treatment were performed. Wells without amended bacteria were used as negative controls, whereas the bacterial suspensions at 10^5 CFU/mL without copper and with nutrient deprivation were used as positive controls. All the experiments were independently repeated.

2.5 Detection of mRNA from gumD in Batch Cultures

Viability of the bacterial population in batch cultures was also evaluated by rt-RT-PCR detection of mRNA from *gumD* gene. Presence of a 445 bp transcript from *gumD* gene was monitored by real time-RT-PCR as previously described [30, 31], before and after bacteria were treated to induce the VBNC state. Total RNA extractions were performed from bacteria in the batch cultures using the RNeasy mini kit (Qiagen, California, USA) and treated with Turbo DNase free following the manufacturer's instructions (Ambion, USA). Purified RNA samples were used for reverse transcriptase rt-RT-PCR reaction or stored at -80 °C until further use.

Amplifications were carried out by adding five µL of the extracted RNA at 0.1 µg/µL concentration to a reaction mixture containing 12.5 µL of 2× Quanti probe master mix (Qiagen), 0.25 µL Quanti RT-mix, one µL of 10 µM of *MG-GumD1F* (CAT TGC AGT GGG CGT CAA GT) and *MG-GumD1R* (TCG ACC AAC GGC GGA TGT AGT) primers, and 0.5 µL of 10 µM of the *J-GumD TaqMan* probe (FAM-AAT GGT TTC CGT GGC GAG ACG–TAMRA) in a total volume of 25 µL. Control RNA samples were not supplemented with reverse transcriptase to check for DNA contamination. Amplifications were run in a SmartCycler (Cepheid, USA) with an initial reverse transcription step of 30 min at 50 °C, an activation step of 15 min at 95 ºC followed by 40 cycles at 94 °C for 15 s and 60 °C for 1 min.

To study the evolution of a *Xcc* population in VBNC condition, total RNA was extracted from 1 mL suspensions from the batch cultures at three stages: immediately after bacterial addition to the batches, when 100% of the bacterial population was confirmed as non culturable (t = 0), and after 10 days (t = 10). Average Ct was obtained from each RNA extraction and statistically analysed to determine differences among exposure times and copper concentrations.

2.6 Resuscitation Assays

Ability of different compounds such as glutamate,

citrate, asparagine, fructose, glucose, EDTA and phosphate buffer, all at 100 μM concentration, or three culture media (Wilbrink, WB [32], YPG or LB), as well as citrus leaf extract, was evaluated for complexing copper ions using Microquant copper kit following manufacturer's instructions (Merck, Darmstadt, Germany). To obtain citrus leaf extract, 100 mg of grapefruit leaf *cv*. Duncan (*Citrus paradisi*), previously washed with sterile water and disinfected with 70% ethanol, were grounded in 5 mL of PBS and thereafter filter sterilized.

The compounds with the highest copper chelating activity were evaluated to reverse the VBNC condition in the batch cultures in which 100% of the bacterial population was confirmed as non cultivable. 1 mL from these batch cultures was added to 9 mL of each compound at 100 μM concentration or culture media or grapefruit leaf extract and incubated at room temperature for one week under shaking at 1.000 g/min, as shown in Table 1.

After seven days, CFU were determined by colony counting on YPGA plates as described above. To demonstrate that the resuscitation was not the result of a regrowth from a few undetected culturable cells, dilution series were performed according to the method described in a previous work [33]. VBNC and resuscitated cells were tested for pathogenicity in assays conducted by injection-infiltration of detached grapefruit leaves as described in Ref. [34]. Negative controls consisted of AB medium without bacteria, whereas positive controls included a suspension of strain 306 of *Xcc* in PBS at 10^7 CFU/mL.

To evaluate the period of time that *Xcc* could persist in a reversible VBNC condition, a time course resuscitation experiment was performed from the batch culture at 5 μM copper sulfate. Cultivability recovery was attempted, as described above, adding as chelating agents LB, YPG or WB culture media, glutamate, citrate or asparagine as chemical compounds or citrus leaf extract from grapefruit.

Cultivability was evaluated 90 days after bacterial batch inoculation, when the entire population was firstly considered in a non cultivable condition and no chelating agent was added, and after adding the chelating agents 3, 5, 10, 20, 30, 60 and 90 days later.

