GUSTAVO MATEUS DA SILVA

Acidovorax citrulli: GENETIC ANALYSES AND PROTOCOL FOR ITS DETECTION IN SEEDS

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitopatologia, para a obtenção do título de Doutor

Orientador

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Aos meus pais Joaquim Donizete da Silva e Adelia dos Santos Silva, incentivadores, razão de minha existência. Exemplos de valores, honestidade e simplicidade.

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DEDICO

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GENERAL ABSTRACT

Bacterial fruit blotch of cucurbits (BFB), caused by the seed borne Gram-negative bacterium *Acidovorax citrulli* is a serious threat to cucurbit industry worldwide. Since late 1980`s after devastating outbreaks in watermelon fields in southern United States, BFB has spread worldwide and has been reported in other cucurbit crops such as melon, pumpkin, cucumber and squash. To date, there is evidence for the existence of at least two genetically and pathogenically distinct populations of *A. citrulli*. In Brazil, the first report of BFB was in 1991, in a watermelon field in São Paulo. Although widespread in the country, BFB has been a major problem to melon production. More precisely, BFB has caused significant yield losses to melon production in northeastern Brazil, which concentrates > 90% of the country`s melon production. Despite the management efforts and the recent advances in *A. citrulli* research, BFB is still a continuous threat to the cucurbit industry, including seed producers, growers and transplant nurseries. To better understand the population structure of *A. citrulli* strains in Brazil, and to provide a basis for the integrated management of BFB, we used pulsed-field gel electrophoresis (PFGE), multilocus sequence analysis (MLSA) of housekeeping and virulence-associated genes and pathogenicity tests on different cucurbit seedlings to characterize a Brazilian population of *A. citrulli* strains from different hosts and regions. Additionally, we conducted for the first time a comparative analysis of the *A. citrulli* group I and II population at genomic level and showed that these two groups differ on their genome sizes due to the presence of eight DNA segments, which are present in group II and absent in group I genomes. We also provide the first evidence to suggest that temperature might be a driver in the ecological adaptation of *A. citrulli* populations under nutrient-rich or -depleted conditions. Finally, in order to improve the routine detection of *A. citrulli* on melon seedlots, we designed a new primer set that is able to detect the different Brazilian haplotypes, thus minimizing the risk of false-negatives on PCR-based seed health testing.

Keywords: *Cucumis melo*. Bacterial Fruit Blotch. Multilocus sequence analysis. Pulsed-field gel electrophoresis. Temperature. Survival

RESUMO GERAL

A mancha aquosa do fruto, causada pela fitobactéria Gram negativa *Acidovorax citrulli,* é uma doença com alto poder de destruição e representa uma constante ameaça à produção mundial de melão e melancia. A doença teve o seu primeiro relato no estado da Georgia nos Estados Unidos em meados da década de 60 com o primeiro isolamento do agente etiológico a partir de lesões necróticas em cotilédones de mudas de melancia. A doença, durante muitos anos, foi considerada ser de pouca importância no campo e de ocorrência restrita a mudas em viveiros. No entanto, ao final da década de 80, com o primeiro surto epidêmico da doença nas Ilhas Mariana, foi que o potencial destrutivo da doença no campo foi então conhecido. A partir da década de 90, devido ao transito de sementes contaminadas, a doença expandiu a sua distribuição geográfica e também a sua gama de hospedeiras, com a sua ocorrência em outras cucurbitáceas além da melancia. Até o presente, dados da pesquisa científica suportam a existência de ao menos dois grandes grupos geneticamente distintos nas populações de *A. citrulli*. No Brasil, o primeiro relato da bacteriose foi em 1991, no estado de São Paulo. Embora já relatada em diversos estados, a mancha aquosa tem sido de maior preocupação aos cultivos de melão na região nordeste, onde concentra-se mais de 80% da produção nacional. Para melhor compreender a variabilidade das populações de *A. citrulli* no Brasil e, gerar conhecimentos que possam auxiliar o manejo integrado da mancha aquosa, uma coleção de isolados brasileiros de *A. citrulli* foi caracterizada por pulsed-field gel electrophoresis (PFGE), Multilocus sequence analysis (MLSA) de genes *housekeeping* e genes associados a virulência e por teste de patogenicidade em diferentes espécies hospedeiras. Adicionalmente, conduziu-se pela primeira vez uma análise comparativa da sequência de DNA genômico de isolados bacterianos, referência dos dois grandes grupos de diversidade genética de *A. cirtrulli* (grupo I e grupo II). Resultados da análise comparativa entre os isolados mostraram que os isolados do grupo I e II diferem quanto ao tamanho de seus genomas, devido a presença de 8 segmentos de DNA que estão presentes no genoma dos isolados do grupo II e ausentes no grupo I. Investigou-se também o efeito da temperatura como um fator determinante para a adaptação de isolados de *A. citrulli*. Por último, com o objetivo de reduzir falso-negativos na detecção de *A. citrulli* em sementes de melão, foi desenhado um novo par de *primers* específicos para diferentes haplotypes de isolados brasileiros de *A. citrulli.*

Palavras-chave: *Cucumis melo*. Mancha aquosa do fruto. Multilocus sequence analysis. Pulsed-field gel electrophoresis. Temperatura. Sobrevivencia.

SUMMARY

CHAPTER I

General introduction

1 GENERAL INTRODUCTION

1.1 Origin and importance of cucurbits

Cucurbits belong to the family *Cucurbitaceae* which comprises 120 genera, including about 800 species widely distributed in tropical and subtropical regions of Africa, Asia, Australia and America (JEFFREY, 2005). The family *Cucurbitaceae* is among the most important plant families that supply humans with edible products and useful fibers. The most cultivated cucurbit crop worldwide is watermelon (*Citrullus lanatus*), followed by melon (*Cucumis melo*), cucumber (*C. sativa*), honeydew (*C. melo inodurus*), pumpkin (*Cucurbita pepo*) and squash (*C. maxima*) (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS - FAOSTAT, 2016).

Watermelon is originated from Africa, more precisely from the dessertic region of Kalahari (the current nations of Namibia and Botswana), and has been cultivated in the Middle East, the transcontinental region centered on western China and Egypt, for thousands of years (WALTERS, 1989). The plant can now be found in the Mediterranean, Asia, Europe, and the new world where it was introduced in the early 1600`s by either the Spanish or African slaves (ROMÃO, 2000). The most ancient records on cultivated melon appear in Egypt mural paintings. Extensive records are also found in Chinese writings from about 2000 BC, and in Greek and Roman documents from the first century BC (WALTERS, 1989).

China is currently the leading producer of melon worldwide, followed by Turkey, Iran and Egypt. In 2013, Brazil was ranked as the fourth and eleventh major producer of watermelon and melon respectively (FAOSTAT, 2016). The Brazilian watermelon production concentrates in the states of Rio Grande do Sul, Goiás, Bahia, Sao Paulo and Rio Grande do Norte, which contribute for more than

60% of the national production. The melon production, on the other hand, is almost exclusively performed in the Northeastern region of Brazil, under a semiarid climate. The major producing states are Rio Grande do Norte and Ceará, which together produce more than 80% of the country's melon production (IBGE, 2012).

The Brazilian melon production is a highly profitable activity that involves a remarkable chain of large and small farmers. Besides the national marked supply, melon is one of the fresh fruits most exported recently (RIBEIRO et al., 2015). However, cucurbit production is constantly threatened by plant pathogens that can incite devastating disease epidemics under favorable environmental conditions (CHALUPOWICZ et al., 2015). For some diseases of cucurbits, seeds are the primary source of inoculum, which makes disease management especially difficult (GITAITIS; WALCOTT, 2007). An important disease posing threats to cucurbit production worldwide is bacterial fruit blotch (BFB), caused by the seedborne Gram negative bacterium *Acidovorax citrulli* (SCHAAD et al., 2008).

1.2 *Acidovorax citrulli*

1.2.1 Emergence and Historical Background

Acidovorax citrulli was first reported as an unidentified, seedborne phytobacterium, isolated from necrotic watermelon cotyledons of different plant introductions (PI) from Turkey, at the US Department of Agriculture Plant Introduction Station in Griffin, GA, USA (WEBB; GOTH, 1965). Symptoms often resulted in premature seedling death and included small irregular shaped, water-soaked spots on leaves that eventually coalesced to form larger blighted areas. The disease was primarily reported to be restricted to seedlings.

The BFB-causing pathogen produces non-fluorescent, round, smooth, and slightly convex colonies on King's B medium, have approximately 0.5 – 1.7 μm in length and a single polar flagellum. Based on its morphological and physiological

characteristics, the pathogen was initially classified as a member of the *Pseudomonadacea* family. The bacterium was thought to be most similar to *Pseudomonas pseudoalcaligenes* and was taxonomically placed as a subspecies of *P. pseudoalcaligenes* (*P. pseudoalcaligenes* subsp. *citrulli*) due to its unique ability to infect watermelon seedlings (SCHAAD et al., 1978).

