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Effect of Furans and a Pyran on Several Quorum Sensing Factors in *Ralstonia solanacearum*

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Abstract

Ralstonia solanacearum race 2 is the causative agent of Bacterial Wilt in bananas, a disease also known as Moko disease. Once this bacterium becomes established within a plant, it causes the death of its host. Currently, no treatment is available for Bacterial Wilt, and the disease is able to destroy 100% of the crops in afflicted areas

This study investigates a non-biocidal control method for *R. solanacearum* that attempts to nullify the pathogenic effects of *R. solanacearum* by interfering with the bacteria's Quorum Sensing (QS) communication system. The effects of several molecules on *R. solanacearum* growth, biofilm formation and production of acylated homoserine lactone (AHL) autoinducers were analyzed. The results suggested that, of the molecules tested, 5,6 dihydro-2(H)-pyran-2-one most effectively inhibited growth, biofilm formation and AHL production. Furfural, 3-methyl-2(5H)-furanone and methyl 2-furoate also effectively inhibited growth. These molecules affected biofilm formation in a concentration-dependent manner. In addition, 3-methyl-2(5H)-furanone and methyl 2-furoate likewise inhibited AHL production. Therefore, all of these molecules could be useful for controlling *R. solanacearum.*

Keywords: Furan; Pyran; Quorum sensing; Ralstonia; Banana; Control

Introduction

Bananas are widely consumed worldwide. In Colombia, bananas are the third most exported farming product, exceeded only by coffee (first) and flowers (second). Currently, two diseases limit banana production: Black Sigatoka and Moko disease. While Black Sigatoka is a fungal disease, Moko disease is caused by the bacteria *Ralstonia solanacearum* race 2 and affects almost all banana crops globally [1]. The presence of *R. solanacearum* in Colombia was first detected in 1954 [2]. There are currently no available treatments for controlling *R. solanacearum*. New chemical and biological treatments are therefore urgently required.

R. solanacearum is a gram-negative soil inhabitant that is motile due to the presence of one to four polar flagella. Additionally, is aerobic and can survive in temperatures ranging from 10°C to 41°C and can be found in tropical, subtropical and temperate zones [3]. This pathogen is known for causing bacterial wilt in economically important crops worldwide and can parasitize approximately 250 species belonging to 54 plant families [4,5]. Five races of *R. solanacearum* have been described; these races differ in their preferred hosts, geographical distribution and ability to survive in various environmental conditions. *R. solanacearum* race 1 infects several plants of the Solanaceae family, including eggplants, tomatoes, tobacco and peppers. Race 2 causes wilt in the Musaceae family as well as species of the Heliconia genus. Race 3 affects potatoes and tomatoes but is weakly virulent in other solanaceous crops. Finally, race 4 affects ginger plants in the Philippines, and race 5 affects mulberry plants in China [3].

R. solanacearum enters plants through wounds or natural openings in roots. The bacterium then makes its way to the xylem, where it multiplies and causes the plant to wilt. Wilting has been primarily attributed to the bacteria's production of extracellular polysaccharides (EPS), which can obstruct the vascular system of the plant, preventing the flow of water and nutrients throughout the plant. The bacterium affects all parts of the plant, causing wilting and dropped leaves, reducing fruit quality and quantity, and usually staining and rotting the pulp. Bacterial pustules as well as rotting of the pseudostem and rhizome can also result [4-6].

Due to microbial resistance to synthetic pesticides and the often deleterious effects of pesticides on humans and the environment, non-biocidal methods that allow for selective control of microbial populations with marginal collateral damage are being considered as alternatives for controlling pathogens. One such non-biocidal method involves the use of molecules that interfere with the bacterial communication system known as Quorum Sensing (QS); this communication allows bacteria to act as a group and to attack their host in concert and is closely related to virulence and has been linked to accelerated bacterial growth, biofilm formation and the synthesis of hydrolytic and proteolytic enzymes [7]. QS involves the synthesis of N-acyl homoserine lactones (AHLs) autoinductors as messenger communication, which modulate gene expression when high cell densities are achieved within a determined space; specifically, *R. solanacearum* produces hexanoyl and octanoyl homoserine lactone [8,9].

