Chapter

Quorum Quenching Bacteria: An Approach for Phytopathogens Control in Citrus Cultivars

Juan Carlos Caicedo and Sonia Villamizar

Abstract

Cell-to-cell communication system quorum sensing (QS) denotes the ability of bacteria to track the population density, in order to coordinate its phenotypic traits to successfully establish and thrive in new ecological niches. Different citrus phytopathogenic bacteria such as: *Xanthomonas citri spp. citri*, *Xillela fastidiosa* and *Pseudomonas syringae* pv. *syringae* regulate several pathogenicity factors through well-established quorum sensing DSF (Diffusible Signal Factor) and AHL (AcylHomoserine Lactone) pathways. The goal of this chapter is to review exophytic and endophytic bacteria able to disrupt quorum sensing communication system in these bacteria in order to reduce the symptomatology of citrus canker, citrus variegated chlorosis and citrus blast. The quorum quenching of phytopathogen bacteria could afford new tools for disease control, thus reducing the overuse of antimicrobial drug and decrease its environmental accumulation, thus relieving the selection pressure of resistant bacterial populations.

Keywords: citrus canker, quorum sensing, auto-inducer, biological control, biofilm

1. Introduction

Nowadays, at the bacterial world, it is widely accepted that there are biological processes that must be pointed by a coordinated behavior of entire population. Factors such as: virulence factors production, biofilm formation, secondary metabolite production, and bioluminescence are fruitless when undertaken by a single bacterium proceeding isolated [1]. A wide variety of bacteria are endowed with encoding genes for components of a cell-to-cell communication system termed as quorum sensing (QS). This QS system enables bacteria to regulate their behavior in a cell density fashion in order to modulate a gene set that enables the bacteria to adapt to environmental challenges [2]. Quorum sensing relays its activity in a production, releasing, and perception of small signal molecules called auto-inducers. In Gram-negative bacteria, the usual auto-inducers are small molecules, i.e., acyl lactone and short-chain fatty acids. The cognate receptor involved in perception of these auto-inducer molecules is: cytoplasmic transcription factor and two components histidine sensor kinases. The complex produced by the auto-inducer and receptor leads the promotion of target genes regulated by quorum sensing [3]. Gram-positive bacteria

use mainly short peptides as auto-inducers, and its related receptors are transmembrane histidine sensor kinase. Usually, the union of auto-inducer and receptor triggers expression of encoding gene for AI (auto-inducer) synthase, which increases the extracellular AI concentration switching on the bacteria quorum sensing mode [4].

Disruption of quorum sensing communication system, which is termed quorum quenching, leads to a reduction in virulence factors expression without compromising bacterial survival [5]. Since a wide diversity of bacterial cells that use QS display a significant competitive advantage over other prokaryotes and eukaryotes with which they coexist in the same ecological niche, it is rational that the contender microorganisms have developed mechanism to disrupt the QS communication systems present in the ecological niche. Interference with the quorum sensing communication system either by natural or synthetic approaches may afford strategies for disease control, by reducing the virulence or turn the pathogens more susceptible to antibiotic therapy. The design, development, and employment of these approaches will depend in great measure upon the knowledge of mechanistic details of quorum sensing pathway such as: auto-inducer synthesis, signal perception, signal transduction, and genes under quorum sensing regulation [6].

Citrus is the most commercialized horticultural product in the world; however, farmers in the last two decades have seen production reduced by average of 65%, due to devastating bacterial diseases such as: Bacterial Citrus Canker (BCC) caused by *Xanthomonas citri* subsp. *citri* (Xcc), Citrus Variegated Chlorosis (CVC) caused by *Xylella fastidiosa*, Citrus Blast caused by *Pseudomonas syringae* pv. *Syringae*, and Citrus Greening or Huanglongbing (HLB) caused by *Candidatus liberibacter* sp. All bacteria aforementioned except the *Candidatus liberibacter* are endowed with quorum sensing systems, which are responsible for the pathogenesis and symptomatology in citrus host. The main objective of this chapter is to describe quorum sensing pathways in these phytopathogen bacteria, as well to review some successful approaches based in quorum sensing disruption in order to decrease disease severity.

