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Biofilm Formation by Phytopathogenic Bacteria *Acidovorax citrulli* and *Ralstonia solanacearum*

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ABSTRACT

Phytopathogenic bacteria are responsible for causing several losses in agriculture economic world. Biofilm formation presents itself as an important structure related to bacterial virulence. The objective of this study was to investigate the biofilm formation by *Ralstonia solanacearum* and *Acidovorax citrulli* isolates. Quantification of biofilm formation was performed by the crystal violet method, using NYD as the standard medium for both bacteria, TZC as a specific medium for *R. solanacearum* and YDC for *A. citrulli*. The biofilm was observed in SEM and CLSM. Under the tested conditions, B5-5, CGH8, CGM10 and CGH26 *R. solanacearum* isolates and Aac1.43 and Aac1.73 *A. citrulli* isolate formed moderately or strongly biofilm in both media tested. However, the amount of biofilm produced by *R. solanacearum* was higher than those produced by *A. citrulli*. The SEM and CLSM revealed structurally distinct biofilms among isolates of *R. solanacearum*, which did not occur for *A. citrulli* isolates. We conclude that *R. solanacearum* is a strong biofilm producer, while *A. citrulli* not seem to be well suited to this condition by not adhere well to the surface. This species depicts potentials to become natural models to study plant biofilm infections due to the high number of host species.

Keywords: bacterial wilt; bacterial fruit blotch; scanning electron microscopy; confocal laser scanning microscopy; bacterial biofilm.

Introduction

In nature microorganisms usually live as adhered cells aggregates, growing on a surface and immersed in an amorphous extracellular matrix known as a biofilm (Ramey et al., 2004; Bogino et al., 2013). The biofilm matrix is a complex structure composed of a variety of extracellular polymeric substances, such as exopolysaccharides, proteins and extracellular DNA. This structure allows the biofilm to be highly resistant to antimicrobial agents (Branda et al., 2005), conferring particular importance in medical, agricultural and industrial environments (Ramey et al., 2004).

The biofilm formation is extremely important for the survival of bacteria, besides contributing for the phytopathogens virulence via several mechanisms, including the block of xylem vessels, the increase of bacterial resistance to antimicrobial compounds and/or the increase of habitats colonization (Mansfield et al., 2012; Bogino et al., 2013). It is known that microbial populations use cellular links to adhere to solid supports, surfaces, and particles where they grow and survive in a natural state. This process is the first stage in the biofilm formation and occurs primarily via nonspecific physicochemical interactions and then by molecular interactions mediated by specific binding of the receptor-ligand type (Dunne, 2002; Trentin & Macedo, 2013). The microbial colonization is regulated dependently of the population density by *quorum sensing* signaling (Whitehead et al., 2001) that, combined with bacterial surface components,

extracellular compounds, and environmental signals, are required for biofilm development (Bogino et al., 2013; Bellenberg et al., 2014).

Several phytopathogenic Gram-negative bacteria are causing significant economic damage to global agriculture since their control methods are still extremely inefficient. *Ralstonia solanacearum*, the cause of bacterial wilt, is a pathogen-derived from soil source that penetrates into the xylem vessels through the root (Safni et al., 2014), where it multiplies and produces polysaccharides which increase the xylem viscosity, causing clogging and consequent wilting and death of the plant. This bacterium has about 450 host species, belonging to over 54 botanical families (Wicker et al., 2007; Garcia et al., 2013), especially species of the Solanaceae family (Hayward, 1991). *Acidovorax citrulli* is the causal agent of bacterial fruit blotch, a disease that leads to decay fruit and often occurs in plants of Cucurbitaceae family (Burdman & Walcott, 2012), especially melon (*Cucumis melo*), watermelon (*Citrullus lanatus*) (Oliveira et al., 2007) and pumpkin (*Cucurbita maxima*) (Nascimento et al., 2004).