2.7 Statistical Analyses

In all assays data of total, viable and culturable cell counts were transformed to log. Mean comparisons in each point were done to determine significant differences ($P < 0.05$) among viable, culturable and total cells in the batch cultures with different copper sulfate concentrations and for the Ct average obtained after *gumD* amplification from RNA of bacterial suspensions at different time periods. Data from the samples were subjected to analysis of variance and the means separated by Student-Newman-Keuls procedure. All statistical analyses were performed using Statgraphics Plus for Windows 4.1 (Statistical Graphics, Rockville, MD, USA).

3. Results

3.1 Induction of VBNC in Xcc

To examine the induction of the VBNC condition,

Table 1 Resuscitation of VBNC cells with chelating agents in different copper concentrations after seven days incubation.

Chelating compounds	Copper concentration (μM)			
	0.5	5	10	50
LB medium	+	+	-	-
YPG medium [27]	+	+	-	-
WB medium [32]	+	+	-	-
EDTA[a]	+	-	-	-
Sodium glutamate	+	+	+	-
Sodium citrate	+	+	+	-
Asparagine	+	+	+	-
Grapefruit leaf extract (1/100)	+	+	+	+
Phosphate buffer	+	-	-	-

+: Appearance of one or more colonies on YPGA plates; -: No colonies on YPGA plates; [a]Ethylene diamine tetraacetic acid.

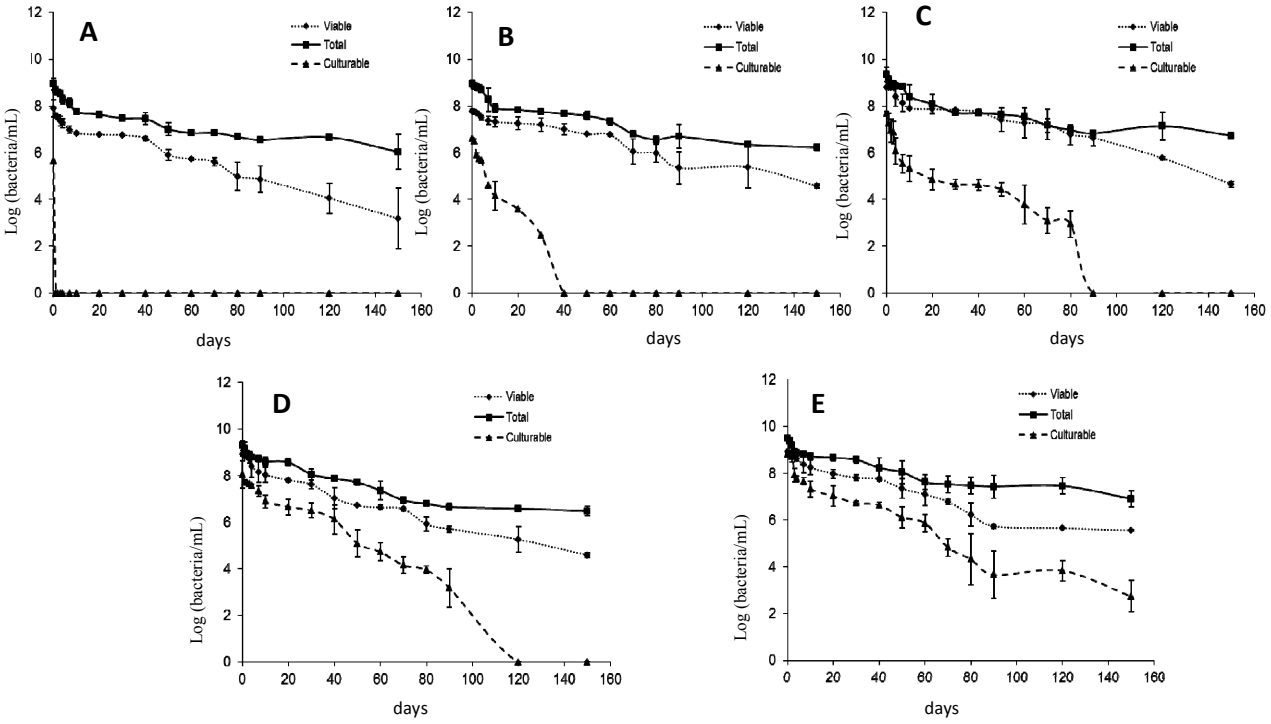

Fig. 1 Five-month time-course of *X. citri* subsp. *citri* population in AB medium with copper sulfate at 50 (A), 10 (B), 5 (C), 0.5 μM (D) concentrations or without copper (E). For culturable cells graphs show the average of the three replicates for each dilution and time. For total and viable cells, each point was the average of the counts of cells in 20 microscopic fields. Error bars represent standard deviation of the means. Data of each time were subjected to analysis of variance to determine significant differences ($P < 0.05$) among viable, culturable and total bacterial populations.

culturable, viable and total bacterial populations were evaluated in batch cultures of two *Xcc* strains grown under different copper concentrations in time course experiments lasting 150 days, as shown in Fig. 1. Since no significant differences were obtained ($P > 0.05$) between the two *Xcc* strains, data for each strain were combined for analysis.