2. Citrus canker and DSF quorum sensing pathway in *X. citri* subsp. *citri*

Bacterial citrus canker (BCC) is one of the major citrus diseases, almost all varieties of citrus crops are affected, and the severity of disease depends on bacterial species and weather conditions [7]. The etiological agent of BCC is the Gram-negative bacterium *X. citri* subsp. *citri* [8]. Nowadays, three types of BCC are recognized, which are: (i) citrus canker type A, also known as Asian citrus canker, is the most widespread disease. The BCC has a pronounced host range producing symptoms in: *Chrysopelea paradisi*, *C. aurantifolii*, *C. sinensis*, and *C. reticulate.* (ii) Citrus canker type B is caused by the bacterium *Xanthomonas fuscans* subsp. *aurantifolii* type B (*XauB*) [9]. The symptomatology development is similar to citrus canker type A; however, because of the *XauB* slower growth rate, the symptoms spent more time to appear. Host range is limited to *C. limon*; however, *XauB* was rarely isolated from *C. sinensis* and *C. paradisi* [10]. Citrus canker type C is produced by *X. fuscans* subsp. *aurantifolii* type C (*XauC*). The symptomatology is similar to citrus canker type A; nevertheless, its host range is restricted to *C. aurantifolii* [9].

Pathognomonic symptoms of BCC type A are the raised corky and spongy lessons surrounded by a water-soaked margin, which are present in leaves and fruits. This lesion results from the hypertrophy and hyperplasia of mesophilic cells. This cell division disorder is induced by the bacterial effector from family AvrBs3/PthA [11].

The bacterium *Xcc* is outfitted with a vast arsenal of organelles responsible for the pathogenic traits in citrus host. The main known are: bacterial attachment, antagonism, effector production, quorum sensing regulation, and biofilm formation. For an in-depth review, please refer to Caicedo and Villamizar [12].

2.1 DSF quorum sensing pathway in Xcc

Xcc bacteria have a quorum sensing system whose auto-inducer molecule (AI) is a short-chain fatty acid belonging to DSF (diffusible signal factor) family. The DSF auto-inducer family modulates the expression of virulence and pathogenicity in several pathogenic bacteria to plants and humans [13]. The DSF molecules display a *cys* unsaturated double bond at position two as a distinguished feature in the family. The DSF family are cis-2-unsaturated fatty acids (**Figure 1**), the cis-11-methyl-2-dodecenoic acid was the auto-inducer molecule characterized to be responsible for the signaling processes in *Xcc* [15].

The discovery of the DSF signaling molecule came within a genomic study that seeks to identify a gene cluster termed *rpf* (regulation of pathogenicity factor) *rpfb-* in the bacterium *Xanthomonas campestris* pv. *campestris*. Researchers found that mutation of components of this gene cluster drives the decrease in extracellular enzyme production and exopolysaccharide as well as reduction in pathogenicity in plant susceptible [16]. Later works established the participation of these genes in the coding of elements belonging to quorum sensing communication system involved in the synthesis and perception of the DSF signal molecule [17]. The gen *rpf*F encodes an enzyme, amino acids sequence of which is related to enoyl CoA hydratase; this enzyme is responsible for DSF synthesis. The gen *rpf*B encodes and Acyl CoA ligase, which participates to a lesser extent in the synthesis of DSF auto-inducer [16].