Subsequently, DNA-rRNA hybridization showed that *P. pseudoalcaligenes* subsp. *citrulli* belonged to the *Acidovorax* rRNA complex in the rRNA superfamily III, and the bacterium was then transferred to the *Acidovorax* genus and renamed *A. avenae* subsp. *citrulli* (WILLEMS et al., 1992). Finally, based on low similarity on DNA/DNA hybridization (less than 50%) to other *A. avenae* subspecies, the pathogen was elevated to species level and renamed as *A. citrulli* (SCHAAD et al., 2008).

The disease was considered to only affect seedlings and to have a low damage potential on watermelon fruits in the field until the late 1980s. In 1987 the first BFB outbreak occurred in the Mariana Islands, and entire watermelon fields were lost (WALL; SANTOS, 1988). Additional outbreaks followed rapidly throughout The United States in the mid-1990s leading to numerous lawsuits filed by growers against seed companies. Thus, small seed companies went out of business and others required that contracts should be signed by growers indicating that the seeds had been tested, but the risk and liability of BFB outbreaks would be assumed by the growers (LATIN; HOPKINS, 1995).

During the 1990s, BFB spread globally and the host range was broadened. To date, BFB has already been reported in many cucurbits, such as honeydew, muskmelon, citron melon, pumpkin, cucumber and squash (LANGSTON et al., 1999; O`BRIEN; MARTIN, 1999; WALCOTT; FESSEHAIE; CASTRO, 2004). Thus far, BFB outbreaks have occurred in the Americas, Asia , Europe, Africa, the Middle and Far East, and Australia (BURDMAN; WALCOTT, 2012).

In Brazil, the first record of BFB outbreak was in Sao Paulo state in a watermelon field in 1991 (ROBBS et al., 1991). Since then, BFB has become a

serious threat to the Brazilian watermelon and melon production and its occurrence has already been reported in the states of Rio Grande do Norte (ASSIS et al., 1999), Ceará (SANTOS; VIANA, 2000), Minas Gerais (MACAGNAN et al., 2003), Rio Grande do Sul (UENO; COUTO; UESUGUI, 2003), Pernambuco (MARIANO; SILVEIRA, 2004), Bahia (MARIANO et al., 2004) and Roraima (HALFELD-VIEIRA; NECHET, 2007). Despite its wide distribution throughout the country, BFB outbreaks have occurred sporadically on watermelon fields. On the other hand, significant yield losses have been frequently reported on melon producing areas during the rainy season in Northeastern Brazil, more precisely in the states of Rio Grande do Norte and Ceará, which together are responsible for over 80% of the country`s melon production (ASSIS et al., 1999; CONCEIÇÃO et al., 2014; MELO et al., 2014; SALES JUNIOR et al., 2007).

1.2.2 BFB Epidemiology

Acidovorax citrulli is a seedborne pathogen and contaminated seeds represent the main source of primary inoculum for BFB outbreaks (LATIN; HOPKINS, 1995). Like many phytobacterial diseases, under favorable environmental conditions, such as high humidity and high temperature, BFB can be devastating (SCHAAD; POSTNIKOVA; RANDHAWA, 2003). Once an infection is established, *A. citrulli* can efficiently be spread by wind-driven rain and overhead irrigation (CHALUPOWICZ et al., 2015). The stomata and wounds play an important role on the invasion of leaves and cotyledons, leading to development of foliar lesions, which are the source of secondary spread in nurseries. Symptomless seedlings harboring epiphytic populations can serve as *A. citrulli* reservoir and contribute to BFB development on fruit (BURDMAN; WALCOTT, 2012).

Watermelon fruit infection through stomata occurs at early stages of fruit

development, by two to three weeks after anthesis. After this period, a waxy layer is formed on the fruit surface and block stomata, preventing from bacterial invasion (FRANKLE; HOPKINS; STALL, 1993). Once the waxy surface is established, the developing fruit can only be externally penetrated through the wounds (SOMODI et al., 1991). Infected fruits do not show BFB symptoms at early stages of fruit development and characteristic water-soaked lesions develop shortly before harvest maturity. Moreover, foliar symptoms are not frequently clear and can be easily confused with abiotic stresses becoming the BFB management even more challenging (BAHAR; BURDMAN, 2010).

Seedborne inoculum is the most important primary inoculum source for BFB epidemic development. Figure 1 illustrates the elements of the cycle. The pathogen is introduced with contaminated seeds (step 1). Seeds for transplant production are planted in a soilless potting medium in plastic trays (step 2). A warm, humid environment (conducive for BFB establishment) is maintained in transplant production facilities used for raising seedlings. Bacteria from infested seed infect the developing seedling as the cotyledons emerge from the seed coat. Through overhead irrigation, *A. citrulli* is efficiently splash-dispersed to neighboring seedlings (step 3). Secondary spread in the transplant house can be responsible for significant proportions of infected seedlings reaching the fields (step 4). As plants grow in the field, the pathogen spreads to new leaves and neighboring plants (step 5). Infected plants are not killed and vines do not collapse. However, lesions on foliage provide a source of inoculum for infection of immature fruits. Characteristic blotch symptoms appear on fruit shortly before they ripen (step 6).

A diseased fruit decay in the field, seeds associated with affected fruit slip to the soil (step 7). These seeds produce infected volunteer seedlings in the following season and may serve as a source of local inoculum (step 8). In addition, some cucurbit weeds occurring naturally at melon producing areas in Northeastern Brazil, have been reported to harbor *A. citrulli* as an alternative host and therefore

being a potential source of inoculum (NASCIMENTO; MARIANO; SILVA, 2004). *Acidovorax citrulli* can efficiently colonize and be asymptomatically transmitted by non-host seeds of tomato and pepper (DUTTA et al., 2014a). The bacterium was isolated and identified in May 1995 and in January 1997, in Israel, from diseased eggplants seedlings grown from imported seeds and from a shipment of tomato seeds imported from India, respectively, at the Israeli quarantine station (ASSOULINE et al., 1997). In fact, the threat posed by *A. citrulli* surviving in infected cucurbit weeds and volunteer seedlings in the field is affected by a variety of environmental factors (step 9); however, detailed studies quantifying the extent of the threat as well as the factors affecting *A. citrulli* survival are lacking.

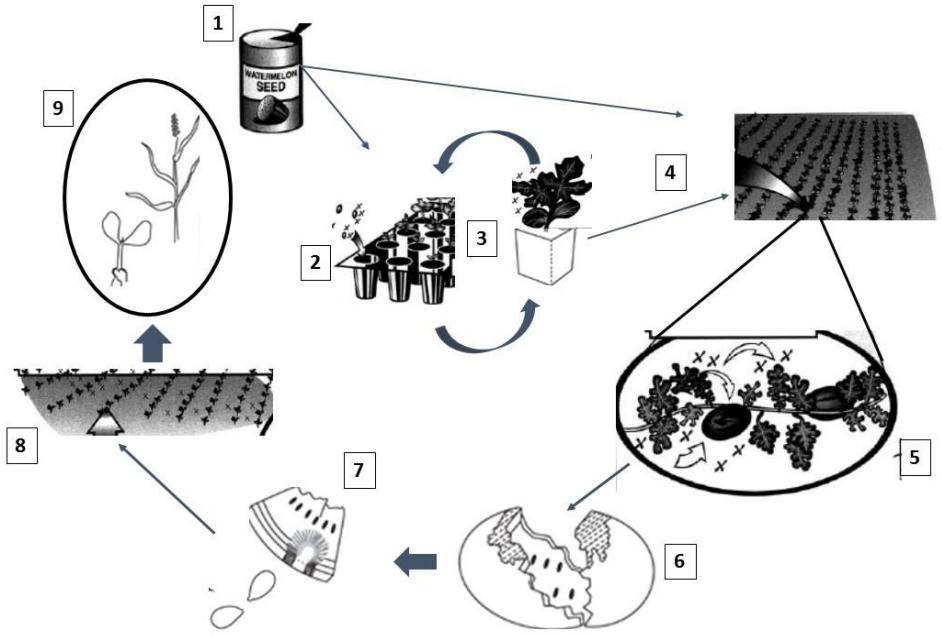

Figure 1 Disease cycle of bacterial fruit blotch. Steps 1 through 9 illustrate the cycle from the pathogen`s introduction with contaminated seeds to its potential survival in local fields.
Adapted from Latin and Hopkins (1995).

1.2.3 Biology of Seed Infection

Contaminated seed lots are the primary source of inoculum for BFB outbreaks in the field and transplant houses (ASSOULINE et al., 1997; BAHAR; BURDMAN, 2010; DUTTA et al., 2012a; LATIN; HOPKINS, 1995; WALCOTT; FESSEHAIE; CASTRO, 2004). However, the interactions between *A. citrulli* and the seed during the early stages of germination is poorly understood. Although Rane and Latin (1992) reported that symptomless fruits produced non-infested seeds when artificially inoculated, it is unlikely that seeds produced in symptomatic fruits account for natural commercial seed infection. To investigate how seeds become infected, Walcott, Gitaitis and Castro (2003) demonstrated the role of blossoms in watermelon seed infestation. This study showed that pollination and inoculation of female watermelon blossoms led to seed infection within symptomless fruits. Seeds produced in this manner transmitted BFB to seedlings once planted. In agreement with these findings, Bahar, Kritzman and Burdman (2009) reported that, under unfavorable conditions for BFB development, over 50 % of seeds within symptomless melon fruits were infested with *A. citrulli*.

