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Chapter

Biological Control of Citrus Canker: New Approach for Disease Control

Sonia Villamizar and Juan Carlos Caicedo

Abstract

Citrus canker is a disease that affects the major types of commercial citrus crops. *Xanthomonas citri* subsp. *citri*, the etiological agent, reaches to mesophyll tissue through the stomata and afterward induces cell hyperplasia. Disease management has been based on both tree eradication and copper spray treatment. Overuse of copper for control of bacterial citrus canker has led to the development and prevalence of copper-resistant strains of Xcc. Several genera of both soil- and plant-associated bacteria became powerful tools in sustainable agriculture for control of Xcc and reduction of citrus canker disease severity. In this chapter we present bacteria able to interfere with quorum sensing as well to display antibacterial activity against *Xcc* by production of secondary metabolite. These bacteria may represent a highly valuable tool in the process of biological control and offer an alternative to the traditional copper treatment currently used for the treatment of citrus canker disease, with significant environmental, economic, and health implications worldwide.

Keywords: quorum quenching, *Pseudomonas*, biofilm, secondary metabolites, *Bacillus*

1. Introduction

The steady increase in global overpopulation has forced the agricultural producer to introduce environmentally aggressive practices (e.g., undiscriminating use of pesticides and chemical fertilizers), in order to respond to the rising request of cultivated crops for food. The growing breach between supply and request and the negative impact on the environment have stimulated researchers to develop alternative strategies, pursuing to promote a sustainable agriculture.

The interactions between plants and their associated microorganisms have generated a huge interest. A deep understanding of these processes allows the implementation of innovative agricultural applications. Plants produce an extensive collection of organic compounds comprising sugars, organic acids, and vitamins, which can be served as nutrients or signals for microbial communities. On the other hand, microorganisms release phytohormones, small molecules, or volatile compounds, which may act directly or indirectly reducing disease severity caused by phytopathogenic agents. Some of these actions are nutrient competition, antibiotic activity, plant immunity activation or plant growth, and morphogenesis activation [1].

Prokaryotes and mainly the bacterial domain are the numerically dominant component of most microbial communities in plants. Numerous genera of both soil- and plant-associated bacteria turn out to be powerful tools in sustainable agriculture, because these bacteria display extremely versatile secondary metabolisms with valuable biological activities, including quorum quenching and antibiotic activity. The aim of this chapter is to present two different approaches for biological control of bacterial citrus canker. This antagonism specifically focus in a quorum quenching of DSF pathway and antibacterial activity by *Pseudomonas* bacteria against *Xanthomonas citri* subsp. *citri* ethological agent of citrus canker disease.

2. Citrus canker disease

One of the most important diseases of citrus is citrus canker, affecting almost all commercial varieties. Bacterium *Xanthomonas citri* subsp. *citri* (*Xcc*) is the etiological agent of citrus canker. In the last decade, citrus canker disease rise as one of the main threats to citrus industry, because of the rise of copper-resistant Xcc strains. Factors such as bacterial species and weather conditions determine the disease severity. The geographical origin of the disease is not clear; some researchers report that the first disease cases appeared at Southern China [2]. However, according to Fawcett and Jenkins in 1933, the disease originated in regions of India and Java [3]. These reports suggest, therefore, that the origin of the disease occurred in tropical areas of Asia, where it is assumed that citrus species originated and has been distributed to other areas through grafting [4]. In America, the first report of the disease occurred in the United States in 1915 [5]. Currently, citrus canker is present in more than 30 countries in Asia, the Indian Ocean and Pacific Islands, South America, and the Southeastern United States [4].

Traditional control of citrus canker disease centered on the application of copper-based products seeks the reduction of bacterial population in leaf surfaces. However, multiple applications are needed in order to obtain a significant reduction in bacterial burden on phyllosphere. Weather conditions, i.e., wind and rain, decrease drastically the effectiveness of copper applications. The drawbacks of the long-term use of copper compounds to control plant pathogens in the field include selection of copper resistance and horizontal transfer in bacterial populations [6].

2.1 Disease cycle and transmission mechanisms

Invasion and colonization of the citrus host by *Xcc* occur by stomata and wounds in plant tissues, infecting leaves, fruits, and stems. The bacterium *Xcc* multiplies within the intercellular spaces in the mesophilic tissue, inducing cellular hyperplasia, leading to rupture of the leaf epidermis and resulting in high and spongy lesions surrounded by a margin soaked in water. Upon leaf epidermis eruption, a great number of bacteria are released to the environment to reach other leaves and plants. Rain, wind, and agriculture tools are the main agents of natural dispersion of disease; the insect larvae of the citrus tree cause extensive wounds in the foliar tissues and greatly increase the spread of the disease. Rainwater collected from foliage with lesions contains between 10^5 and 10^8 cfu/ml [4].