Despite the importance of these diseases, the pathogens ability to form biofilms and the mechanisms used by pathogenic bacteria to infect their hosts are still little known. This study aimed to investigate biofilm formation by isolates of *R. solanacearum* and *A. citrulli* on surfaces and to evaluate the influence of the growth medium on biofilm formation by these bacteria.

Material and Methods

Bacterial isolates

A total of 13 isolates were used in this study, six of *R. solanacearum* of pepper (Garcia et al., 2013) and seven of *A. citrulli* of melon (Silva et al., 2016). These isolates were obtained by Culture Collection of the Phytobacteriology Laboratory of the Agronomic Department of the Universidade Federal Rural de Pernambuco (UFRPE), Brazil, where they were isolated, identified and biochemically characterized. The isolate identity was confirmed by PCR of the 16S region, conducted at the Laboratory of Molecular Biology of the Biochemistry Department of Universidade Federal de Pernambuco (UFPE), Brazil.

Media

Two culture media broth were tested for each species. The NYD medium (5 $g.L^{-1}$ yeast extract, 3 $g.L^{-1}$ meat extract, 5 $g.L^{-1}$ peptone, and 10 $g.L^{-1}$ dextrose) was used as a standard medium for both species. The TZC medium (5 $g.L^{-1}$ dextrose, 1 $g.L^{-1}$ casamino acid, 10 $g.L^{-1}$ peptone and 1 % triphenyltetrazolium) was employed as specific medium for *R. solanacearum* (Kelman 1983) and the YDC medium (10 $g.L^{-1}$ extract yeast, 20 $g.L^{-1}$ dextrose and 20 $g.L^{-1}$ calcium carbonate) for *A. citrulli* (Schaad et al. 2001). All media were autoclaved for 15 min at 121°C.

Quantification of biofilm formation

Biofilm production quantification in microplates was based on the adapted method described by Trentin et al. (2011). In this study, sterile polystyrene 96 well microplates (Costar 3599) were purchased from Corning Inc. (USA). The isolates were cultured in NYDA medium (NYD added 18 $g.L^{-1}$ agar) for 24 h at 28°C and a bacterial suspension in saline (0.9 % NaCl), corresponding to McFarland scale (3 x 10^8 $CFU.mL^{-1}$), was used in the tests. One hundred and forty microliters of medium (NYD, TZC or YDC), 40 µL of bacterial suspension and 20 µL of distilled water were used for the biofilm formation. Rifampicin (32 µM) was used as an antibiotic control. The plates were aerobically incubated for 24 h, at 28°C. The experiment was made at least in triplicate for each isolate.

Afterward, the content of the plate was discarded, and the plates were washed three times with 200 µL of the sterile saline solution. The remaining adherent bacteria were heat-set at 60°C for 1 h. Two hundred microliters of crystal violet (0.4 %) were used to evidence the adhesive layer of the formed biofilm, for 15 min at room temperature, and the colorant excess was removed with distilled water. The bound cell stain was solubilized with ethanol (99.5 %) for 30 min, and the optical density (OD) was measured at 570 nm (Spectramax M2e multimode Microplate Reader, Molecular Devices, USA).

Thus, biofilm formation was evaluated by OD cut (ODc), following the classification of Stepanovic et al. (2000), which consists of three standard deviations above the mean OD of the negative control, where: $OD \leq ODc$ - no biofilm producer, $ODc < OD \leq (2 \times ODc)$ – weak biofilm producer, $(2 \times ODc) < OD \leq (4 \times ODc)$ - moderate biofilm producer and $(4 \times ODc) < OD$ - strong biofilm producer. Biofilms formed by isolates that presented as moderate or strong producers were observed in Scanning Electron Microscopy and Confocal Laser Scanning Microscopy.

Scanning Electron Microscopy (SEM)

Biofilms were cultured in 96 well plate microplates (as described in item 2.3) with a PermanoxTM slide (NalgeNunc International, USA) in each well. After 24 h incubation at 28°C,

the samples were fixed in glutaraldehyde (2.5 %) for 2.5 h, washed with cacodylate buffer (100 mM, pH 7.2) and dehydrated in increasing concentrations of acetone. The PermanoxTM slides were dried by CO_2 critical point technique (CPD 030 Balzers, Liechtenstein), fixed on aluminum bases, covered with platinum film and examined in a scanning electron microscope (JEOL JSM-5800).