Acidovorax citrulli rapidly colonizes watermelon stigmas, and there is a strong linear relationship between blossom inoculum dose and seed infection (LESSL; FESSEHAIE; WALCOTT, 2007). It has also been shown that the bacterium penetrates through the style and enters the ovary by 24 h after pollination (DUTTA et al., 2012a). Additionally, in seeds infested via blossom inoculation, *A. citrulli* cells are deposited deep inside the seeds (under the perisperm-endosperm layer), when compared with seeds that are infested within symptomatic fruits (bacteria just under the seed coat). These findings indicate that blossom invasion by *A. citrulli* can lead to seed infection, even in the absence of BFB fruit symptoms. Despite the relevance of these studies, the epidemiological

significance of blossom invasion in seed infection remains to be determined under seed production fields conditions (BURDMAN; WALCOTT, 2012).

1.2.4 Diagnosis and control of Bacterial Fruit Blotch

Currently, there are no commercial cucurbit cultivars with resistance to BFB. Therefore, effective BFB management requires the integration of a range of approaches in seed, transplant and fruit production. As seeds are the most important source of primary inoculum and the main vehicle for long distances dissemination of *A. citrulli*, efforts to prevent seed infection are critical (RANE; LATIN, 1992). Cucurbit seeds are generally produced in regions of cool dry climate or during the dry periods of the year, which is unfavorable for the BFB development (BAHAR; BURDMAN, 2010). Additionally, only *A. citrulli*-free stock seeds should be used for commercial seed production and seed producing fields visually inspected for fruit and foliar BFB symptoms. Seed producing fields once presenting symptomatic plants, must not be used for seed production (LATIN; HOPKINS, 1995).

A variety of chemical compounds have been suggested for the seed treatment of cucurbit crops; however, they have shown a limited efficacy against *A. citrulli*. The factors that influence the effectiveness of seed treatments include: i) the ability of seed treatments to penetrate the seed coat; and ii) the location of the bacteria (on the seed surface or deep inside) (BURDMAN; WALCOTT, 2012). Although seed treatments, including streptomycin sulphate and NaOCl, have been reported to reduce BFB seedling transmission, they generally fail to eradicate the bacterium from within the seed (DUTTA et al., 2012b). Other seed treatments have also been proposed, such as exposure to chlorine gas (STEPHENS et al., 2008) and acidic electrolyzed water (FENG et al., 2009a). However, moderately effective, once the bacteria is located inside the seed.

Since BFB tolerance in seedling transplant houses is zero, seed health testing is critical for disease management. Seeds infested/infected with *A. citrulli*, as the majority of seed-transmitted diseases, do not show any visible symptoms. Therefore, seeds need to be inspected using both traditional and molecular tests, for instance, agar plating on semiselective media, seedling grow-out assay, serology and PCR-based techniques (FENG et al., 2013). For *A. citrulli* detection, several semiselective media have been developed, including ethanol bromocresol purple/brilliant blue R (EBB) (ZHAO et al., 2009). Generally, antibiotics such as ampicillin and kanamycin are employed to limit growth of seed saprophytes. Quality assurance protocols are often based on the isolation of bacteria from seed or plant extracts by culturing on semiselective media, followed by physiological, biochemical and pathogenicity tests for final confirmation (BURDMAN et al., 2005).

The seedling grow-out test is a widely used technique for detecting *A. citrulli* in seeds under conducive conditions (21 to 35°C and 55 to 90% of relative humidity) (BURDMAN; WALCOTT, 2012). Negative and positive controls for this assay include uninfected and artificially infested seed samples, respectively, to ensure that the environmental conditions are favorable for BFB symptoms development. Seedlings are visually inspected daily for 18-25 days after planting for water-soaked lesions along the veins of cotyledons and leaves. Subsequently, confirmatory laboratory tests are performed on suspect seedlings, as needed (ZHAO et al., 2009).

Serology-based assays, originally developed to detect viruses, have been used for *A. citrulli* detection. Commercial ELISA (enzyme-linked immunosorbance assay) kits are available for *A. citrulli* (Agdia, Elkhart, IN and ADGEN, Ayr, Scotland), and polyclonal and monoclonal antisera have also been developed and used in numerous protocols (TIAN et al., 2013; WALCOTT et al., 2006; WALCOTT; GITAITIS, 2000). However, serology-based techniques should only be used as preliminary steps, since false-positive and negative results may

occur. Moreover, the detection threshold given by serology generally does not meet the requirements for *A. citrulli* detection in seeds (WALCOTT et al., 2006).

Ever since the PCR development, DNA-based techniques have rapidly become the preferred tool for detection and identification of plant pathogenic bacteria. PCR offers many advantages over traditional isolation and serological methods, for example, high levels of specificity and sensibility in a timely fashion way (BAHAR et al., 2008; FENG et al., 2013; HA et al., 2009; TIAN et al., 2013; WALCOTT; GITAITIS, 2000). Although PCR is widely employed for detection of plant pathogens, it cannot distinguish between viable and non-viable cells (ZHAO et al., 2009). The DNA extracted from non-viable cells in natural conditions or from dead cells still serve as template for PCR amplification. As an alternative, by allowing the target organism to grow in liquid or solid media prior to PCR detection, BIO-PCR has been a powerful tool for a cost-effective *A. citrulli* detection (SCHAAD et al., 1995; ZHAO et al., 2009).

As a prophylactic measure for the BFB management in the field, cooper-based chemicals are commonly used combined or not with resistance inducing compounds (CABRAL et al., 2010; SALES JUNIOR et al., 2007). Cooper-based treatments when applied weekly, starting from the early stages of flowering until fruit maturity, have shown significant reduction of BFB incidence (BAHAR; BURDMAN, 2010; SALES JUNIOR et al., 2007). However, the excessive use of cooper can lead to the selection of resistant population and environment contamination.

A promising strategy for plant diseases management is the use of resistant cultivars. Unfortunately, screening cucurbitaceous cultivars for BFB resistance is a very difficult task. Several reasons can lead to unsuccessful results. Primarily, the pathogen can attack the plant at different stages of growth. Secondly, the genetic and pathogenic variability of *A. citrulli* is high, and therefore a specific strain used on cultivar screening may not induce the same response that others. Finally, BFB

is greatly affected by environmental conditions and, consequently results from field assays may differ between seasons (LATIN; HOPKINS, 1995).

An alternative for BFB management is the use of biocontrol agents. Medeiros et al. (2009), studied the ability of plant growth-promoting rhizobacteria to protect melon seeds and leaves from BFB and the *in vitro* antibiosis against *A. citrulli*. The biocontrol agents showed antibiosis activity against the pathogen and a significant reduction on disease progress when spray-inoculated or seed-treated. The efficiency of biological control agents on seed treatment and watermelon blossoms protection was accessed by Fessehaie and Walcott (2005). Seeds treated with the beneficial agents showed a significant reduction on BFB transmission under greenhouse conditions. Additionally, watermelon blossom protection reduced the seed infection by *A. citrulli*. Recently, melon seedlings treated with antagonistic yeasts showed a protective effect against *A. citrulli* by inducing defense responses (CONCEIÇÃO et al., 2014). These findings reinforce the use of biological control as a sustainable alternative for the integrated management of BFB.

1.2.5 Genetic and phytopathogenic variability of *Acidovorax citrulli*

Acidovorax citrulli strains were initially thought to comprise a homogeneous population and, one reason is that most of the initial BFB outbreaks in Georgia, US were limited to watermelon seedlings (SCHAAD et al., 1978). However, with the BFB outbreaks in Florida, US in the late 1980s, it became evident that there was a genetic variability amongst *A. citrulli* strains. Supporting this observation, Somodi et al. (1991) reported that the *A. citrulli* strains recovered from the BFB outbreaks in Florida were related to the original strains recovered from Georgia, but they differed on their ability to induce HR on tobacco. More specifically, the original strains recovered from Georgia failed to induce positive reactions, while the Florida strains did not.

Subsequently, O'Brien and Martin (1999) observed that *A. citrulli* strains recovered from melons in North Queensland Australia were more aggressive on rockmelon, than strains recovered from watermelons in South Queensland. Strains from North and South Queensland Australia could also be distinguished based on substrate utilization profiles. More specifically, *A. citrulli* strains from watermelon plants in south Queensland utilized L-leucine while strain from melon in north Queensland did not. The Australian strains were further characterized as group I *A. citrulli* strains by Walcott et al. (2000) in an independent study.

Using pulse field gel electrophoresis (PFGE) DNA fingerprinting and gas chromatography-fatty acid methyl ester (GC-FAME) to analyze a global population of 121 *A. citrulli* strains, from a range of cucurbit hosts, Walcott et al. (2000) provided evidence for two genetically and physiologically distinct groups of *A. citrulli*. Group I strains included the American Type Culture Collection *A. citrulli* strain (ATCC type strain), and strains that were mainly isolated from non-watermelon cucurbit hosts. In contrast, group II strains were mainly isolated from watermelon. In a subsequent study, Walcott, Fessehaie and Castro (2004) confirmed the existence of two genetically distinct groups by repetitive PCR. In addition, they confirmed that the group I strains were moderately aggressive on a range of cucurbit hosts. On the other hand, group II strains, isolated primarily from watermelon, were highly aggressive on watermelon and mildly aggressive on nonwatermelon cucurbit hosts.