DSF perception and signal transduction are encoded by an *rpfGHC* gene operon. *rpfC* encodes the receptor RpfC protein. This protein has a transmembrane domain, which is involved in the perception of DSF auto-inducer and the cytoplasmic domains: His-Kinase A (phosphoacceptor), His-Kinase-like ATPase, REC domain (receiver

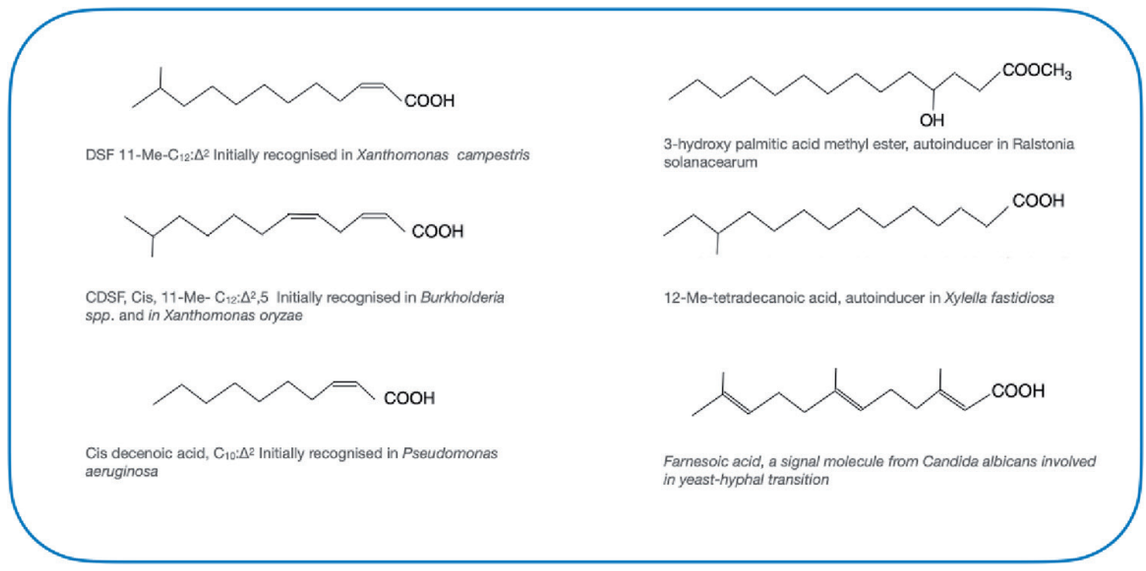

Figure 1.
(Left) Structure of the main molecules from DSF auto-inducers family and some related putative auto-inducers. (Right) Related molecules with signaling activity. Notice that these molecules lack the cys unsaturated double bond at position two. (Adapted from Ryan and Dow [14]).