Confocal Laser Scanning Microscopy (CLSM)

Bacteria biofilms were cultured in CEELviewTM glass bottom dish plates (35/10 mm, 4 compartments) under the same conditions of item 2.3 for 24 h, at 28°C and after this period the free bacteria were washed with PBS buffer (10 mM). The Live/Dead BacLight Kit (Invitrogen) was used to visualize the presence of live and dead bacteria, in green and red respectively, according to the specifications of the manufacturer, and Concanavalin A tetramethylrhodamine conjugate (Invitrogen) was used to evidence the formation of microbial matrix (Strathmann et al., 2002). The experiment was performed in duplicate and visualized in a confocal microscope (Olympus Fluoview 1000 - FV1000) with excitation light at 473 nm for green and 559 nm for red, under the objective of 60x.

Table 1. Pure culture biofilm formation assay in NYD media and TZC media for *Ralstonia solanacearum* and YDC media for *Acidovorax citrulli*. Isolates were classified according to Stepanovic et al. (2000) as not produced (NP), weak (WP), moderate (MP) and strong (SP) biofilm producers. The values represent the mean ± standard deviation of the absorbance at 570 nm (n = 24). a – No different significantly from the control; b-d – different significantly from the control ($p<0.05$).

Strain	Medium			
	Standard		Specific	
	OD 570 nm	Classification	OD 570 nm	Classification
Aac1.12	0.287 ± 0.043^{a}	NP	0.163 ± 0.020^{a}	NP
Aac1.43	0.443 ± 0.087^{c}	MP	0.549 ± 0.049^{c}	MP
Aac1.5	0.129 ± 0.006^{a}	NP	0.180 ± 0.220^{b}	WP
Aac1.73	1.260 ± 0.155^{d}	SP	0.578 ± 0.071^{c}	MP
Aac1.78	0.178 ± 0.034^{a}	NP	0.202 ± 0.031^{b}	WP
Aac5.16	0.192 ± 0.037^{b}	WP	0.198 ± 0.030^{b}	WP
Aac5.3	0.214 ± 0.020^{b}	WP	0.223 ± 0.025^{b}	WP
RsB5-5	0.421 ± 0.126^{c}	MP	0.359 ± 0.076^{c}	MP
RsCGM10	0.787 ± 0.207^{c}	MP	1.340 ± 0.096^{d}	SP
RsCGM6	0.249 ± 0.049^{b}	WP	0.367 ± 0.076^{b}	WP
RsCGH8	1.804 ± 0.362^{d}	SP	1.061 ± 0.174^{d}	SP
RsSCN21	0.244 ± 0.062^{b}	WP	0.400 ± 0.145^{b}	WP
RsCGH26	1.530 ± 0.211^{d}	SP	0.593 ± 0.059^{c}	MP

Results

The experiments performed in this study allowed us to measure the adhesion rate and subsequent biofilm formation of the tested bacteria. The results of the biofilm formation can be verified in table 1. *R. solanacearum* isolates cultured in standard and specific media revealed that only CGM6 and SCN21 isolates showed weak biofilm formation in both media tested. B5-5 formed biofilm moderately only in NYD, and other isolates showed moderate and strong biofilm formation under the tested conditions. Only two isolates of *A. citrulli* were biofilm producers, Aac1.43 that formed moderately biofilm in both media tested and Aac1.73 that was a reliable producer in NYD and moderate producer in YDC. The amount of biofilm produced by *R. solanacearum* was higher than those produced by *A. citrulli*. In the presence of rifampicin (32 µg/ml), the growth of the bacterial isolates was inhibited.