At present, yield losses attributed to BFB in Brazil are more significant for melon than watermelon, since the occurrence of BFB outbreaks on watermelon are sporadic (CONCEIÇÃO et al., 2014). Variability among Brazilian *A. citrulli* populations have been reported with regards to physiological and biochemical properties (OLIVEIRA et al., 2007), aggressiveness on different cucurbit seedlings and genetic relatedness by rep-PCR (MELO et al., 2014). These studies were conducted primarily with Brazilian strains and did not report the genetic relatedness of Brazilian population in accordance with the two well-documented

and genetically distinct groups of *A. citrulli* (ECKSHTAIN-LEVI et al., 2014; FENG et al., 2009b; YAN et al., 2013; WALCOTT; FESSEHAIE; CASTRO, 2004). Considering that contaminated seeds are still globally traded, a more detailed study on genetic diversity that allow a comprehensive analysis of the Brazilian population structure in the global scenario is needed.

1.2.6 Pathogenicity and virulence factors

Despite the fact that BFB is an economically important disease of cucurbit crops worldwide, the basic aspects of biology and pathogenesis of *A.citrulli* are still scarce. The genome sequences of the group II *A. citrulli* strain, AAC00-1, released in 2007 by the Joint Genome Institue (JGI; GenbankNC_008752) and more recently, other group I strains (pslb65 and TW6; Genbank accession numbers JYHM01000000 and JXDJ01000000 respectively) will allow a more detailed investigation of the molecular basis of BFB pathogenesis.

The AAC00-1 genome comprises a single circular chromosome of 5.3 Mb, putatively encoding 4858 genes. The group I *A. citrulli* strains pslb65 and TW6 have 4.9 Mb and 5.0 Mb of genome size, respectively (WANG et al., 2015; WANG; YANG; ZHAO, 2015). Comparative studies between the group I and II *A. citrulli* genomes, combined with suitable experimental approaches, will provide the identification of the genetic factors that influence virulence and govern the host preferences of the two *A. citrulli* groups, and thus, enlarge the current knowledge of *A. citrulli* pathogenesis.

1.2.6.1 Type III secreted effectors

Many Gram-negative plant-pathogenic bacteria utilize a functional type III secretion system (T3SS) to secrete protein effectors directly into the host cell (MANSFIELD, 2009). The genes encoding the type III secretion apparatus are

named hypersensitive response and pathogenicity (*hrp*) genes, since they are utilized for pathogenicity and hypersensitive response (HR) induction in susceptible and resistant plants respectively (ALFANO; COLLMER, 2004). The *hrp* genes are generally located in large clusters (20-to-25 Kb). On the basis of gene organization, sequence analysis and regulation, *hrp* gene clusters are divided into two classes: class I contains the clusters of *Pseudomonas syringae* and enteric plant-pathogenic bacteria, while class II contains the *hrp* clusters of *Xanthomonas* species and *Ralstonia solanacearum* (BÜTTNER; BONAS, 2002).

The genome sequence of the *A. citrulli* group II strain AAC00-1 revealed the existence of a class II Hrp-T3SS. Mutagenesis analysis showed that *A. citrulli* requires a functional T3SS to infect cucurbit plants and to elicit HR in tomato and tobacco plants (BAHAR; BURDMAN, 2010; JOHNSON et al., 2011). The presence of at least 11 putative type III secreted effector proteins have been confirmed in the *A. citrulli* genome, based on their homology to other plant-pathogenic bacterial genes, more specifically *Xanthomonas* spp and *Ralstonia solanacearum*. In addition, comparative analysis of the 11 effector genes from a collection of *A. citrulli* strains, revealed that group I and II strains differ on their effector repertoire. This data supports the idea that these effector genes were acquired by horizontal gene transfer and may influence the host preference association of *A. citrulli* strains (ECKSHTAIN-LEVI et al., 2014). The functional analysis of *A. citrulli* effectors has not been published to date.

1.2.6.2 Polar flagellum

Flagella are filamentous protein structures commonly found on the surface of many bacteria (MACNAB, 2003). These structures mediate bacterial motility and are involved in various processes, such as adhesion to and colonization of biotic and abiotic surfaces, and virulence in animal or plant hosts (DUAN et al., 2013). Using a random mutagenesis approach combined with virulence screens of

A. citrulli transposon library, a mutant impaired in *fliR* gene displayed reduced virulence in seed transmission assays. The *fliR* gene encodes a flagellar biosynthetic protein which is involved in flagellin secretion. Further analysis of an *A. citrulli* mutant impaired in *fliC*, which encodes flagellin, confirmed the role of polar flagellum as a virulence factor of *A. citrulli*. Specifically, the *A. citrulli fliC* mutant was less virulent than its wild-type in seed transmission assays and when inoculated in seedlings stem and foliage BAHAR; LEVI; BURDMAN, 2011).

1.2.6.3 Quorum sensing

Quorum sensing (QS) is a mechanism of cell-to-cell communication used by bacteria to coordinate the gene expression and behavior in a density-dependent manner (WATERS; BASSLER, 2005). In QS, bacterial cells respond to a stimulatory concentration of extracellular autoinducers. Gram-negative bacteria produce acylated homoserine lactones (AHLs) as autoinducers (BASSLER, 1999; MILLER; BASSLER, 2001). The role of QS in the virulence of plant-pathogenic bacteria has been extensively investigated in several species, including *Rhizobium radiobacter* (formerly known *Agrobacterium tumefaciens*), *Pseudomonas syringae*, *Pantoea stewartii*, *Pectobacterium sp*., *Ralstonia solanacearum*, *Xylella fastidiosa* and *Xanthomonas sp*. (BODMAN; BAUER; COPLIN, 2003; CHATTERJEE; WISTROM; LINDOW, 2008; VENTURI, 2006). Among the traits the have been reported to be regulated by QS, are included the production of extracellular polysaccharides, degradative enzymes, siderophores, antibiotics, biofilm formation and epiphytic fitness. As QS is generally associated with pathogenesis, it becomes a target for the development of strategies for plant disease control (BODMAN; BAUER; COPLIN, 2003).

Acidovorax citrulli genome (AAC00-1; Genbank NC_008752) contains homologous *luxI* and *luxR* genes, putatively encoding the AHL synthase and the AHL-dependent transcriptional protein respectively. Mutagenesis analysis

generated a mutant *A. citrulli* strain impaired in the *luxI* homologous gene (FAN et al., 2011). This mutant showed reduced virulence on watermelon fruits and melon seedlings compared to the wild-type strain. The authors reported that the autoinducer molecule of *A. citrulli* is N-3-oxo-octanoyl-L-homoserine Lactone (3-oxo-C8-HSL) and that the mutant was unable to produce it.

Investigating the role of QS in watermelon seed colonization and seed-to-seedling transmission, Johnson and Walcott (2013) showed that *aacR* (*luxR*) and *aacI* (*luxI*) mutants of AAC00-1 colonized watermelon seeds at wild-type level; however seed-to-seedling transmission was affected in a cell density-dependent manner. When seed inoculum was reduced from 10^6 to 10^3 CFU/seed, the seed-to-seedling transmission of *aacI* mutant was significantly reduced. In contrast, BFB seed-to-seedling transmission for the *aacR* mutant was significantly the same as the wild-type strain. Further exploration of QS in seed-to-seedling transmission could enhance disease management.

1.3 Multilocus sequence analysis

Multilocus sequence analysis (MLSA) is currently a widely used genotypic method for phylogenetic classification and diversity analysis of prokaryotic taxa (GLAESER; KAMPFER, 2015). MLSA is based on multilocus sequence typing (MLST), which was first introduced by Maiden et al. (1998). MLST is derived from the concept of Multi-locus enzyme electrophoresis (MLEE), a molecular typing method applied to populations and epidemiological studies of bacterial species (URWIN; MAIDEN, 2003). For MLST, a portion of a coding-protein gene (5 to 10 genes) is sequenced and the differing sequences are assigned an allele number; each strain is characterized (for *n* loci) and represented by a set of *n* numbers defining the alleles at each locus. Organisms with identical alleles in each sequenced locus, are given the same allele profile designation referred to as sequence type (ST) (MAIDEN et al., 1998). The term MLSA (Multilocus sequence

analysis) is used when instead of STs, the concatenated set of obtained DNA sequence is used for analysis (DIGGLE; CLARKE, 2002).

A critical point for MLSA is the selection of genes. Housekeeping genes coding for proteins with essential functions should be considered because they are conserved with regard to rapid genetic modifications (URWIN; MAIDEN, 2003). This technique indexes nucleotide sequence of fragments of housekeeping genes, typically seven in number and each of ~ 450 bp in length (DIDELOT; MAIDEN, 2010). Genes often analyzed are those coding for subunits of ubiquitous enzymes, such as the β-subunit of DNA gyrase (*gyrB*), the β-subunit of RNA polymerase (*rpoB*), the sigma 70 (Sigma D) factor of RNA polymerase (*rpoD*), recombinase A (*recA*), the β-subunit of ATP synthase (*atpD*) and translation initiation factor (*infB*) (BRADY et al., 2008; DIGGLE; CLARKE, 2002; FENG et al., 2009b; PARKER; HAVIRD; DE LA FUENTE, 2012).