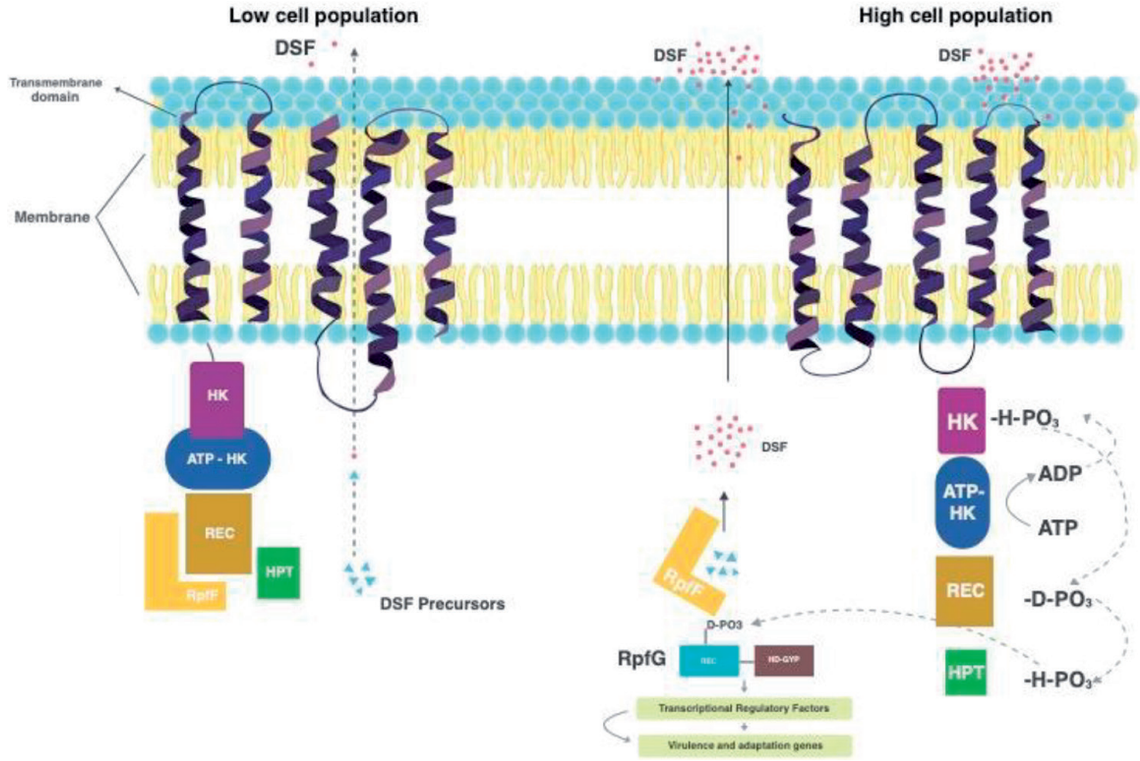

Figure 2.
At low cell population condition, RpfF the DSF synthase remains bound to REC domain from RpfC sensor, which maintains compact conformation. At high cell population condition, the DSF binds to RpfC sensor domain and induces a conformational change releasing the synthase RpfF, thus triggering an autophosphorylation and phosphorelay and the subsequent phosphotransfer to the REC domain of response regulator RpfG. The phosphorylation of RpfG, it triggers the activation of RpfG as a cyclic di-GMP phosphodiesterase reducing the level of cyclic di-GMP and releasing Clp that promotes the synthesis of extracellular enzymes and EPS (take it from Caicedo et al. [18]).

domain), and finally, the HTP domain histidine phosphotransfer (**Figure 2**). The gene *rpfG* encodes for response regulator protein, this protein include two domains: (i) an REC domain, which receives the phosphate of HTP domain from the RpfC; and (ii) the HD- GYP, which displays phosphodiesterase activity responsible for degradation of second messenger cyclic di-GMP. At physiological levels of cyclic di-GMP, the transcriptional activator cAMP-receptor-like-protein Clp remains bound to second messenger cyclic di-GMP. Consequently, reduction of cyclic di-GMP via DSF in *Xcc* leads to the release of Clp, thus allowing Clp to activate the expression of several gene and proteins involved in virulence such as: extracellular enzyme production, EPS production, biofilm formation, motility, iron uptake [19]. Furthermore, DSF signaling also positively controls the expression of *clp* gene, which suggests a supplementary regulatory complexity [14]. Summarizing, the perception of auto-inducer molecule DSF in *Xcc* by the receptor RpfC triggers the expression of its virulome. For all afore-mentioned, the disruption of this quorum sensing communication system could turn into a valuable tool for reducing citrus canker severity.

3. Variegated chlorosis, *Xylella fastidiosa and* quorum sensing

CVC (Citrus Variegated Chlorosis) is a disease that affects sweet orange (*Citrus sinensis*) and grapefruit (Citrus x paradise); no other citrus are susceptible to disease

[20]. CVC is caused by the Gram-negative bacterium *X. fastidiosa (Xf).* Transmission paths of CVC are: (i) *Propagative vegetative material*: CVC is a paradigm of accidental long-distance propagation of phytopathogen; this is mainly due to the movement made by agriculture workers of infected plant material [21]. (ii) *Insect vector borne:* vectors of *Xf* are xylem sap sucking insects belonging to the Hemiptera order including sharpshooter, leafhoppers, and spittlebugs [22].

CVC symptomatology in susceptible host sweet orange initiates as a small chlorosis, which extents irregularly on the upper surface of mature leaves, the affected leaves display a consequent brownish gum-like material on the lower surface. At this disease stage, the lesions are present only in one or two branches. At the later disease stage, bacteria became systemic spreading in the plant canopy, symptoms become apparent between 3 and 6 months [22].