2.2 Types of disease

There are three different types of citrus canker caused by two species of *Xanthomonas*, citrus canker type: A, B, and C. The differentiation of these types is mainly based on the geographical distribution and pathogen host range [7].

The Asian type of canker (canker A) is caused by *Xanthomonas citri* subsp. *citri*. Canker A is the most common and widespread disease, and its geographical distribution continues increasing. The disease is endemic in more than 30 countries: Asia, in the Pacific of India, Pakistan, the Indian Ocean islands, Southeast Asia, South America, Southeast China, and Japan. The bacterium Xcc has a wide range of host and produces the disease in the great majority of the citrus species as *C. paradisi*, *C. aurantifoli*, *C. sinensis*, and *C. reticulata* [8]. Type B canker is caused by the bacterium *Xanthomonas fuscans* subsp. *aurantifolii* type B (*XauB*) [9]. Type B canker has similar symptoms to type A canker; however, the symptoms take longer to appear as a consequence of the slower growth rate of XauB, and the host range is restricted to *C. limon* but has also been sporadically isolated from *C. sinensis and C. paradisi* [10]. C-type canker has only been identified in the state of São Paulo, Brazil [11], and has the same symptoms as type A citrus canker, caused by *Xanthomonas fuscans* subsp. *aurantifolii* type C (XauC) and only infects *C. aurantifolii* [9].

2.3 Symptomatology

The diseased plants are characterized by the occurrence of conspicuous raised necrotic lesions that develop on leaves, branches, and fruits. In the leaves, the first appearance is circular patches of 2–10 mm in diameter; their appearance is oily and usually appears on the abaxial surface reflecting stomatal entrance. The lesions are often similar in shape and size. Subsequently, both epidermal surfaces may become ruptured by pathogen-induced tissue hyperplasia. In the leaves, stems, thorns, and fruits, circular lesions became like a raised boil, growing in spongy white or yellow pustules. These pustules then darken and thicken brown cork type, which is rough to the touch. Often, a watery swell develops around the necrotic tissue and is easily visualized with transmitted light (**Figure 1**).

2.4 Management and treatment

Bacterial citrus canker management involves different approaches ranging from strict quarantine measures to chemical control. Quarantining is a practical usually used in Brazil and United States of America. Extinction of infected and adjacent

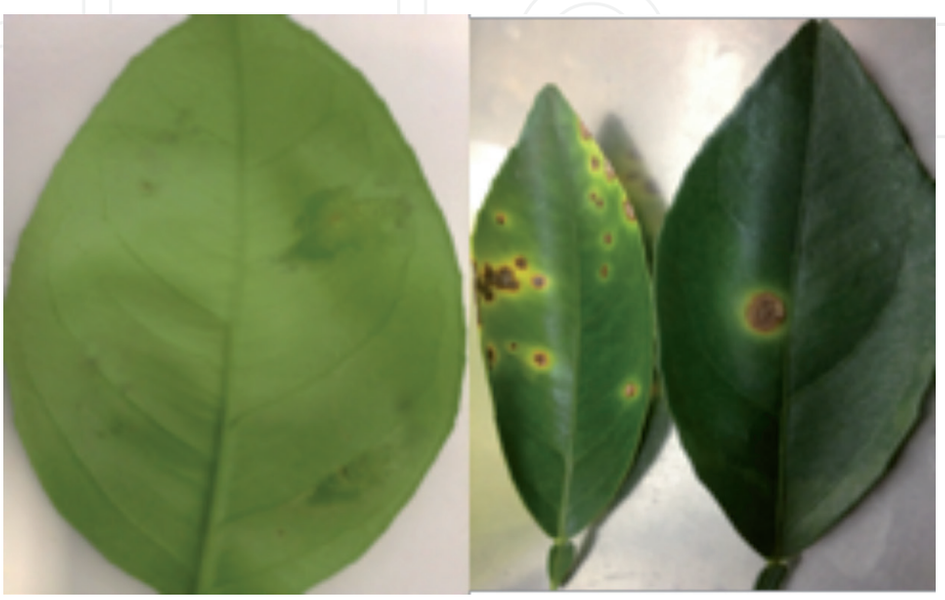


Figure 1.
Symptoms of citrus canker. Left, early stage of the disease. Right, hyperplasia and rupture of the foliar tissue.

trees is one of the major prophylactic measures against citrus canker in commercial citrus crops. Once a symptomatic tree is identified, it is uprooted, stacked, and burned, as prophylactic measure surrounding trees is destroyed as mentioned before [12].