MLSA has deciphered the phylogenetic relationship of numerous plant pathogenic bacteria (BRADY et al., 2008; FENG et al., 2009b; JACQUES et al., 2012; PARKER; HAVIRD; DE LA FUENTE, 2012) and, has also shown to be a robust tool for accessing relationships within complex species, such as *Agrobacterium tumefaciens* (AUJOULAT et al., 2011) specie complex and *Ralstonia solanacearum* (WICKER et al., 2012). At present, there are more than 50 MLSA schemes available in public databases via the Internet, that can be easily replicated between laboratories (GLAESER; KAMPFER, 2015). In addition, comparisons at public databases can be made with prior data.

1.4 Factors affecting survival of plant pathogens

The survival of plant pathogens between cropping seasons and its effective dispersal to non-infected plants are crucial aspects of the plant disease cycle (AGRIOS, 2005). Most pathogens possess mechanisms to survive intercrop periods or periods of unfavorable environmental conditions (DEAN et al., 2012).

Phytopathogenic bacteria often survive by associating with seeds, infected plant organs, crop debris and epi- or endophytically in crops, volunteer plants or weeds (SCHUSTER; COYNE, 1974). Bacterial survival is affected by multiple factors, such as temperature, humidity, pH, ultraviolet radiation and antagonistic interactions with the resident microbiota (SUNDIN; JACOBS, 1999).

Plant pathogenic bacteria are not adapted to survive as free cells in the soil for long periods of time, since they do not form endospores and are not as competitors as the soil microbiota. Moreover, as the organic matter increases, the microbiota activity accelerates the production of antibiotic compounds (KOCKS et al., 1998). In field conditions, under mild temperatures (27 to 30°C) and low rainfall (< 5 mm), *Xanthomonas axonopodis* pv. *phaseoli* survived for 65 to 180 days in common bean leaflets on the soil surface, and for 30 to 120 days on those incorporated in the soil at 15 cm of depth. When higher temperatures (30 to 35°C) and rainfall (30 mm) occurred, the survival was from 45 to 60 days on leaflets on the soil surface and from 30 to 45 days (TORRES; MARINGONI; SILVA JUNIOR, 2009).

Acidovorax citrulli may survive/overwinter citron melon (cucurbitaceous weed) seeds for at least 7 years (DUTTA et al., 2014b). Nascimento, Mariano and Silva (2004) observed that tomato plants and cucurbitaceous weeds can serve as alternative hosts for *A.citrulli* in the field. These studies highlight the importance of crop rotation recommendations and eradication of volunteer seedlings and weeds that serve as a potential source of inoculum. Studies covering *A. citrulli* ecology and the role of alternative hosts and volunteer plants on BFB epidemiology are still lacking.

REFERENCES

AGRIOS, G. **Plant pathology**. 5th ed. San Diego: Academic, 2005. 922 p.

ALFANO, J. R.; COLLMER, A. Type III secretion system effector proteins: double agents in bacterial disease and plant defense. **Annual Review of Phytopathology**, Palo Alto, v. 42, p. 385-414, 2004.

ASSIS, S. M. P. et al. Mancha aquosa do melão causada por *Acidovorax avenae* subsp. *citrulli*, no Estado do Rio Grande do Norte. **Fitopatologia Brasileira**, Brasília, v. 24, n. 2, p. 191, 1999.

ASSOULINE, I. et al. *Acidovorax avenae* subsp *citrulli* transmitted by solanaceous seeds. **Pytoparasitica**, Heidelbergh, v. 25, n. 2, p. 117-118, 1997.

AUJOULAT, F. et al. Multilocus sequence-based analysis delineates a clonal population of *Agrobacterium (Rhizobium) radiobacter (Agrobacterium tumefaciens)* of human origin. **Journal of Bacteriology**, Washington, v. 193, n. 10, p. 2608-2618, Mar. 2011.

BAHAR, O.; BURDMAN, S. Bacterial fruit blotch: a threat to the cucurbit industry. **Israel Journal of Plant Sciences**, Tel Aviv, v. 58, n. 1, p. 19-31, Apr. 2010.

BAHAR, O. et al. New subspecies-specific polymerase chain reaction-based assay for the detection of *Acidovorax avenae* subsp. *citrulli*. **Plant Pathology**, Honolulu, v. 57, n. 4, p. 754-763, Aug. 2011.

BAHAR, O.; KRITZMAN, G.; BURDMAN, S. Bacterial fruit blotch of melon: screens for disease tolerance and role of seed transmission in pathogenicity. **European Journal of Plant Pathology**, Dordrecht, v. 123, n. 1, p. 71-83, July 2009.

BAHAR, O.; LEVI, N.; BURDMAN, S. The cucurbit pathogenic bacterium *Acidovorax citrulli* requires a polar flagellum for full virulence before and after host-tissue penetration. **Molecular Plant-Microbe Interactions**, Saint Paul, v. 24, n. 9, p. 1040-1050, Sept. 2011.

BASSLER, B. L. How bacteria talk to each other: regulation of gene expression by quorum sensing. **Current Opinion in Microbiology**, Oxford, v. 2, p. 582-587, 1999.

BODMAN, S. B. von; BAUER, W. D.; COPLIN, D. L. Quorum sensing in plant-

pathogenic bacteria. **Annual Review of Phytopathology**, Palo Alto, v. 41, p. 455-482, 2003.

BRADY, C. et al. Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). **Systematic and Applied Microbiology**, Stuttgart, v. 31, n. 6, p. 447-460, Dec. 2008.

BURDMAN, S. et al. Molecular, physiological, and host-range characterization of *Acidovorax avenae* subsp . *citrulli* isolates from Watermelon and Melon in Israel. **Plant Disease**, Quebec, v. 89, n. 12, p. 1339-1347, Dec. 2005.

BURDMAN, S.; WALCOTT, R. Pathogen profile *Acidovorax citrulli*: generating basic and applied knowledge to tackle a global threat to the cucurbit industry. **Molecular Plant Pathology**, London, v. 13, n. 8, p. 805-815, Oct. 2012.

BÜTTNER, D.; BONAS, U. Getting across--bacterial type III effector proteins on their way to the plant cell. **The EMBO Journal**, Oxford, v. 21, n. 20, p. 5313-5322, Oct. 2002.

CABRAL, C. P. et al. Effect of acibenzolar-S-methyl, mannano-oligosaccharides and citrus bioflavonoids on the control of bacterial fruit blotch and growth of melon plant. **Tropical Plant Pathology**, Brasília, v. 35, n. 2, p. 119-123, Apr. 2010.

CHALUPOWICZ, L. et al. Cotyledons are the main source of secondary spread of *Acidovorax citrulli* in melon nurseries. **Plant Pathology**, Honolulu, v. 64, n. 3, p. 528-536, June 2015.

CHATTERJEE, S.; WISTROM, C.; LINDOW, S. E. A cell-cell signaling sensor is required for virulence and insect transmission of *Xylella fastidiosa*. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 105, n. 7, p. 2670-2675, Feb. 2008.

CONCEIÇÃO, C. S. et al. Combined effect of yeast and silicon on the control of bacterial fruit blotch in melon. **Scientia Horticulturae**, Amsterdam, v. 174, n. 1, p. 164-170, July 2014.

DEAN, R. et al. The Top 10 fungal pathogens in molecular plant pathology. **Molecular Plant Pathology**, London, v. 13, n. 4, p. 414-430, May 2012.

DIDELOT, X.; MAIDEN, M. C. J. Impact of recombination on bacterial evolution. **Trends in Microbiology**, Cambridge, v. 18, n. 7, p. 315-322, July 2010.

DIGGLE, M. A.; CLARKE, S. C. Rapid assignment of nucleotide sequence data to allele types for multi-locus sequence analysis (MLSA) of bacteria using an adapted database and modified alignment program. **Journal of Molecular Microbiology and Biotechnology**, Basel, v. 4, n. 6, p. 515-517, Nov. 2002.

DUAN, Q. et al. Flagella and bacterial pathogenicity. **Journal of Basic Microbiology**, Malden, v. 53, n. 1, p. 1-8, Jan. 2013.

DUTTA, B. et al. *Acidovorax citrulli* seed inoculum load affects seedling transmission and spread of bacterial fruit blotch of watermelon under greenhouse conditions. **Plant Disease**, Quebec, v. 96, n. 5, p. 705-711, May 2012a.

DUTTA, B. et al. Interactions of seedborne bacterial pathogens with host and non-host plants in relation to seed infestation and seedling transmission. **PLoS ONE**, San Francisco, v. 9, n. 6, p. e99215, June 2014a.

DUTTA, B. et al. Location of *Acidovorax citrulli* in infested watermelon seeds is influenced by the pathway of bacterial invasion. **Phytopathology**, Saint Paul, v. 102, n. 5, p. 461-468, May 2012b.