From an initial concentration of 10^8 bacteria/mL of viable *Xcc* cells added to the batch, around 10^2 CFU/mL became unculturable immediately after exposure to 50 μM of copper sulfate. No culturable cells were detected 24 h later, although green fluorescent bacteria, and therefore putative VBNC cells at 10^7 cells/mL concentration, were observed under fluorescence microscopy (Fig. 1A). After 150 days at 50 μM of copper sulfate, VBNC cells were still detected at a concentration of around 10^3 cells/mL. In the entire batch cultures amended with different copper sulfate concentrations at different time periods 100% of no culturable cells were detected. However, viability of bacteria was confirmed by membrane integrity and respiratory activity from all the batch cultures after 150 days (Fig. 1). Moreover, culturable bacteria were found from the cultures treated with copper sulfate at concentrations of 10, 5 and 0.5 μM and from the control without copper sulfate after 30, 80, 90 and 150 days, respectively (Figs. 1B-1E). Therefore, viable bacterial populations, at those time periods and copper sulfate concentrations, were encompassed by culturable and VBNC cells in variable proportions. In addition, at 50, 10 and 5 μM of copper sulfate, significant differences ($P < 0.05$) were observed between the average number of viable and culturable cells immediately after inoculation, demonstrating rapid induction of VBNC in a fraction of the bacterial population (Figs. 1A-1C). At the lowest concentration (0.5 μM copper sulfate), the

appearance of VBNC cells was observed after 24 hours (Fig. 1D) and even without copper sulfate in the medium, the presence of VBNC cells was observed after 72 h (Fig. 1E).

3.2 Resuscitation of VBNC Xcc Cells

Low nutrient supply or copper ions acted as VBNC inducers; consequently, the addition of nutrients as well as reducing the copper concentration or its removal might reverse the VBNC state to a culturable condition. To test this hypothesis, different chelating compounds and various culture media were tested in order to induce resuscitation of VBNC cells.

First, the potential of several compounds as chelating agents to complex copper ions was evaluated using the Microquant kit. All compounds analyzed were able to complex copper, reducing the amount of this ion in solution. Citrus leaf extract and several culture media were able to bind 100% of the soluble copper in concentrations from 0.5 μM to 50 μM. Percentage of copper capture was variable for other compounds: glutamate was able to remove from 20% to 70% of copper, fructose and glucose around 20%, and phosphate buffer was able to bind 50% of soluble copper, as shown in Fig. 2.

Culture media and those compounds that showed higher chelating capacity were selected to recover *Xcc* cells from the VBNC condition. In addition, other chelating agents such as EDTA, asparagine and citrate, previously used in other studies on *E. amylovora* resuscitation [16], were also evaluated. Cell resuscitation was always completed when total bacterial population in the batches was established to be in a VBNC state by colony counting on culture plates and viability confirmed by membrane integrity and respiratory assays. Consequently, samples from each batch containing different copper concentrations were analyzed at different time periods, as shown in Table 1.

Resuscitation from batch cultures amended with copper sulfate at concentrations of 0.5 μM and 5 μM was observed using LB, YPG and WB culture media. Using EDTA, culturable bacterial cells were recovered only from 0.5 μM copper sulfate batch cultures. Glutamate, citrate and asparagine resuscitated *Xcc* from batches with copper sulfate at 0.5, 5 and 10 μM concentration. Interestingly, grapefruit leaf extract was the most effective for reversing VBNC condition since culturable cells could be recovered from batches treated even with 50 μM of copper sulfate.