The bacterium *X. fastidiosa* belongs to the gammaproteobacteria group, *Xanthomonadaceae* family. *Xf* is a xylem-limited bacterium, which is obligatory colonizer of plants and insect vectors [21]. There are three major monophyletic subspecies of *X. fastidiosa: Xf* subsp. *multiplex, Xf* subsp. *Fastidiosa,* and *Xf* subsp. *pauca*, endemic respectively in North, Central, and South America [23–25]. Nowadays, it is widely accepted that *Xf* has a commensal relationship with a huge number of plant species; however, just a few numbers of clades and specific bacterial genotypes are associated as phytopathogens. Contrasting with the relationship between *X. fastidiosa* and the plant, the association of *X. fastidiosa* with insect vector is independent of plant-pathogen mixtures. Vectorial transmission is the only natural mechanism spread. There exist two principal xylem-sap feeders insect vector groups: the sharpshooter leafhoppers (Cicadellidae subfamily Cicadellinae) and spittlebugs (Cercopoidea, families Aphrophoridae, Cercopidae, and Clastopteridae) [26, 27]. Actually it is extensively known that all insect vectors aforementioned display the ability to transmit all genotypes of *X. fastidiosa* without any specificity [28]. *Xyllela fastidiosa* colonize plants gradually, the initial stage is a vessel obstruction because of bacterial multiplication, reduction of sap flow in the xylem system is due to plant response (i.e., tylose) and bacterial dissemination between vessel via pit membrane [29].

3.1 Quorum sensing in *Xyllela fastidiosa*

As mention before, the virulence of *X. fastidiosa* is related with its capability to travel and to multiply within xylem vessels, and symptoms could basically be an unintended effect caused by effective colonization that restricts with xylem sap flow. *X. fastidiosa* as similar to related phyto-pathogenes Xanthomonas and Stenotrophomonas use small molecules from DSF family in order to coordinate its behavior in a cell-density-dependent fashion [30]. Cell-to-cell communication system in *X. fastidiosa* involving the production of DSF *rpfF* gene is responsible for the DSF production as same of Xanthomonas bacteria. Unlike, *Xanthomonas* bacteria, in which the disruption of DSF quorum sensing pathway reduces its virulence and pathogenicity in *X. fastidiosa,* mutants of *rpfF* and subsequently deficient in DSF production show an hypervirulent phenotype behavior in a susceptible host such as sweet orange and grapefruit [31]. In *X. fastidiosa,* the DSF molecule is 12 methyl tetradecanoic acid *xf*DSF (**Figure 1**). The whole mechanistic details in DSF pathway are not yet completely understood. Previous studies have proposed the existence of two different types of receptors in *X. fastidiosa:* the first one RpfC transmembrane has a high sequence similarity with the RpfC of *Xanthomonas campestris*, the only difference lies that in the *X. fastidiosa* is truncated at the N terminus, apparently its function is to

bind DSF and in that way to execute a negative feedback regulation in DSF production [32]. Another potential DSF sensor has an intracytoplasmic localization, and its function could be the perception of DSF accumulated within the cell. Once DSF bound to intracellular DSF receptor the autophosphorylation and phosphorelay to a response regulator as RPFG is triggered. It allows the expression of genes involved in attachment and biofilm formation [32].

Previous studies have shown that *rpfF* mutants of *X. fastidiosa* display a hypervirulent phenotype in a susceptible plant host, and this mutant strain was incapable to colonize and be spread by insect vectors. These observations arose the hypothesis that DSF signaling is used as a lifestyle dependent switch, because *rpfF* promotes the genes expression involved in attachment and biofilm formation in the xylem vessels. By contrast, *rpfF* in *X. fastidiosa* represses the expression of genes intricated in motility and hydrolytic enzyme production, which are responsible for the cell migration and pit membrane disruption. *X. fastidiosa* xylem vessels attached cells display reduced pathogenicity and a phenotype highly favorable to be acquired by the insect vector [33]. On the other hand, *rpfC* deletion mutants displayed an avirulent phenotype, because the great DSF production, these mutants were capable to successfully colonize the insect vector, whoever these bacterial cells display an impaired ability to be transferred to another susceptible plants. Finally, similar to Xanthomonas bacteria, DSF-dependent signaling regulates decyclic di-GMP in *X. fastidiosa* [32].