DUTTA, B. et al. Long-term survival of *Acidovorax citrulli* in citron melon (*Citrullus lanatus* var. *citroides*) seeds. **Plant Pathology**, Honolulu, v. 63, n. 5, p. 1130-1137, Jan. 2014b.

ECKSHTAIN-LEVI, N. et al. Comparative analysis of type III secreted effector genes reflects divergence of *Acidovorax citrulli* strains into three distinct lineages. **Phytopathology**, Saint Paul, v. 104, n. 11, p. 1152-1162, Nov. 2014.

FAN, J. et al. The acyl-homoserine lactone (AHL)-type quorum sensing system affects growth rate, swimming motility and virulence in *Acidovorax avenae* subsp. *citrulli*. **World Journal of Microbiology and Biotechnology**, Oxford, v. 27, n. 5, p. 1155-1166, May 2011.

FENG, J. et al. Evaluation of seed treatments for the eradication of *Acidovorax avenae* subsp *citrulli* from melon and watermelon seeds. **Canadian Journal of Plant Pathology**, Ottawa, v. 31, n. 2, p. 180-185, Apr. 2009a.

FENG, J. et al. Multilocus sequence typing reveals two evolutionary lineages of *Acidovorax avenae* subsp. *citrulli*. **Phytopathology**, Saint Paul, v. 99, n. 8, p. 913-920, Aug. 2009b.

FENG, J. J. et al. Advances in detection of *Acidovorax citrulli*, the causal agent of

bacterial fruit blotch of cucurbits. **Seed Science and Technology**, Bassersdorf, v. 41, n. 1, p. 1-15, Jan. 2013.

FESSEHAIE, A.; WALCOTT, R. R. Biological control to protect watermelon blossoms and seed from infection by *Acidovorax avenae* subsp. *citrulli*. **Phytopathology**, Saint Paul, v. 95, n. 4, p. 413-419, Apr. 2005.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS. Available from: <http://faostat.fao.org/>. Accessed: Feb. 15, 2016.

FRANKLE, W. G.; HOPKINS, D. L.; STALL, R. E. Ingress of the watermelon fruit blotch bacterium into fruit. **Plant Disease**, Quebec, v. 77, p. 1090-1092, 1993.

GITAITIS, R.; WALCOTT, R. The epidemiology and management of seedborne bacterial diseases. **Annual Review of Phytopathology**, Palo Alto, v. 45, p. 371-397, 2007.

GLAESER, S. P.; KAMPFER, P. Multilocus sequence analysis (MLSA) in prokaryotic taxonomy. **Systematic and Applied Microbiology**, Stuttgart, v. 38, n. 4, p. 237-245, June 2015.

HA, Y. et al. Simultaneous detection of *Acidovorax avenae* subsp. *citrulli* and *Didymella bryoniae* in cucurbit seedlots using magnetic capture hybridization and real-time polymerase chain reaction. **Phytopathology**, Saint Paul, v. 99, n. 6, p. 666-678, June 2009.

HALFELD-VIEIRA, B. A.; NECHET, K. L. Mancha-aquosa da melancia em Roraima. **Fitopatologia Brasileira**, Brasília, v. 32, n. 4, p. 26, jun. 2007.

INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA. 2012. Available from: <http://www.ibge.gov.br/home/estatistica/economia/pam/2012/PAM2012_Publicacao_compta.pdf>. Accessed: Sept. 12, 2015.

JACQUES, M. A. et al. Phylogenetic analysis and polyphasic characterization of *Clavibacter michiganensis* strains isolated from tomato seeds reveal that nonpathogenic strains are distinct from *C. michiganensis* subsp. *michiganensis*. **Applied and Environmental Microbiology**, Washington, v. 78, n. 23, p. 8388-8402, Dec. 2012.

JEFFREY, C. A new system of Cucurbitaceae. **Botaniceskii Zhurnal**, Moscow, v. 90, n. 3, p. 332-335, 2005.

JOHNSON, K. L. et al. Efficacy of a nonpathogenic *Acidovorax citrulli* strain as a biocontrol seed treatment for bacterial fruit blotch of cucurbits. **Plant Disease**, Quebec, v. 95, n. 6, p. 697-704, June 2012.

JOHNSON, K. L.; WALCOTT, R. R. Quorum sensing contributes to seed-to-seedling transmission of *Acidovorax citrulli* on watermelon. **Journal of Phytopathology**, Berlin, v. 161, n. 8, p. 562-573, Aug. 2013.

KOCKS, C. G. et al. Survival and Extinction of *Xanthomonas campestris* pv *campestris* in soil. **European Journal of Plant Pathology**, Dordrecht, v. 104, p. 911-923, 1998.

LANGSTON, D. et al. First report of a fruit rot of pumpkin caused by *Acidivorax avenae* subsp. *citrulli* in Georgia. **Plant Disease**, Quebec, v. 83, n. 2, p. 199-219, Feb. 1999.

LATIN, R. X.; HOPKINS, C. L. Bacterial fruit blotch of watermelon, the hypothetical exam question becomes reality. **Plant Disease**, Quebec, v. 79, p. 761-765, 1995.

LESSL, J. T.; FESSEHAIE, A.; WALCOTT, R. R. Colonization of female watermelon blossoms by *Acidovorax avenae* ssp. *citrulli* and the relationship between blossom inoculum dosage and seed infestation. **Journal of Phytopathology**, Berlin, v. 155, n. 2, p. 114-121, Feb. 2007.

MACAGNAN, D. et al. Mancha bacteriana da melancia: uma nova bacteriose no Estado de Minas Gerais. **Summa Phytopathologica**, Jaguariúna, v. 29, n. 3, p. 286-287, nov. 2003.

MACNAB, R. M. How bacteria assemble flagella. **Annual Review of Microbiology**, Palo Alto, v. 57, p. 77-100, 2003.

MAIDEN, M. C. et al. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. **Proceedings of the National Academy of Sciences of the United States of America**, Washington, v. 95, p. 3140-3145, 1998.

MANSFIELD, J. W. From bacterial avirulence genes to effector functions via the hrp delivery system: An overview of 25 years of progress in our understanding of plant innate immunity. **Molecular Plant Pathology**, London, v. 10, n. 6, p. 721-734, Nov. 2009.

MARIANO, R. L. R. et al. Ocorrência da mancha-aquosa do melão na Bahia. **Fitopatologia Brasileira**, Brasília, v. 29, n. 5, p. 147-148, ago. 2004.

MARIANO, R. L. R.; SILVEIRA, E. B. Mancha-aquosa: Importante bacteriose do meloeiro no Brasil. **Anais da Academia Pernambucana de Ciência Agronômica**, Recife, v. 1, n. 1, p. 79-88, jan. 2004.

MEDEIROS, F. H. V. et al. Management of melon bacterial blotch by plant beneficial bacteria. **Phytoparasitica**, Bet Dagan, v. 37, n. 5, p. 453-460, Nov. 2009.

MELO, L. A. et al. Comparing *Acidovorax citrulli* strains from melon and watermelon: phenotypic characteristics, pathogenicity and genetic diversity. **Tropical Plant Pathology**, Brasília, v. 39, n. 2, p. 154-162, mar. 2014.

MILLER, M. B.; BASSLER, B. L. Quorum sensing in bacteria. **Annual Review of Microbiology**, Palo Alto, v. 55, p. 165-199, 2001.

NASCIMENTO, A. R. P.; MARIANO, R. L. R.; SILVA, E. I. Hospedeiros alternativos de *Acidovorax avenae* subsp. *citrulli*. **Horticultura Brasileira**, Brasília, v. 22, n. 3, p. 345-349, set. 2004.

O'BRIEN, R. G.; MARTIN, H. L. Bacterial blotch of melons caused by strains of *Acidovorax avenae* subsp. *citrulli*. **Australian Journal of Experimental Agriculture**, Melbourne, v. 39, p. 479-485, 1999.

OLIVEIRA, J. C. et al. Caracterização de isolados de *Acidovorax avenae* subsp. *citrulli*. **Fitopatologia Brasileira**, Brasília, v. 32, n. 6, p. 480-487, dez. 2007.

PARKER, J. K.; HAVIRD, J. C.; DE LA FUENTE, L. Differentiation of *Xylella fastidiosa* strains via multilocus sequence analysis of environmentally mediated genes (MLSA-E). **Applied and Environmental Microbiology**, Washington, v. 78, n. 5, p. 1385-1396, Mar. 2012.

RANE, K.; LATIN, R. Bacterial fruit blotch of watermelon: association of the pathogen with seed. **Plant Disease**, Quebec, v. 76, p. 509-512, 1992.

RIBEIRO, M. F. et al. Honey bees (*Apis mellifera*) visiting flowers of yellow melon (*Cucumis melo*) using different number of hives. **Ciência Rural**, Santa Maria, v. 45, n. 10, p. 1768-1773, out. 2015.

ROBBS, C. F. et al. Mancha bacteriana da melancia no estado de São Paulo, causada por *Pseudomonas pseudoalcaligenes* subsp. *citrulli*. **Fitopatologia Brasileira**, Brasília, v. 16, n. 2, p. 48, 1991. Abstract.