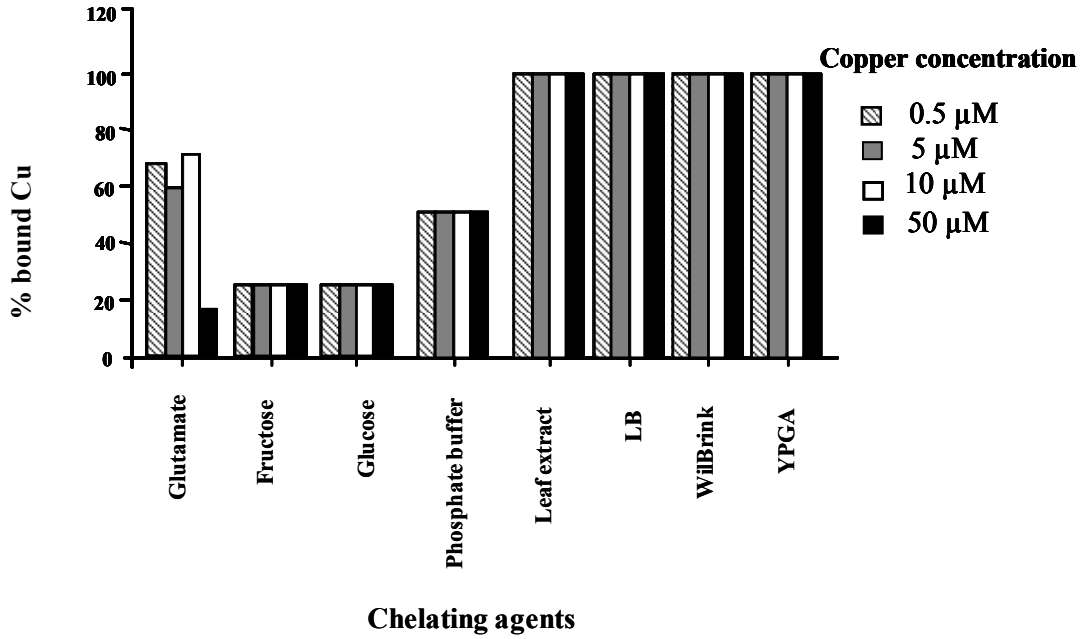

Fig. 2 Copper complexing ability of several compounds at 0.5, 5, 10 and 50 μM copper concentrations in AB medium.

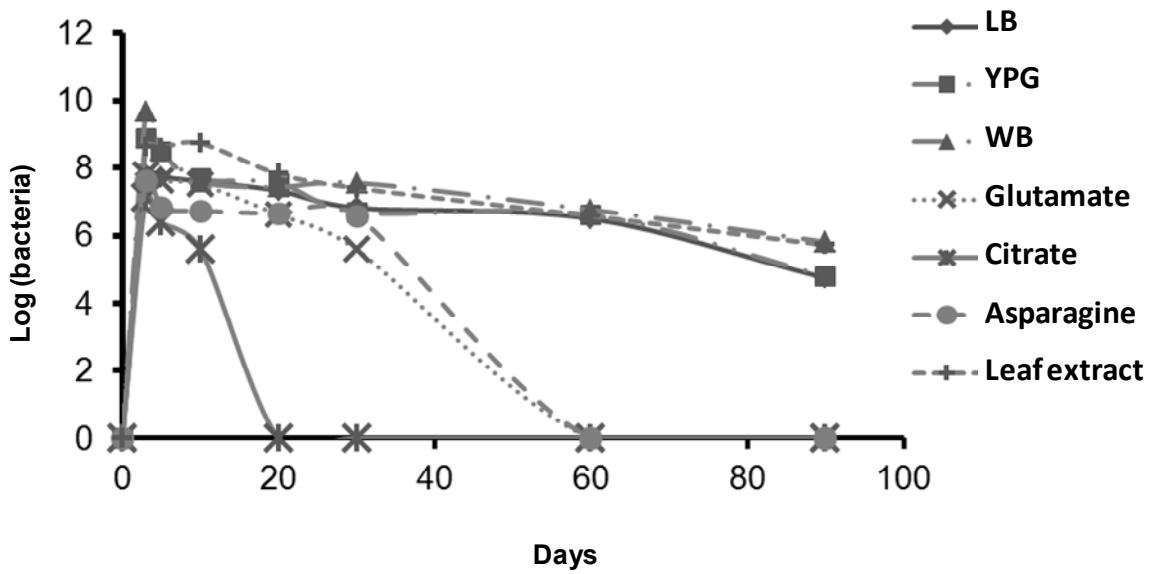

Fig. 3 Recovery of cultivability from *X. citri* subsp. *citri* VBNC cells treated with copper sulfate at 5 μM concentration after the addition of several chelating agents and culture media. At time 0 the bacterial population was 100% non-culturable and no chelating agents were added.