SEM analysis showed biofilm structures produced by the different isolates in NYD medium (Figure 1). It was possible to observe that the isolates of *R. solanacearum* were morphologically distinct from each other. B5-5 showed a filamentous pattern biofilm, where the bacteria were joined together forming long filaments of attached cells (Figure 1 panel 1). In contrast, CGH26 isolate (Figure 1 panel 4) showed a dense and uniform biofilm with increased formation of multilayers and a matrix around of bacteria. The CGM10 and CGH8 isolates (Figure 1 panels 2 and 3, respectively)

possess a very similar pattern of biofilm formation between them, with the formation of a monolayer biofilm, which bacterial aggregates isolated were formed after 24h. *A. citrulli* showed structurally, dense, uniform and multilayered biofilms, more similar between the isolates. The images obtained by SEM were by the OD 570 nm biofilm quantification.

The biofilm was analyzed by CLSM after 24 hours using concanavalin A marking matrix and the staining Live (green)/Dead (red) to the bacteria (Figure 2). It was observed the matrix formation in CGM10, CGH8, CGH26, and Aac1.43; contrary, it was not possible to detect the presence of the biofilm matrix in B5-5 and Aac1.73 isolates. Live/Dead staining pattern showed a heterogeneous distribution among isolates. One can observe that B5-5 had the same filamentous pattern obtained by SEM, but with a considerable number of viable cells. The other isolates showed a considerable amount of adhered cells. The CGH8 showed an equal Live/Dead cells proportion while the CGH26 showed fundamentally viable cells. Further, Aac1.43 and Aac1.73 isolates showed few adherent cells, but only Aac1.73 had a viable cell monolayer adhesion. These results were discordant from those obtained by SEM.