In addition, other type of molecules has been reported to be involved in QS in *R. solanacearum*. One such molecule is 3-hydroxypalmitic acid methyl ester (3-OHPAME) [10]. *R. solanacearum* also produces 2-hydroxy-4-(methylamino-phenyl-methyl) cyclopentanone, which has been implicated in biofilm production. While the production of these molecules has been shown to be regulated by 3-OH PAME their function is currently unknown [11,12].

The neutralization of QS is known as Quorum Quenching (QQ) and has been proposed like an alternative to modulate the virulence of pathogen in a non-biocidal manner [13]. Therefore, in this research we wish to analyze the control of *R. solanacearum* with several furans and

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a pyran, since previously halogenated furanones displayed high QQ activity [14-16].

The following sequence was used to evaluate this compound. In first instance the effect of the molecule on *R. solanacearum* growth was determined and then viability tests were performed at high molecule concentrations to avoid biocidal activities at the studied concentrations. Finally, the effect of the molecule on biofilm formation and the production of homoserine lactone-type molecules was evaluated using *Chromobacterium violaceum* CV026 as a biosensor.

Materials and Methods

General experimental procedures

Bacteria: *Ralstonia solanacearum* was obtained from Grupo de Biocontrol y Microbiología Ambiental (BIOMA) from Universidad de Antioquia (Colombia); *Chromobacterium violaceum* Bergonzini ATCC®12472 from ATCC (Rockville, USA). *Chromobacterium violaceum* CV026, supplied by Universidad Nacional de Rio Cuarto (Argentina).

Assayed molecules: 2-methyltetrahydro-3-furanone (1), 3-methyl-2(5H)-furanone (2), 2(5H)-furanone (3), furfural (4), L-homoserine (5),5,6 dihydro-2(H)-pyran-2-one (6), methyl-2-furoate (7), 5-hydroxymethyl-2-furaldehyde (8) and 2-pentylfurane (9), were purchased from SIGMA-ALDRICH (San Louis, MSS, USA).They were assayed at 500, 250, 125, 62.5, 31.25 y 15.62 ppm to determine growth inhibition and at 10, 1.0 y 0.1 ppm to establish effects on biofilm formation and at 50 ppm to analyze effect in the AHLs synthesis (Figure 1).

Assessment of the effect of several molecules on *R. solanacearum* growth: The method reported by Othman *et al.* was used to determine the effect of the furans and the pyran on *R. solanacearum* growth [17]. Molecules were evaluated using 96-well microplates containing (per liter): meat extract (5.0 g), yeast extract (1.0 g), bacteriological peptone (5.0 g), sucrose (5.0 g) and $MgSO_4.7H_2O$ (0.49 g). To generate the inoculum, *R. solanacearum* was seeded on 10% tryptic soy agar (TSA) consisting of 4.0 g/L tryptic soy broth, 1.0 g/L sucrose and 15 g/L agar. A 5 ml volume of 1% 2,3,5-triphenyltetrazolium chloride was added to the mixture, allowing the identification of mucoid colonies via the presence of a red center.

The inoculum was prepared by suspending colonies in 3.0 ml physiological saline solution until the desired turbidity was reached. Turbidity was determined via comparison to a 0.5 McFarlan standard. The suspension was then diluted 1:4 with culture medium. Three substances were assessed in each microplate, using three wells for each concentration. Each well contained a volume of 200 µl. Two rows were used as controls: one row was used as a bacterial growth control, with each well containing 100 µl medium and 100 µl inoculum, and the second row was used as a sterility control. Each well of the second row contained 200 µl culture medium. Initial readings of optical densities (OD) at 600 nm and final readings following 21 h of incubation at 28°C were performed for each microplate. A 200-1000 nm Varioskan Flash microplate reader was used for OD measurements. For each substance, the percent inhibition compared to the control was calculated from OD readings. Growth was estimated for each concentration and respective controls by subtracting final OD readings from initial OD readings ($OD_{600}=OD_{final}-OD_{initial}$). The amount of growth and inhibition were then calculated as follows [18,19].

$$\%\ \text{growth} = \frac{OD_{600}\ \text{wells containing substance}}{OD_{600}\ \text{control wells}} \times 100$$

% inhibition=100-% growth.