ROMÃO, R. L. Northeast Brazil: a secundary center of diversity for watermelon. **Genetic Resources and Crop Evolution**, Dordrecht, v. 47, n. 2, p. 207-213, Apr. 2000.

SALES JUNIOR, R. et al. Eficiência de Acibenzolar-S-Methyl e Oxicloreto de Cobre no controle de *Acidovorax avenae* subsp . *citrulli* , agente causal da "Mancha-Aquosa" no meloeiro. **Revista de Biologia e Ciências da Terra**, Campina Grande, v. 7, n. 1, p. 66-70, jul. 2007.

SANTOS, A. A.; VIANA, F. M. **Mancha-aquosa do melão**. Fortaleza: EMBRAPA-SPI, 2000. 12 p.

SCHAAD, N.; POSTNIKOVA, E.; RANDHAWA, P. Emergence of *Acidovorax avenae* subsp *citrulli* as a crop threatening disease of watermelon and melon. In: _____. **Pseudomonas syringae and related pathogens:** biology and genetics. Heidelberg: Springer, 2003. p. 573-581.

SCHAAD, N. W. et al. A combined biological and enzymatic amplification (BIO-PCR) technique to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed extracts. **Phytopathology**, Saint Paul, v. 85, p. 243-248, 1995.

SCHAAD, N. W. et al. *Pseudomonas pseudoalcaligenes* subsp. *citrulli* subsp. nov. **International Journal of Systematic Bacteriology**, Reading, v. 28, p. 117-125, 1978.

SCHAAD, N. W. et al. Reclassification of subspecies of *Acidovorax avenae* as *A. avenae* (Manns 1905) emend.; *A. cattleyae* (Pavarino, 1911) comb. nov.; *A. citrulli* Schaad et al.; 1978) comb. nov.; and proposal of *A. oryzae* sp. nov. **Systematic and Applied Microbiology**, Stuttgart, v. 31, n. 8, p. 434-446, Dec. 2008.

SCHUSTER, M. L.; COYNE, D. P. Survival mechanisms of phytopathogenic bacteria. **Annual Review of Phytopathology**, Palo Alto, v. 12, p. 199-221, 1974.

SOMODI, G. C. et al. Occurrence of a bacterial watermelon fruit blotch in florida. **Plant Disease**, Quebec, v. 75, p. 1053-1056, 1991.

STEPHENS, D. et al. A procedure, based on exposure to chlorine gas, for disinfesting watermelon seeds. **Phytopathology**, Saint Paul, v. 98, p. S150-S151, 2008. Supplement.

SUNDIN, G. W.; JACOBS, J. L. Ultraviolet radiation (UVR) sensitivity analysis and UVR survival strategies of a bacterial community from the phyllosphere of field-grown peanut (*Arachis hypogeae* L.). **Microbial Ecology**, New York, v. 38, n. 1, p. 27-38, July 1999.

TIAN, Y. et al. Reliable and sensitive detection of *Acidovorax citrulli* in cucurbit seed using a Padlock-Probe-Based assay. **Plant Disease**, Quebec, v. 97, n. 7, p. 961-966, July 2013.

TORRES, J. P.; MARINGONI, A. C.; SILVA JUNIOR, T. A. F. Survival of *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* in common bean leaflets on soil. **Journal of Plant Pathology**, Wageningen, v. 91, n. 1, p. 195-198, Mar. 2009.

UENO, B.; COUTO, M. E. O.; UESUGUI, C. H. Ocorrência de Mancha-aquosa em melão no Estado do Rio Grande do Sul. **Fitopatologia Brasileira**, Brasília, v. 28, n. 3, p. S246, ago. 2003. Resumo.

URWIN, R.; MAIDEN, M. C. J. Multi-locus sequence typing: a tool for global epidemiology. **Trends in Microbiology**, Cambridge, v. 11, n. 10, p. 479-487, Oct. 2003.

VENTURI, V. Regulation of quorum sensing in *Pseudomonas*. **FEMS Microbiology Reviews**, Amsterdam, v. 30, n. 2, p. 274-291, Mar. 2006.

WALCOTT, R. R. et al. Investigating intraspecific variation of *Acidovorax avenae* subsp. *citrulli* using DNA fingerprinting and whole cell fatty acid analysis. **Phytopathology**, Saint Paul, v. 90, n. 2, p. 191-196, Feb. 2000.

WALCOTT, R. R. et al. Progress towards a commercial PCR-based seed assay for *Acidovorax avenae* subsp. *citrulli*. **Seed Science and Technology**, Bassersdorf, v. 34, n. 1, p. 101-116, Apr. 2006.

WALCOTT, R. R.; FESSEHAIE, A.; CASTRO, A. C. Differences in pathogenicity between two genetically distinct groups of *Acidovorax avenae* subsp. *citrulli* on cucurbit Hosts. **Journal of Phytopathology**, Berlin, v. 152, n. 5, p. 277-285, May 2004.

WALCOTT, R. R.; GITAITIS, R. D. Detection of *Acidovorax avenae* subsp. *citrulli* in watermelon seed using immunomagnetic separation and the polymerase chain reaction. **Plant Disease**, Quebec, v. 84, n. 4, p. 470-474, Apr. 2000.

WALCOTT, R. R.; GITAITIS, R. D.; CASTRO, A. C. Role of blossoms in

watermelon seed infestation by *Acidovorax avenae* subsp. *citrulli*. **Phytopathology**, Saint Paul, v. 93, n. 5, p. 528-534, May 2003.

WALL, G. C.; SANTOS, V. M. A new bacterial disease on watermelon in the Mariana Islands. **Phytopathology**, Saint Paul, v. 78, p. 1605, 1988.

WALTERS, T. W. Historical overview on domesticated plants in China with special emphasis on the *Cucurbitaceae*. **Economic Botany**, New York, v. 43, n. 3, p. 297-313, July 1989.

WANG, T. et al. Genome Sequence of *Acidovorax citrulli* group 1 strain pslb65 causing bacterial fruit blotch of melons. **Genome Announcements**, Washington, v. 3, n. 2, p. e00327-15, Apr. 2015.

WANG, T.; YANG, Y.; ZHAO, T. Genome sequence of a copper-resistant strain of *Acidovorax citrulli* causing bacterial fruit blotch of melons. **Genome Announcements**, Washington, v. 3, n. 2, p. e00310-15, Apr. 2015.

WATERS, C. M.; BASSLER, B. L. Quorum sensing: cell-to-cell communication in bacteria. **Annual Review of Cell Developmental Biology**, Palo Alto, v. 21, n. 1, p. 319-346, Nov. 2005.

WEBB, R. E.; GOTH, R. W. A seedborne bacterium isolated from watermelon. **Plant Disease Reporter**, Washington, v. 49, p. 818-821, 1965.

WICKER, E. et al. Contrasting recombination patterns and demographic histories of the plant pathogen *Ralstonia solanacearum* inferred from MLSA. **The ISME Journal**, New York, v. 6, n. 5, p. 961-974, Nov. 2012.

WILLEMS, A. et al. Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov.; comb. nov.; *Acidovorax avenae* subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax konjaci*. **International Journal of Systematic Bacteriology**, Reading, v. 42, p. 107-119, 1992.

YAN, S. et al. Genetic diversity analysis of *Acidovorax citrulli* in China. **European Journal of Plant Pathology**, Dordrecht, v. 136, n. 1, p. 171-181, May 2013.

ZHAO, T. et al. An improved assay for detection of *Acidovorax citrulli* in watermelon and melon seed. **Seed Science and Technology**, Bassersdorf, v. 37, n. 2, p. 337-349, July 2009.

CHAPTER II

Assessment of the genetic diversity of Brazilian *Acidovorax citrulli* population and comparative analysis of genome sequences

ABSTRACT

Bacterial fruit blotch (BFB) caused by the seedborne pathogen *Acidovorax citrulli*, is an economically important threat to cucurbitaceous crops worldwide. Since the first report of BFB in Brazil in 1990, outbreaks have occurred sporadically on watermelon, and more frequently on melon, resulting in significant yield losses. At present, the genetic diversity and the population structure of *A. citrulli* strains in Brazil remain unclear. A collection of 74 *A. citrulli* strains isolated from naturally infected fruits of different hosts in Brazil between 2000 and 2014 and 18 representative group I and group II *A. citrulli* strains from other countries were compared by pulsed-field gel electrophoresis (PFGE), multilocus sequence analysis (MLSA) of housekeeping and virulence-associated genes and pathogenicity tests on different cucurbit seedling hosts. The Brazilian population was comprised predominantly of group I strains (98%), regardless of the year of isolation, geographical region or host. Whole genome restriction digestion and PFGE analysis revealed that three unique and previously unreported *A. citrulli* haplotypes (assigned as haplotypes B22, B23 and B24) occurred in Brazil. The greatest diversity of *A. citrulli* (4 haplotypes) was found among strains collected from the Northeastern region of Brazil, which accounts for more than 90% of the country`s melon production. Multilocus sequence analysis clearly distinguished *A. citrulli* strains into two well supported clades, in agreement with observations based on PFGE analysis. Five Brazilian *A. citrulli* strains, representing different group I haplotypes, were moderately aggressive on watermelon seedlings compared to four group II strains that were highly aggressive. In contrast, no significant differences in BFB severity were observed between group I and II *A. citrulli* strains on melon and squash seedlings. Finally, based on preliminary results from the genome sequencing of the group I strain M6, we compared the genome sequences of group I and II *A. citrulli* strains and observed that group I genomes are relatively shorter than group II (~ 400 Kb). This difference in genome size is mainly explained by the presence of eight fragments distributed throughout the genome of group II strains, that are absent in the group I strains, and may have been gradually acquired by group II strains through horizontal gene transfer events from other bacterial species. These results contribute to a better understanding of the genetic diversity of *A. citrulli* associated with BFB outbreaks in Brazil, and reinforce the efficiency of MLSA and PFGE analysis for assessing population structure. This study also provides the first comprehensive comparison at the genomic level between the two major groups of *A. citrulli*. Further investigation is needed to sharpen the genetic markers of group I and II strains.