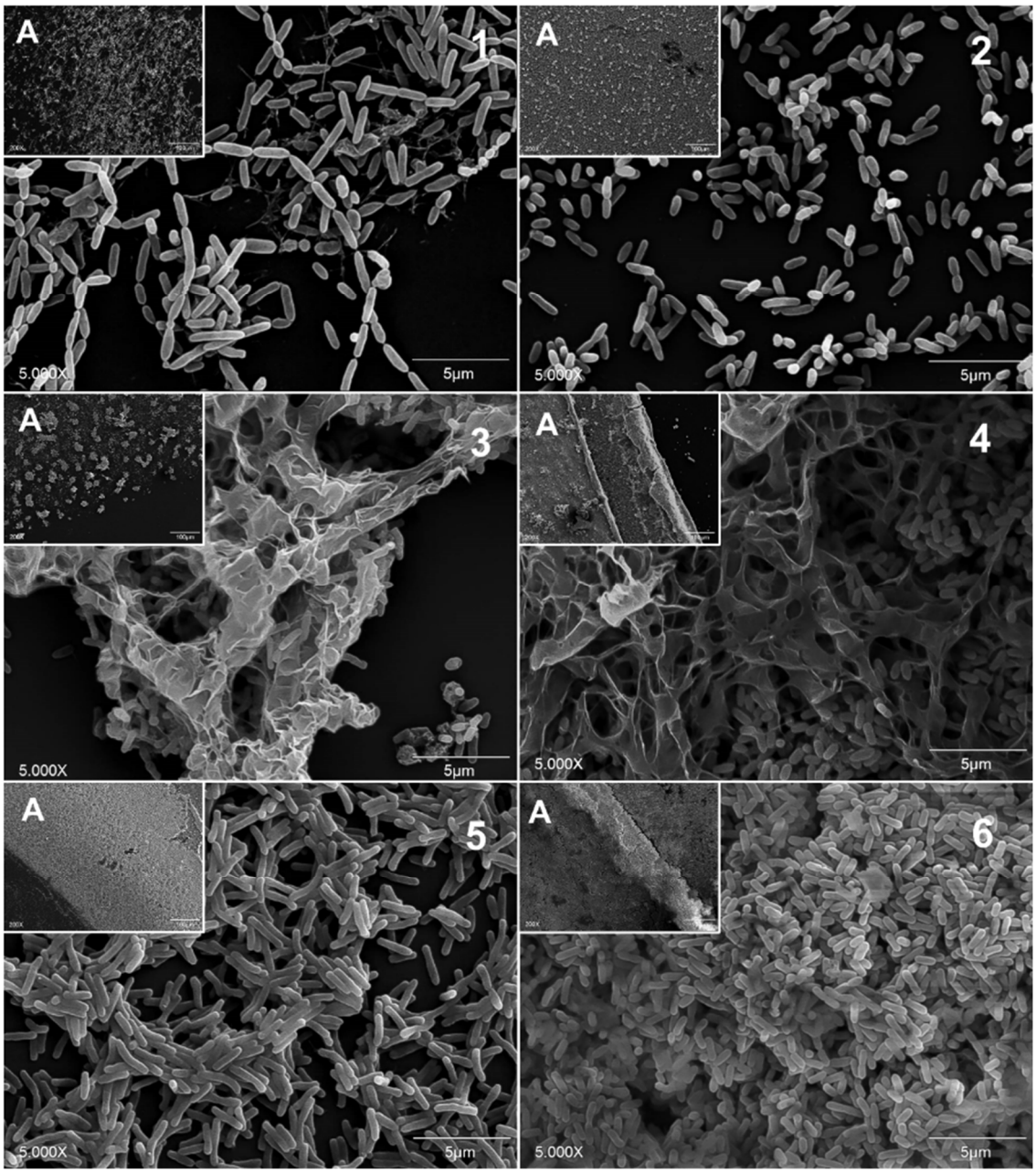

Figure 1. SEM of biofilms in media NYD on PermanoxTM: *Ralstonia solanacearum* B5-5 (1), CGM10 (2), CGH8 (3), CGH26 (4) and *Acidovorax citrulli* Aac1.43 (5), Aac1.73 (6) at 5.000× (Inserts A at 200x).