Prevention of primary infection in the new sprouts perhaps is the major effective approach to reduce citrus canker spread. The eradication methodology comprises conducting periodic surveys of the orchard, identifying and eliminating the outbreaks of the disease before its proliferation. Brazilian regulation stipulates that any field that has a number of diseased trees greater than 0.5% of the total orchard must be eliminated. After eradication, the contaminated field should be sprayed with copper fungicide based on 1.5 kg of metallic copper per 1 mL of water (0.15% of metallic copper). The contaminated plantations are prohibited and are forbidden from marketing the production until eradication works are completed.

The use of bactericidal products based on copper by spray application is a practice widely used for more than two decades for the bacterial citrus canker control. The prolonged exposure of bacterial strains to copper has led to the rise of resistant strains in endemic areas. Behlau et al. reported that the genes *copAB* and *cohAB* may encode copper-binding proteins responsible for the copper resistance in *Xanthomonas citri* subsp. *citri* [13].

3. *Xanthomonas citri* subsp. *citri*

The genus *Xanthomonas* includes a vast group of phytopathogenic bacteria belonging to the group of γ proteobacteria. *Xanthomonas* infects 124 species of monocotyledonous and 268 dicotyledonous plants [14]. *Xanthomonas* are Gram-negative bacillus endowed with a sole polar flagellum. After 24-hour incubation at 29°C, yellow and shiny colonies appear in a culture media. Xanthomonadin is an unique pigment, and it is responsible for the yellow color of bacterial colonies; the biological role is explained in detail below. The exopolysaccharide known as xanthan gum gives the shiny appearance to colonies [15]. Although the genus itself has a very broad host range, individual members are often specialized to cause disease in a limited number of taxonomically related hosts as mentioned above.

3.1 Isolation and identification

The bacterium *Xcc* can be isolated from symptomatic plants and its diverse infected tissues. Xcc grows easily in regular microbiological culture media. In order to isolate Xcc, infected tissues must be excised and washed, and subsequently the surface must be sterilized for 3 minutes in a 10% NaClO solution. The water-soaked tissue at the lesion margin is streaked across agar medium containing 50 ppm kasugamycin. *X. citri* strains grow easily on regular nutrient agar media containing 0.5% tryptone, 0.3% yeast extract, 0.09% $CaCl_2$, 0.05% K_2HPO_4, and 1.5% agar in tap water, pH 7.2 [16]. After 48 hours of incubation at 29°C, mucoid yellow colonies begin to appear in microbiological medium.

3.2 Determinants of virulence in *Xanthomonas citri* subsp. *citri*

3.2.1 Adhesins

An essential stage in bacterial host colonization is its attachment ability. Adhesins are bacterial surface structures that facilitate the attachment to host tissues. The

nature of these structures is mainly polysaccharidic, e.g., lipopolysaccharides and exopolysaccharides. However, some of these structures share a proteinaceous nature (type IV pili, chaperone/usher pili, two-partner secretion) [17].

3.2.2 Protein secretion systems and their effectors

Bacteria inside in *Xanthomonas* genus exhibit at least six different types of protein secretion system (i.e., T1SS to T6SS), which vary in their arrangement, function, and in a recognition of secretion substrates [18]. Like many other Gram-negative phytopathogenic bacteria, *Xcc* employs mainly secretion systems T3SS, T4SS, and T5SS and their effectors as effective tools in an attempt to invade and to multiply in a susceptible host.

Protein transport from bacterial periplasm to the extracellular environment occurs mainly by T2SS secretion system. Extracellular enzymes as lipases, proteases, and cell wall-degrading enzymes are translocated using this secretion system. Possibly the major enzymes responsible for the degradation of the plant cell wall are secreted by T2SS. T2SS translocator apparatus is composed of up to 12–15 constituents, most of which are linked to the bacterial inner membrane [19].