Viability test: This test allows substances that have biocidal, bacteriostatic or no effect on the pathogen to be differentiated. The viability test involves mixing an aliquot of bacterium with a test substance, incubating the mixture for a period of time and then transferring a small volume of the mixture to fresh culture medium devoid of the test molecule. If colonies are observed on solid medium following a suitable incubation period, it can be concluded that the tested substance has a bacteriostatic and non-biocidal effect. Therefore, this substance can likely act as a QQ molecule, interfering with bacterial communication at subinhibitory concentrations. For viability testing, 5.0 µl from wells containing mixtures of bacteria and test molecules at their maximum concentrations was seeded onto 10% TSA. Cultures were then incubated for 72 h.

Effect of substances on biofilm production: Biofilms are bacterial communities embedded in a polysaccharide matrix and adhered on a surface; thus, this structure is a protective against environmental changes and antibacterial agents [20]. The effect of several substances on the inhibition of biofilm formation was determined according to the method described by Shiva *et al.* with some modifications [17]. For this purpose, 96-well microtiter plates and 72 h cultures in 10% TSA broth at an $OD_{600\ nm}$ of 0.2–0.3 were used. A total of 100 µl bacteria in culture and 100 µl medium containing the test substance was added to each well. Eight wells were used as viability and biofilm formation controls. Another eight wells were used as sterility controls (medium only). Plates were incubated at 28°C for 7 days.

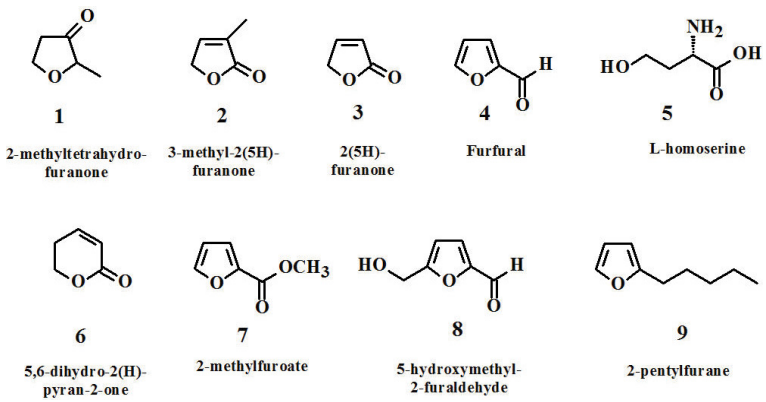


Figure 1: Assayed substance.

After the incubation period, supernatants were discarded, and two washes with distillated water were performed. Plates were then incubated at 50°C to allow for fixing of the biofilm. Following incubation, 200 µL of a 0.1% crystal violet solution was added to each well. Wells were then incubated at room temperature for 15 min, washed again with distillated water and left to dry. Next, 200 µl of 96% ethanol was added, and the plates were incubated for 30 min at room temperature. Absorbances were then measured at 585 nm using a 200-1000 nm Varioskan Flash microplate reader. Each substance was tested at concentrations of 10, 1.0 and 0.1 ppm and four wells were used for each concentration. The percentage of inhibition was calculated as described above.

Effect of substances on the production of homoserine lactones (AHLs) by thin layer chromatography (TLC): Hexanoyl homoserine lactone (C_6-AHL) and octanoyl homoserine lactone (C_8-AHL) have been reported as autoinducers of QS in *R. solanacearum*. To determine the effect of furans and pyrone in the production of AHLs, the method described by Shiva et al. was used, with some modifications [17]. Briefly, *R. solanacearum* was cultured in 50 ml broth with meat extract containing different substances at 50 ppm. Cultures of *R. solanacearum* in broth without any test substance and cultures of *C. violaceum* ATCC® 12472, a known producer of AHL in LB broth, were used as controls. Cultures of *R. solanacearum* were incubated at room temperature for 72 h with agitation at 110 rpm; cultures of *C. violaceum* were incubated under similar conditions for 48 h. After incubation, cultures were centrifuged at 10,000 rpm for 15 min. Supernatants were then extracted thrice with 50 ml of ethyl acetate. The ethyl acetate extract was then dried in a rotary evaporator.