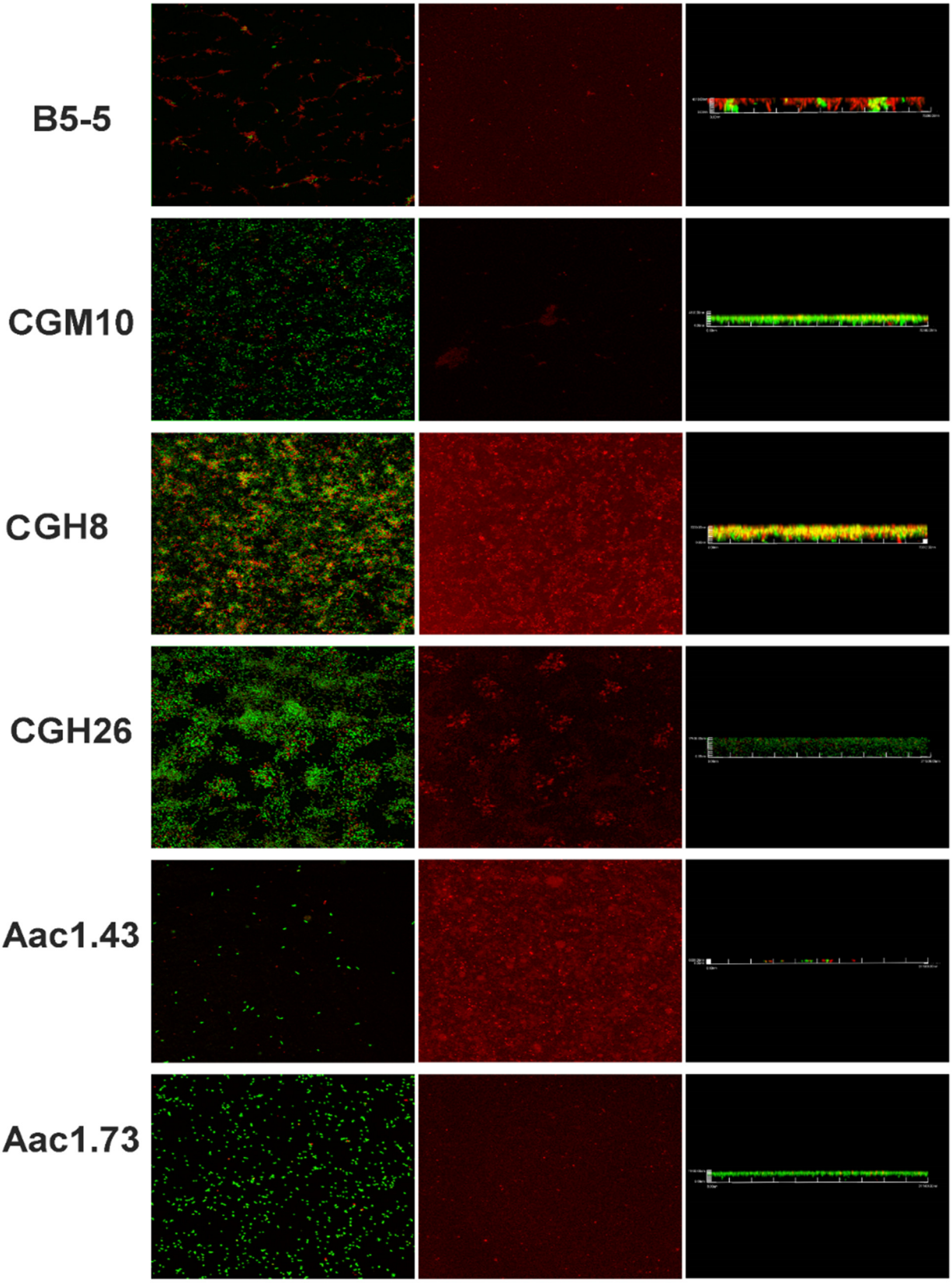

Figure 2. Biofilm structure for *Ralstonia solanacearum* (B5-5, CGM10, CGH26, and CGH8) and *Acidovorax citrulli* (Aac1.43 and Aac1.73), after 24 h of growth in liquid culture medium NYD analyzed by Confocal Laser Scanning Microscopy (CLSM). Stained with A. Live/Dead BacLight (Invitrogen); B. concanavalin A (Invitrogen); C. biofilm thickness observed with Live/Dead stain.

Discussion

Currently, it is known that in most environments the microorganisms may change from a life-free state to a sessile lifestyle in order to form biofilms, exhibiting specific properties such as increased tolerance to adverse conditions

including desiccation resistance and high concentrations of antimicrobial agents (Lebeaux et al. 2013). Further, in several plant pathogens such as *Xylella fastidiosa* (Silva et al., 2011), *Xanthomonas campestris* pv. *campestris* (Dow et al., 2003; Tao et al., 2010), *Pantoea stewartii* subsp. *stewartii* (Leigh & Coplin, 1992; Burbank et al., 2015) and *Clavibacter michiganensis* (Marques et al., 2002; Chalupowicz et al., 2012), biofilm formation has been reported as an important factor associated with virulence, since it allows a higher resistance to plant defenses and competition with other species (Ramey et al., 2004).

Garcia et al. (2013) observed that *R. solanacearum* isolates were actively virulent against pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*), eggplant (*S. melongena*) and tobacco (*Nicotiana tabacum*), confirming the results obtained in our study for the same isolates, since most of they were moderate or strong biofilm producers. Also, our results confirm previous studies that reported the biofilm formation on abiotic surfaces by *R. solanacearum* (Yao & Allen, 2007). However, for CGM6 and SCN21 isolates, which were poor producers of biofilm in both media tested, there was not a direct correlation between virulence and biofilm formation. According to Silva et al. (2016), *A. citrulli* Aac5.3 isolate shows low virulence in melon fruits while Aac1.43 has moderate virulence and the others isolated used in this study had high virulence and was not observed a correlation between these data and the biofilm formation.