The T3SS secretion system also known as "needle" delivers effectors directly into host cells. These act as virulence factors influencing cell host activities [20]. In the Xcc genome, 24 effectors have been identified [21]. One of the main effectors delivered by the T3SS in *Xcc* belongs to the AvrBs3/PthA family. Xcc contains four PthA genes that encode transcription activator-like effector (TALE); of these four genes, pthA4 is responsible for the formation of citrus canker lesions. In citrus host the gene known as CsLOB is targeted by the TALE encoded by the Xcc gene pthA4; this gene was assessed in two susceptible host to Xcc infection, i.e., grape fruit and sweet orange [22]. CsLOB1-specific function still remains unclear; some previous studies suggest that CsLOB1 is involved in the regulation of development of lateral organ and metabolism of nitrogen and anthocyanin. Some plant hormones such as auxin, gibberellin, and cytokines also have proven to exert an effect on CsLOB1 gene [23].

T4SS secretion system is an important virulence factor in a wide range of bacterial pathogens. This secretion system involves the secretion of protein or DNA into the host cells [24]. Xcc harbors two gene arrays encoding for T4SS components [25]; one of them has chromosomal location, and the other one is located at the plasmid pXAC64. Proteins VirB1–VirB11 and VirD4 make up the T4SS translocator apparatus. Nowadays, the structural disposition is well established:

(i). Three ATPases (VirB4, VirB11, and VirD4) located at the cytoplasm. These enzymes have been involved in the process of providing the necessary energy for the secretion process.

(ii). Fourteen repetitions of VirB7-VirB9-virB10 trimer. These repetitions form the periplasmic core. It is noteworthy that VirB10 is anchored on both inner and outer membranes; on the other hand, VirB7 is a lipoprotein located at the outer membrane.

(iii). An inner membrane complex formed by VirB3, VirB6, and VirB8.

(iv). An extracellular pili formed by VirB2 and VirB5.

(v). VirB1 which is a periplasmic transglycosylase [26].

A recent study has shown that T4SS in Xcc displays the ability to secrete toxins; these toxins are known as VirD4-interacting proteins (XVIPs), and they are recruited by VirD4. The biological role of XVIPs is targeting and destabilizing the peptidoglycan layer in the cell wall of bacterial contenders in the ecological niche. This feature is distinctive in *Xcc*, and the protein VirD4XAC2623 endows the bacterium with an extra ability to compete in the phyllosphere [27].

4. Biological control of Xcc approaches

4.1 Biological control based on DSF quorum quencher pathway

A wide majority of bacterial genera have developed a cell-to-cell communication system known as quorum sensing (QS). This communication system is based on a signal translation mechanism whose objective is to coordinate the expression of genes at the population level in order to respond and fit to environmental changes. The cell-to-cell communication system is based on the production, secretion, and perception of small molecules known as autoinducers. A basal quantity of autoinducers are produced by every single cell, subsequently, which is secreted to extracellular milieu reflecting the bacterial population density. At high population density, the autoinducers reach a critical concentration and enable to cognate receptor to sense them. Consequently, this biological event results in triggering a cascade of diverse cell functions [28]. In the *Xanthomonas* genus, the bacteria display a quorum sensing system in which the autoinducer molecule is a short acid fat called diffusible signal factor (DSF). The DSF autoinducer family is cis-2-unsaturated fatty acids. In Xcc this DSF was characterized as cis-11-methyl-2-dodecenoic acid. The gene cluster that encodes element of quorum sensing system in *Xanthomonas* genus is the *rpf* cluster [29].

Since quorum sensing helps to coordinate community-based bacterial behavior, it is not essential for bacterial survival; therefore, the inhibition of QS interrupts only the desired phenotype, i.e., virulence, biofilm formation, and bacterial resistance to different antibiotics. Interference with QS can provide a route for disease control. This interference may involve signal degradation (quorum quenching) or excess signal production (pathogen confusion) [30]. Quorum quenching is a mechanism adopted by a number of bacteria to break the QS signaling of competitors, giving these organisms an advantage within a particular habitat [31]. It is rational that microorganisms can develop mechanisms to disarm the QS systems of competing organisms in order to increase their competitive strength in an ecosystem [32].

We have conducted a recent study that allows the isolation and identification of bacteria isolated from citrus leaves belonging to plant of field crops with and without citrus canker symptoms. From a total of 114 isolates recovered, 7 bacteria able to disrupt DSF quorum sensing pathway in *Xac* (quorum quencher bacteria) were identified. These bacteria were identified by API kits (bioMérieux's API®) and sequencing of PCR-mediated amplification products of the 16S rRNA genes as *Bacillus amyloliquefaciens*, *Bacillus vallismortis*, *Pseudomonas oryzihabitans, Pseudomonas aeruginosa, Raoultella planticola*, *Kosakonia cowanii*, and *Citrobacter freundii* [33].