In all assays, identification of the resuscitated bacteria was confirmed by recovery of colonies on culture media and detection of the 445 bp *gumD* fragment by real-time PCR [31]. In addition, pathogenicity of resuscitated cells was confirmed in detached grapefruit leaves. Moreover, *Xcc* cells from batches at 0.5, 5 and 10 μM copper sulfate concentrations and in the VBNC state were able to induce symptoms in plant leaves after addition of chelating agents.

As shown in Fig. 3, cultivable cells were recovered after the three months experiment when culture media or leaf extract were used for resuscitation, although a significant reduction in recovery of cultivable bacteria was shown through the time. Using citrate and asparagine for resuscitation, cultivability could be recovered only 30 days after VBNC induction and with glutamate just after 10 days (Fig. 3).

3.3 Determination of Bacterial Population Viability by Detection of a GumD RNA Fragment by rt-RT-PCR

The *gumD* fragment of 445 bp from the mRNA transcript was always detected in batch cultures in which the presence of VBNC cells had been onfirmed by membrane integrity and respiratory assays (Fig. 4). Variation in threshold cycles (Ct), and therefore in viable bacteria concentration, was related to the period of time exposed to copper as well as to the copper concentration in the batch. Cts obtained by rt-RT-PCR from bacterial RNA in batch cultures were always lower at the initial stage of the assay, when culturable bacteria were still present, revealing a higher concentration of viable cells (Fig. 4). Significantly higher Ct average ($P < 0.05$) and therefore, a lower concentration of viable bacteria was shown in all the batches when culturable cells were not found. No significant differences were found between Cts obtained from the sample at t = 0 (first time 100% bacterial population in the batch was identified as non cultivable) and t = 10 days from the batches without copper or with 0.5 μM and 5 μM copper concentration, indicating stabilization of the *Xcc* population in the VBNC condition. However, differences in Ct averages were observed between t = 0 and t = 10 days when bacteria were exposed to 10 μM and 50 μM of copper sulfate, suggesting mortality of a proportion of the bacterial population under these copper concentrations (Fig. 4).

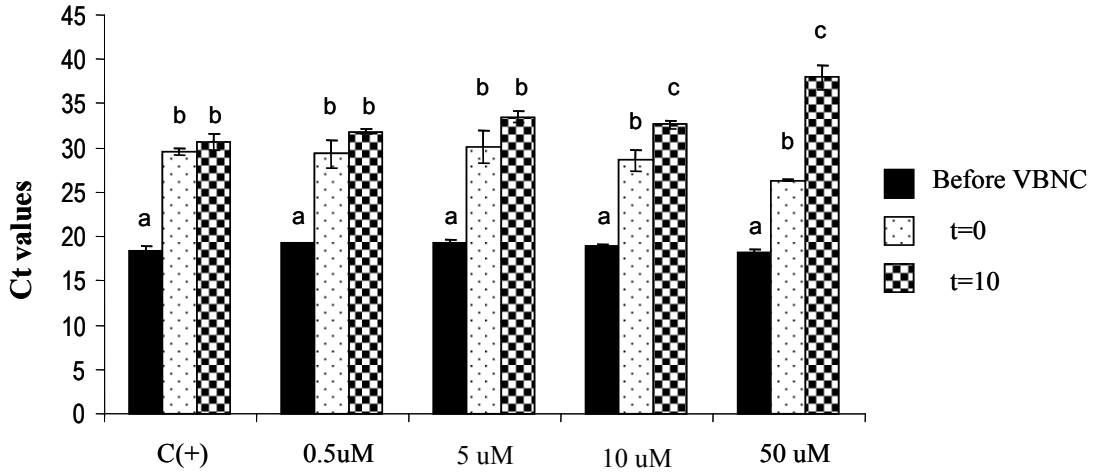

Fig. 4 Average threshold cycles (Ct) resulting after rt-RT-PCR from *gumD* mRNA obtained from *X. citri* subsp. *citri* batches with copper at different concentrations. rt-RT-PCR was performed from RNA extracted immediately after bacteria addition to the batch (before VBNC), when the total population was first considered non cuturable (t = 0) and 10 days after this stage (t = 10). The graph shows the average of at least two PCR reactions for each sample and error bars display standard deviation. Means with the same letter within a sample do not differ significantly according to the Student-Newman-Keuls multiple range test ($P < 0.05$).