The presence of lactones in the extracts was determined using thin layer chromatography plates (Merck 0.25 mm). The previously obtained ethyl acetate extracts were separated using a mixture of dichloromethane/methanol (1.0:0.1, v/v) as the mobile phase. After the plates were developed, they were placed in sterilized Petri dishes and 3 ml LB medium containing 0.8% agar and *C. violaceum* CV026 was added to each plate. Plates were incubated at 28°C for 48 h. Besides, to determine direct effects of pure substances on AHL production, they were subjected to TLC, then covered with LB medium containing *C. violaceum* CV026 or *C. violaceum* ATCC®12472.

Results and Discussion

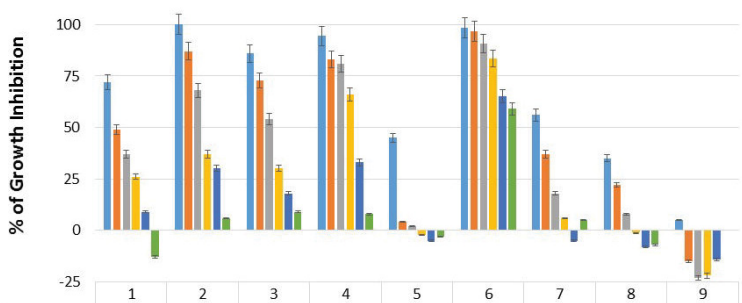
Evaluation of growth inhibitory activity

The tested substances can be grouped into three groups: Those that seem to show a clear biocidal effect, characterized by a high inhibition even at low concentrations; those that have very poor growth inhibition activity; and those have almost no inhibitory effect or paradoxically seem to promote bacterial growth. Some molecules, 2, 4 and the pyrone 6 displayed strong inhibitory activity (close to 100%) at the highest assessed concentration; the last exhibited high inhibitory activity (59%) even at the lowest concentration tested (15.6 ppm). High inhibitory activities of 72% and 86% were observed for 1 and 3, respectively. Medium activities at the highest concentration tested were observed for 5 (45%) and 7 (56%). No effect on growth was observed at lower concentrations. *R. solanacearum* was weakly inhibited by 8 (35%) and not inhibited at all by 9 (5%), which appears to be a growth inducer at the lower concentrations tested (Figure 2).

Viability test: The total inhibition of bacterial growth caused by some substances can be interpreted as a biocidal effect. However, a bacteriostatic effect could be related to inhibition of cell reproduction via mechanisms such as QS disruption. Positive viabilities were observed for the molecules that exhibited lower or no inhibitory activities; also displayed positive viabilities the furans 2, 3, furfural 4 and the pyran 6 which demonstrated the highest inhibitory activities in the assay described previously. Therefore, these substances are non-biocidal.

Effect of substances on biofilm production: Biofilm formation is important for the development of plant diseases and virulence process. The biofilm structure allows phytopathogens to protect themselves against harmful external agents such as antibiotics, among others. Almost all of the substances inhibited biofilm formation at concentrations of 1.0 ppm and above: inhibition percentages of 31%-35% were observed for furans 1, 5, 8 and pyran 6; again, the last inhibited biofilm formation at all of the tested concentrations. Substance 9, 2-pentylfuran, did not significantly affect biofilm formation. This result is normal in QS-mediated phenomena, since in general, while practically no biocidal effect is observed at low substance concentrations, net biocidal effects can be seen at high concentrations (Figure 3).