Virulence assays were conducted under controlled growth conditions, and canker lesions were quantified at 21 days post inoculation. These assays demonstrated that, when citrus leaves were inoculated with mixtures of Xcc and quorum quencher bacteria, the number of cancer lesions decreased significantly reducing the severity disease (**Figure 2**).



Figure 2.
Virulence assay. Leaves infected by spray method at the same concentration 1 × 10^6 UFC/mL. Left, Xcc wild type. Right, Xcc plus Pseudomonas oryzihabitans. Picture was taken after 21 days of infection.

Quorum quencher bacteria impaired the attachment and biofilm formation of Xcc to leave the surface. These are essential steps in the maintenance, survival, and initial establishment of tissue pathogenicity in citrus canker. In fact, it is completely accepted that QS plays an important, if not an essential, role in the formation of bacterial biofilm [34]. Studies of scanning electron microscopy SEM confirmed the substantial reduction in the adherence ability of Xcc after 10 hours when it was co-infected with quorum quencher bacteria relative to the control used, i.e., the leaves infected with Xcc alone. After 7 days post-infection with Xcc and the inhibitory bacteria of DSF, SEM has shown the absence of biofilm formation on the surface of leaves co-inoculated with *P. oryzihabitans* and *B. amyloliquefaciens*, relative to the control used, i.e., the infected leaves just with *Xcc*.

A possible mechanism for explaining the modification or degradation of DSF molecule produced by Xcc could be the quorum quencher bacteria using the DSF molecule as a possible substrate for the UDP-sugar transferase enzyme. The addition of one unit of sugar (from UDP-sugars, i.e., UDP-glucose or UDP-galactose to the short chain of fatty acid impossible the recognition of this version of modified DSF molecule by sensor RpfC. These UDP-sugar pools are produced by the activity of carbamoyl phosphate synthetase enzyme, which is encoded by *carA* and *carB* genes. The nucleotide sequence of the *carAB* locus in the DSF inhibitory bacteria *Pseudomonas oryzihabitans* and *Bacillus amyloliquefaciens* has a strong similarity to the sequences of *carAB* genes present in the *Pseudomonas* G strain isolated and identified as efficient quorum quencher bacteria in *Xanthomonas campestris* [35].

4.2 Biological control based on antibacterial activity of *Pseudomonas* strains

Pseudomonas species show traits that allow them to act as effective biological control agents (BCAs) against several phytopathogens. Among these traits the most common shared by a broad range of *Pseudomonas* strains are (a) pronounced colonizing ability of plant surfaces, internal plant tissues, and phytopathogen structures [36]; (b) the ability for production of numerous kinds of antibiotic providing additional advantage in antagonism with local microbiota and phytopathogens [37]; and (c) the ability to trigger resistance responses in host plants [38]. Thus, mechanisms of direct antagonism as antibiosis or indirect mechanisms such

as competition for nutrients (e.g., siderophore production), besides induction of systemic resistance responses, actively participate in phytopathogenic disease suppression by the pseudomonads [39]. The *Pseudomonas* strains most usually recognized for their biocontrol activity against both eukaryotic and prokaryotic phytopathogenic microorganisms are *P. fluorescens*, *P. protegens*, *P. chlororaphis*, and *P. putida* [40].

In recent study (in press), we have isolated and identified from soil samples added with a compost five *Pseudomonas* strains which displayed a strong activity against Xcc. Virulence assays in very susceptible citrus host using these strains result in a deep decrease of canker lesions, which suggest a great reduction in citrus canker severity. This effect could be attributed to the great production of secondary metabolites by the *Pseudomonas* strains isolated.

5. Conclusions

Quorum sensing is an important target for prophylactic and therapeutic interventions. Identification of new bacteria species as ABC could be a new alternative for the treatment of copper traditionally used for the treatment of citrus canker disease, thus reducing selection pressure for copper resistance. We believe that the search for microorganisms that act as inhibitors of quorum sensing in phytopathogenic bacteria also as antagonist agent could be an effective strategy in a broader context. Since the organisms characterized here were originally isolated from the citrus phylloplane, the present study also contributes to an understanding of the potential interactions of bacteria on leaf surfaces.

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Conflict of interest

The authors declare no conflict of interest.

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