4. Discussion

Many factors have been described to induce the VBNC condition in bacteria [11, 12, 22]. Exposure to chemical substances, such as copper, commonly used to control citrus bacterial diseases [3, 35], has been reported to induce VBNC in several Gram negative plant pathogenic bacteria [14, 16] including *Xcc* [17]. However, in this bacterium VBNC has been described after short-term treatments with copper at high concentrations, equivalent to three times the free soluble copper concentration applied in regular field treatments [17]. Moreover, reversibility of the putative VBNC condition in *Xcc* was not assessed in the aforementioned study. Hence the two most important assumptions to consider VBNC as a survival strategy were not fulfilled in the CBC pathogen. By contrast, our work demonstrates that the non culturable state of *Xcc* cells, induced by starvation or low copper exposure, endured while the adverse conditions continued but could be reversed when the environmental stress stopped. The ability of copper sulfate to induce the VBNC condition in *Xcc* was demonstrated and monitored for a five-month period and the reversion of this state demonstrated after 90 days, when the stressful conditions were removed by the addition of chelating components or supplementary nutrients. Furthermore, in the absence of copper, low nutrient availability alone was enough to induce the VBNC state in *Xcc*, although copper clearly sped up the metabolic adjustment to this condition. The higher the concentration of copper in the medium, the faster *Xcc* entered the VBNC state and slighter the culturability recovery. This effect of copper combined with starvation was confirmed for *Xcc* as previously described for *X. campestris* pv. *campestris* [14].

It is important to point out that the studies on VBNC condition were performed with copper concentrations more likely to be encountered by *Xcc* in the field than those concentrations previously employed by del Campo et al. [17]. Moreover, and in order to avoid overestimation of VBNC population, cultivability was assayed on media plates incubated for more than four days, considering that *Xcc* colonies

under stress conditions may need longer to develop as compared to bacteria without environmental pressure. It is also remarkable that, in this study, viability of bacterial cells in the batches was evaluated by three independent methodology approaches: membrane integrity, respiratory assays [28, 29] and detection of an mRNA fragment from *gumD* gene, recently described as a viability marker for *Xcc* [31]. The results with *gumD* also showed that part of the putative VBNC population had to be considered as simply a prior step to cell death, as described in other models. The proportion of cells in actual VBNC state or in a prior step to cell death was influenced by copper concentration.

As the resuscitation of a proportion of non-culturable cells in the batch cultures was achieved by the addition of nutrients or removal of copper with several compounds and leaf extracts, the existence of an authentic VBNC condition for *Xcc* was confirmed. The reversal of the VBNC state was apparently due to sequestration of copper ion. Both citrus leaf extract and culture media were able to facilitate viable bacteria to recover culturability after the total population entered into the VBNC. As described in *E. amylovora* [16] this result may be due, at least partially, to a combined effect of the complexing activity and nutrient availability of the plant extract or culture media to the bacteria. Herein this synergistic effect was demonstrated by the fact that some compounds with lower complexion ability, such as glutamate, were more effective than others like EDTA with a higher copper-ion capturing efficiency [16]. In addition, not all the substances were able to chelate 100% yet showed the same effect on recovery from VBNC; for example the culture media tested did not produce the same effect as citrus leaf extracts, despite having the same copper-ion capturing ability or being a clear source of bacterial nutrients. The results indicate that reversion of the VBNC condition in *Xcc* is complex and based on the combined effect of complexing of copper and nutrient availability. On the other hand, the ability of citrus leaf extract to act as a strong copper chelating agent is interesting because it suggests that *Xcc* and other bacteria may be exposed to lower amounts of copper ions than initially expected after plant or fruit control treatments employed in the citrus industry.

5. Conclusion

Copper compounds in concentrations similar to those employed in the field, as well as low nutrient conditions, have proven to be inducers of a long term VBNC state in *Xcc*. The VBNC state has been confirmed as a reversible survival strategy that can last for months, giving the pathogen a powerful tool to overcome stressful conditions. The reversibility and persistence of the VBNC condition has been demonstrated in this work as a first step, although the epidemiological significance of this state in *Xcc* in an ecologically relevant context, ie., in situ, on plant surfaces, or even in survival structures such as biofilms, described for *Xcc* [36], need to be evaluated further. Such information will help to establish effective preventive measures to avoid the introduction of *Xcc* in CBC-free areas, and must be considered for new control strategies in areas where the disease is endemic.

Acknowledgments

This research was supported by INIA projects RTA2006-00149 and RTA2008-00048. The authors thank R. Leite for sending *Xcc* strain 306 and E. Marco-Noales and M. Ordax for helpful advice. M. Golmohammadi had a fellowship from the Government of Iran, and P. Llop had a contract of the Ministry of Science and CCAA of Spain with the support of the European Community.

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