4. Citrus blast, black pit, *Pseudomonas syringae* pv. *Syringae,* and quorum sensing

Citrus blast is an important bacterial disease that affects commercially important citrus fruits such as sweet orange (*C. sinensis*) and mandarin (*Citrus reticulata*). Black pit is a disease that affects sweet orange. Both diseases are caused by the bacterium *Pseusomonas syringae* pv. *syringae. P. syringae* pv. *syringae* becomes especially pathogenic for citrus fruits, when the prevailing environmental conditions are high humidity and temperatures around 18°C, which coupled with damage to shoots or fruits by wind, thorns, and hail [34]. Pathognomonic symptoms of disease are water-soaked lesions, which extend from the midrib to the minor ones that surround the base of the petiole. At the last phase of the disease, leaf desiccation and curling are observed. This trait is mainly present in the leaves that remain attached to the stem and finally fall. Necrotic area in twigs expands and finally dies after 4 weeks.

Within that vast number of bacteria that compound the plant microbiome (either rhizosphere or phyllosphere), Pseudomonas bacteria are the most versatile and metabolically varied. As mentioned above, due to its enormous genetic and metabolic plasticity, many species from Pseudomonas genus are successful colonizers of rhizosphere and phyllosphere inducing beneficial effects to the host plants. The most recognized effects are: plant growth promoters, biological control agents, and resistance auto-inducers [35]. Only one species of Pseudomonas is known to be pathogenic for a wide variety of plants; this species is *P. syringae*. P. *syringae* shows a high host plant specificity. Because of this specificity, these strains have been considered as pathovars within the *P. syringae* complex, depending on the type of plant in which the bacterium acts as a pathogen and produces the disease, actually around 50 pathovars are recognized to act as ethological agents in 180 different plant types [36]. *P. syringae* is a leaf-borne commensal bacterium in a wide variety of crop plants, fruit trees, vegetables, and ornamental plants. These bacteria are epiphytic colonizers, which could

reach the internal leaf tissue, once in the apoplast bacteria begins its multiplication producing disease symptoms. Therefore, the pathogenesis development is a multi-step procedure such as: (i) entry to internal tissues plant to reaching the intercellular place the apoplast, (ii) to evade plants' resistance responses, and (iii) inducing disease and generating symptoms by particular invasive approaches and molecules [37].

4.1 Quorum sensing in *P. syringae* pv. *syringae*

The quorum sensing system in *P. syringae* uses as auto-inducer a molecule from AHL signaling family: 3-oxo-hexanoyl-homoserine lactone (3-oxo-C6-HSL) molecule. Production of 3-oxo-C6-HSL is dependent on gene *ahlI* that encodes the synthase AhlI. The other component of the quorum sensing circuit is the gene *ahlR*, the signal regulator [38]. When the auto-inducer precursor is available, the synthase AhlI catalyzes the formation of 3-oxo-C6-HSL. Subsequently, the signal regulator AhlR forms a stable complex with 3-oxo-C6-HSL and promotes the transcription of *ahlI* via positive feedback increasing the concentration of 3-oxo-C6-HSL proportionally to cell population density. Additionally, this quorum sensing AhlI/R pathway is subject to effect of regulatory proteins as AefR, this protein actively participates in *ahlI* transcription. A novel regulator, GacA, displays a similar effect in the process of auto-induction. Together AefR and GacA seem to have participated in the activation of the AhlI-AhlR quorum sensing system via independent pathways [38]. The AHL quorum sensing system in *P. syringae* regulates the alginate production, the main component of EPS in *P. syringae. aefR, ahlI, and ahlR* deletion mutant strains display limited survival ability on dry leaves, which is due to the EPS helping epiphytic fitness and desiccation tolerance [39]. Motility is considered an indispensable epiphytic fitness trait, and swarming motility in *P. syringae* is coordinated by a bacterial social behavior [40]. In *P. syringae* swarming motility is regulated by AhlI-AhlR quorum sensing system and AefR. Deletion mutant strains of *aefR* and *ahlI– ahlR–* double mutant display a hypermotile phenotype compared with the wild-type strain [41].