The biofilm formation is a complex process regulated by several environmental features. One of the most important factors influencing its formation is the surface properties that allow bacterial adhesion, the first step of the process (Whitehead & Verran, 2015), and the cell surface hydrophobicity also affects bacterial adhesion (Liu et al., 2008). It was previously shown that microorganisms, including *Pseudomonas syringae* isolates, adhere in higher numbers to hydrophobic materials (Laue et al., 2006), which in our work seems to have a positive influence to the biofilm formation of *R. solanacearum* and negative influence to *A. citrulli*. Further, the medium composition also influences the biofilm formation. Nutritive media can influence the persistence of planktonic lifestyle, while the poorest media favor the biofilm formation due to stress condition that the bacteria are submitted (Stepanović et al., 2004). It may also have influenced the absence of biofilm formation by most of the *A. citrulli* isolates that were tested in this study.

In *R. solanacearum* SEM analysis, the CGM10, CGH8 and CGH26 isolates show the monolayer pattern for the biofilm formation, in which a cell layer adheres to the surface. This type of structure is favored when cell surface interactions are stronger than the cell-cell interactions (Karatan & Watnick, 2009). This process was shown in Figure 2 (panels 1, 2 and 3), in which bacteria aggregation and multiplication promotes the biofilm formation. The same was observed for *A. citrulli* isolates that developed multilayered biofilm, which bacteria were able to adhere to a surface and also another bacterial cell. This process is known as cell recruitment by *quorum sensing* and involves the release of attraction factors by the cells adhered to a surface, favoring the deposition of others bacteria, which multiply, thus promoting the increase of biomass (Karatan & Watnick, 2009; Absalon et al., 2011).

CLSM analysis showed the same biofilm structure observed by SEM for CGM10, CGH8 and CGH26 isolates, with viable cells inside of the extracellular polymeric matrix (EPM). Cell adhesion and cohesion, nutrition, and protection are some of the several advantages of life in biofilm (Flemming & Wingender, 2010), however not all biofilm cells profit these features, as noted in competitive relations between polymer producers and non-producers. The evolutionary advantage of the active producers is probably due to the formation of polymers which allows access of the cells to oxygen-rich environments (Flemming & Wingender, 2010).

Bacterial surface structures and components, such as flagella, pili, fimbriae, and lipopolysaccharide (LPS) display a crucial role in the physical processes, during the primary stages of the biofilm formation (interaction to the surface), and the virulence development as observed in *Xylella fastidiosa* (De La Fuente et al., 2007) and *Xanthomonas oryzae* pv. *oryzicola* (Wang et al., 2007). It was shown in previous studies the presence of pili type IV in *R. solanacearum* (Yao & Allen, 2007) and *A. citulli* (Bahar et al., 2010) which connected to adhesion of these species, and LPSs mutations linked to LPS synthesis in phytopathogenic bacteria, such as *P. syringae* (Deng et al., 2010), *Xanthomonas axonopodis* (Li & Wang, 2011a) and *Xanthomonas citri* (Li & Wang, 2011b) caused reductions in biofilm formation and in virulence.

Although in CLSM, B5-5 has shown cell adhesion, there was no matrix formation. Further, it was observed a low adhesion and biofilm matrix formation by *A. citrulli* isolates, probably due to

the hydrophilic characteristics of glass slides surface, which negatively influenced the biofilm formation in many species, since that hydrophobic interaction is essential for the initial adhesion (Trentin & Macedo, 2013).

Conclusion

According to the results, we conclude that, despite being an essential factor in the virulence of any pathogens, the biofilm formation does not appear to be strictly related to the tested species. We also conclude that the biofilm appears structurally distinct such as observed for isolates of *R. solanacearum*. Most isolates of *A. citrulli* seem to be well suited to adhesion and biofilm formation on hydrophobic abiotic surfaces. The biofilm formation is an essential feature of bacteria due to their natural formation and influence in the surrounding environment, giving to the microorganisms' high tolerance to adversity. *R. solanacearum* and *A. citrulli* depict a high potential to become natural models to study plant biofilm infections due to the high number of host species. The recognition of the biofilm concept in plant pathology is likely to have a profound impact in the field, as it had in medical microbiology, with vast implications in the development of novel strategies to prevent and eliminate these and other plant diseases.

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