Detection of homoserine lactones (AHLs) by thin layer chromatography (TLC): To determine whether the observed inhibition of growth and biofilm formation resulted from changes



	1	2	3	4	5	6	7	8	9
500 ppm	72	100	86	95	45	98	56	35	5
250 ppm	49	87	73	83	4	97	37	22	-15
125 ppm	37	68	54	81	2	91	18	8	-23
62,5 ppm	26	37	30	66	-2	84	6	-1	-22
31,25 ppm	9	30	18	33	-5	65	-5	-8	-14
15,6 ppm	-13	6	9	8	-3	59	5	-7	0

Figure 2: Effect of molecules on *R. solanacearum* growth by microdilution in broth.

in the production of AHL autoinducers, the AHL contents of *R. solanacearum* extracts treated with different substances were evaluated using thin layer chromatography. *C. violaceum* CV026 was used as a biosensor for AHLs, due to while this bacterium is unable to produce AHLs, it indicates the presence of exogenous AHLs via the production of violacein. The results indicated that, at 50 ppm furans 1, 2, 7 and pyran 6, inhibited AHL production, whereas substances 3, 4 and 8 were nearly as effective as before mentioned. In contrast, substances 5 and 9 seemed to increase AHL production, as increased pigmentation was observed on plates (Figure 4).

The search for new molecules and methods to generate substances with improved antibiotic properties is essential for humanity, as microbial resistance causes tremendous losses of human life and agricultural productivity. The use of rational methods based on biochemical knowledge of bacteria communication, known as Quorum Sensing, can help address this need; thus, disruption of the chemical mechanism or Quorum Quenching, can yield positive results and this strategy addresses the microbial resistance problem from a non-biocidal point of view.

This investigation analyzed the effects of several furans and a pyran on three important components of the microbial Quorum Sensing. The first one was the microbial growth inhibition that is the preliminary phase of virulence; secondly the biofilm formation, to produce a multicolony resistant to external stress and antibiotics, and finally, the inhibition of lactone autoinducer synthesis, involves in other bacterial process related to virulence. All these factors, which are associated with the production of proteolytic and hydrolytic enzymes and the production of toxins, together constitute the virulence of a pathogen. Besides, the temperature has a high influence in the virulence; thus, all assays were made at 28°C since pathosystem Ralstonia-Banana (which is our main future focus) express its maxima virulence above this temperature.

For this work, a number of furan ring analogs were chosen, as it has been reported that natural brominated substances of marine origin containing the furan ring are strong QS inhibitors; additionally, other synthetic furans have been exhibited this antibiotic effect in other bacteria [16,21-23].

Initially a kinetic of bacterial growth was determined and maximal growth was obtained after 21 h of incubation. All determinations of the effects were made in this time. As a consequence, molecules were classified in three types: total growth inhibitors (biocide compounds), compounds without effect (inactive compounds) and growth inhibitors with activity less than 50% (similar to bacteriostatic) (Figure 2).

The pyran 6 was the most active, followed by furfural 4, 2(5H)-furanone 3, and 3-methyl-2(5H)-furanone 2. On the other hands 2-metiltetrahidro-3-furanona 1 exhibited moderate activity, while L-homoserine 5, methyl-2-furoate 7 and 5-hydroxymethyll-2-furaldehyde 8 showed low activities. Substance 9, 2-pentylfuran,

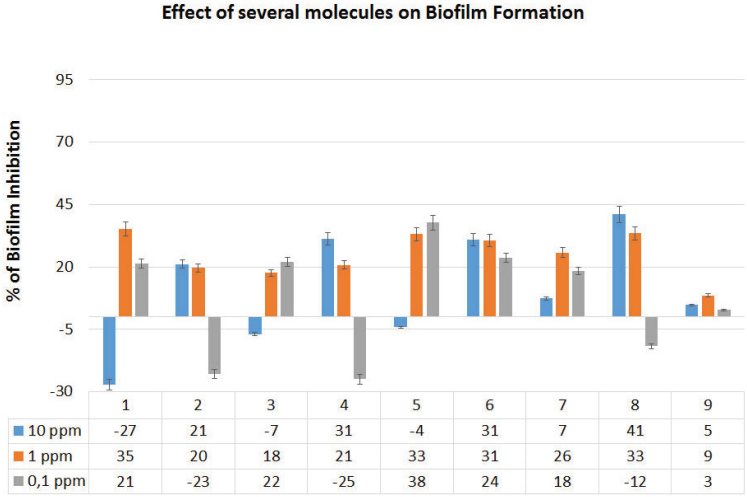


Figure 3: Effect of molecules on biofilm production.