5. Quorum sensing silencing in bacteria: a valuable tool for phytopathogens control

Quorum sensing is a cell-to-cell communication system that depends on population density. This communication system favors the adaptation to new ecological niches, promotes the exploitation of new metabolic resources, and affords competitive advantages to the bacteria that use it. All the aforementioned is directly related to the regulation of virulence, bacterial resistance, and biofilm formation among other phenotypes observed in bacterial population. Quorum sensing disruption is an alternative to reduce the pathogenicity in bacteria. There are two main approaches in order to silence the quorum sensing system in phytopathogenic bacteria: (i) signal degradation/modification termed quorum quenching, (ii) signal overproduction termed pathogen confusion [42]. Quorum quenching is a mechanism approved by several bacteria groups in order to disrupt the QS signaling of contenders, offering to these bacterial cells an additional benefit within a specific niche. It is rational that microorganisms can develop mechanisms to neutralize the QS systems of competing organisms in order to increase their competitive strength in an ecosystem. In a previous study, we have isolated and identified bacterial from citrus phylloplane that display the ability to modify the structure of DSF signal molecule

the cis-11-methyl-2-dodecenoic acid in *Xanthomonas citri* subsp. *citri* the etiological agent of bacterial citrus canker. The bacteria *Bacillus vallismortis*, *Pseudomonas oryzihabitans*, *Pseudomonas aeruginosa*, *Raoultella planticola*, *Kosakonia cowanii*, and *Citrobacter freundii* were characterized by molecular technics and display the ability to reduce DSF/rpf communication pathway [43]. We show that these quorum quenching bacteria use a DSF molecule as a substrate for the UDP-sugar transferase enzyme. These bacteria added to DSF molecule one unit of sugar from UDP sugar pools. Thus, the recognition for the RpfC sensor of this modified DSF molecule was impaired. Subsequently, a substantial reduction in the canker lesions was obtained in *Citrus sinesis.*

A previous study used the pathogen confusion approach expressing rpfF from *X. fastidiosa* in sweet orange (C. sinensis L. Osb.) using *agrobacterium tumefaciens* in order to reduce citrus canker disease severity. Ectopic expression of xfDSF molecule in C. sinensis reduces its susceptibility to *Xcc.* Transgenic plants display a reduction in the number of citrus lesions presumably for the effect on its motility and attachment, also genes involved in the flagella function, pili formation, and T3SS were downregulated in Xcc when they were infiltrated into the leaves of transgenic plants [44].

The bacteria *P. syringae* strain B728a displays the ability to degrade enzymatically different types of AHL. The HacA and HacB are acylases that cleavage the amide bonds of AHL. These enzymes do not have any affect over 3OC6-HSL endogenous accumulation. The heterologous expression of the secreted HacA acylase produced in *P. syringae* strain B728a could become a potential tool in biological control agents, because it might enable the quorum sensing disruption in phytopathogenic bacteria [45].

6. Conclusions

Bacterial coordinated behaviors such as virulence factors production, motility, biofilm formation, and antibiotic resistance are regulated by cell–cell communication system often called quorum sensing. For all aforementioned, quorum sensing silencing has arisen as a good-looking approach to reduce the disease spread and severity. The major bacteria that affect citrus cultivar are endowed with several quorum sensing pathways. The quorum quenching and pathogen confusion implementation approaches could afford new and environmental-friendly strategies for control of this bacterial disease.

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Conflict of interest

The authors declare no conflict of interest.

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Author details

Juan Carlos Caicedo* and Sonia Villamizar
Independent Researcher, Colombia

*Address all correspondence to: caicedocepeda@gmail.com

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