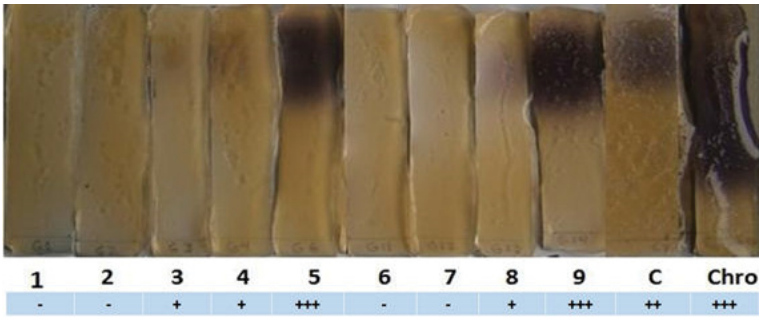


Figure 4: Effect of several molecules on the production of homoserine lactone in *R. solanacearum* accordingly thin layer chromatography.
C: control culture of *R. solanacearum*; Chro: culture of *C. violaceum* ATCC® 12472, an AHL producer strain; (-) indicates the visual absence of violacein and (+ to +++) the presence compared to the control (C)

displayed growth-promoting activity. So, a b-unsaturated lactone or aldehyde conjugate group seems to be essential to the QQ effect; however, a conjugated ester or di-substituted rings practically lacks of activity. More interesting is the promoting effect of pentylfurane, acting like a Quorum Sensing, instead of a Quorum Quenching molecule, and which has not been reported before. Moreover, the viability assay demonstrates that some molecules are not actually bactericidal. In particular 2, 3, 4 and 6, substances that displayed high growth inhibitory activities, exhibited positive viabilities. Because bacteria look as if temporarily lose their reproductive capacity in the presence of these substances appear acting via a QQ-type mechanism.

Concerning to biofilm these results are also consistent with the substances that were found to inhibit biofilm formation at and above 1.0 ppm, 1, 5, 8.The pyran 6 inhibited biofilm formation at all of the assayed concentrations. In contrast molecule 9, 2-pentylfuran, did not significantly affect biofilm formation at any of the tested concentrations. *The use of compounds which can inhibit the formation of biofilms allow dispose of a very efficient tool of control of a plant pathogenic microorganism.*

Related to lactone production, at 50 ppm compounds 1, 2, 6, 7, 3, 4 and 8 affected the production of AHL autoinducers, but 9, 2-pentylfuran, promoted AHL synthesis in *R. solanacearum*, a behavior that is consistent with the growth promotion effect observed in the first part of this research, and therefore be classified as a QS substance, while the other substances can be classified as QQ substances.

Some of these molecules have already been reported to have QQ effects. Shobharani and Agrawal assayed 2(5H)-furanone and found that the addition of the compound to fermented milk inhibited growth, AHL production and expression of virulence genes in *Pseudomonas* sp., increasing the shelf life of the product; besides 3-methyl-2(5H)-furanone was studied by Souza et al. on *Hafnia alvei*, an opportunistic human pathogen; biofilm production, was found to be affected by this compound [24,25].

Finally, the use of bacterial biosensors to determine the inhibition or promotion of AHL may be useful. However, molecules reported to be QS or QQ in *Chromobacterium violaceum* do not necessarily exhibit similar properties in other bacteria. In this investigation although many of the molecules investigated in this study affected AHL production in *R. solanacearum*, none of the assessed molecules exhibited QS or QQ effects in the biosensor bacterium.

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Disclosure Statement

No potential conflict of interest was reported by the authors.

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