Keywords: DNA. Bacterial fruit blotch. *Cucumis melo*. PFGE. MLSA.

INTRODUCTION

Bacterial fruit blotch (BFB), caused by the Gram-negative bacterium *Acidovorax citrulli*, is an economically important disease of cucurbitaceous crops worldwide (BAHAR; BURDMAN, 2010; BURDMAN; WALCOTT, 2012; WALL; SANTOS, 1988; YAN et al., 2013). The pathogen was first reported in Georgia, USA in the 1960s, causing necrotic lesions on watermelon cotyledons of different plant introductions (PI) from Turkey (WEBB; GOTH, 1965). Initially, the disease was reported to only affect the seedling stage with limited damage potential on fruit, and the causal agent was subsequently classified as *Pseudomonas pseudoalcaligenes* subsp. *citrulli* (SCHAAD et al., 1978). More recently the pathogen was renamed *Acidovorax citrulli* (syn. *Acidovorax avenae* subsp. *citrulli*) (SCHAAD et al., 2008; WILLEMS et al., 1992). The economic impact of BFB was realized in 1987, when the first outbreak occurred in the Mariana Islands, causing close to 100% watermelon yield losses (WALL; SANTOS, 1988). To date, BFB management has been problematic as there are no commercially available sources of disease resistance and chemical control has limited efficacy in warm and wet field conditions (BURDMAN; WALCOTT, 2012).

Interestingly, the American Type Culture Collection *A. citrulli* type strain (ATCC 29625), recovered from seedlings at the USDA plant introduction station Georgia in 1965, did not induce a hypersensitive response (HR) on tobacco leaves and was unable to cause watermelon fruit rot (WEBB; GOTH, 1965). In contrast, *A. citrulli* strains recovered from BFB outbreaks in the USA were highly aggressive on watermelon and induced an HR on non-host tomato and tobacco leaves (WALCOTT et al., 2000). These observation, suggested that there was genetic diversity amongst *A. citrulli* populations. To further support these observation, O'Brien and Martin observed that *A. citrulli* strains recovered from melons in North Queensland, Australia were more aggressive on rockmelon, than

strains recovered from watermelons in South Queensland. Strains from North and South Queensland, Australia could also be distinguished based on substrate utilization profiles. More specifically, *A. citrulli* strains from watermelon plants in south Queensland utilized L-leucine while strains from melon in north Queensland did not (O'BRIEN; MARTIN, 1999).

Currently, *A. citrulli* strains can be divided into at least two genetically distinct groups (BURDMAN; WALCOTT, 2012; ECKSHTAIN-LEVI et al., 2014; FENG et al., 2009; WALCOTT et al., 2000; WALCOTT; FESSEHAIE; CASTRO, 2004). These include group I strains that have been isolated from a range of cucurbit hosts, and group II strains that are more aggressive to, and have been isolated predominantly from watermelon. Group I and II *A. citrulli* strains can be clearly distinguished by restriction enzyme (*Spe*I) digestion and pulsed-field gel electrophoresis (PFGE), gas chromatography–fatty acid methyl ester (GC-FAME) profiles, rep-PCR and multilocus sequence typing of housekeeping genes (FENG et al., 2009; WALCOTT et al., 2000). A recent comparative analysis of putative type III secreted effector genes revealed that group I and II *A. citrulli* strains differed in their effector protein repertoires, which may contribute to their host preference association (ECKSHTAIN-LEVI et al., 2014). Moreover, this analysis led to the identification of a third, weakly virulent *A. citrulli* group.

Since the first major BFB outbreak in 1990, in a watermelon field located in Sao Paulo state (ROBBS et al., 1991), BFB has become a serious threat to Brazilian watermelon and melon production. The disease has occurred in the states of Rio Grande do Norte (ASSIS et al., 1999), Ceará (SANTOS; VIANA, 2000), Minas Gerais (MACAGNAN et al., 2003), Rio Grande do Sul (UENO; COUTO; UESUGUI, 2003), Pernambuco (MARIANO; SILVEIRA, 2004), Bahia (MARIANO et al., 2004) and Roraima (HALFELD-VIEIRA; NECHET, 2007). Despite its wide distribution, BFB outbreaks have only occurred sporadically in watermelon fields. On the other hand, significant yield losses have been common in melon producing areas during the rainy season in the northeastern region of

Brazil. More precisely, BFB outbreaks in melon have been common in the states of Rio Grande do Norte and Ceará, which account for > 80% of Brazil's melon production (ASSIS et al., 1999; CONCEIÇÃO et al., 2014; MELO et al., 2014).

Previous studies have reported variability among Brazilian *A. citrulli* strains with regards to aggressiveness on melon and watermelon seedlings, biochemical properties and genetic relatedness based on rep-PCR analysis (MELO et al., 2014; OLIVEIRA et al., 2007). However, none have reported the genetic relatedness of Brazilian strains in accordance with the two well-documented and genetically distinct groups of *A. citrulli* (ECKSHTAIN-LEVI et al., 2014; FENG et al., 2009; WALCOTT et al., 2000; WALCOTT; FESSEHAIE; CASTRO, 2004). Since these studies were conducted primarily with Brazilian *A. citrulli* strains, it is important to clarify the genetic diversity of *A. citrulli* strains in Brazil by comparison with well characterized reference strains.

To further examine the population structure of *A. citrulli* in Brazil, develop a fingerprinting database and provide a basis for the integrated management of BFB, we used PFGE, multilocus sequence analysis of housekeeping and virulence-associated genes and pathogenicity tests on different cucurbit seedlings to characterize a Brazilian population of *A. citrulli* strains collected from different hosts, and geographical regions between 2000 and 2014. We also compare the genome sequences of representative group I and II *A. citrulli* strains.

MATERIALS AND METHODS

Bacterial strains. *Acidovorax citrulli* strains used in this study are listed in table 1. Seventy-four *A. citrulli* strains were isolated from naturally infected curcurbit fruits during BFB outbreaks in Brazil between 2000 and 2014. Additionally, some Brazilian strains were provided by collaborators. Well characterized reference strains from international sources were provided by a range of sources (Table 2). *Acidovorax citrulli* strains were routinely grown on King's B

medium (KING; WARD; RANEY, 1954) at 28°C for 48 h, and single colonies were confirmed as *A. citrulli* by PCR assay with primers WFB1/WFB2 (WALCOTT; GITAITIS, 2000), BX-S/BX-L (BAHAR et al., 2008) and BOXAACF/AACR2 (HA et al., 2009). Bacterial DNA was extracted using the UltraClean Microbial DNA isolation kit (MO BIO laboratories, Inc., Carlsbad, CA. Strains were stored in 15% sterile glycerol at -80°C at the Seed Pathology laboratory at The University of Georgia, Athens, GA.

DNA fingerprinting by *Spe*I digestion and pulsed-field gel electrophoresis (PFGE). DNA extraction, *SpeI* restriction enzyme digestion and PFGE were conducted according to previously described methods (WALCOTT et al., 2000). After electrophoresis, agarose gels were stained with a 0.5 µg/ml ethidium bromide solution for 30 min, destained with distilled water for 30 min and, digital images were captured in tagged image file format (TIF) under ultraviolet transillumination with an Eagle Eye II Still Video System (Stratagene, La Jolla, CA, USA). DNA fingerprint profiles for each unique haplotype were compared using Dice's (1945) coefficient of analysis with the aid of the BioNumerics software package (Applied Math, Kortrijk, Belgium) and the unweighted pairwise group method with arithmetic mean (UPGMA) algorithm was used to generate a dendogram indicating strain relatedness.

Multilocus sequence analysis (MLSA). Seven genes, representing a total of 5,681 bp, were sequenced for MLSA. The genes were chosen based on a previous MLST scheme developed for *A. citrulli* (FENG et al., 2009). Additional genes were chosen by comparing two sequenced *A. citrulli* genomes, the reference group II strain, AAC00-1 (NCBI accession number NC_008752) and the draft genome of M6 (ECKSHTAIN-LEVI et al., 2016), the reference group I strain, using Geneious software version 8.1.7 (Biomatters Ltd., Auckland, New Zealand; http://www.geneious.com). Four of these genes were housekeeping genes (*adk*, *gyrB*, *pilT* and *gltA*) and three were virulence-associated genes (*luxR* homologue, *avrRx01* and *Aave_1548*) (Table 3). In selecting the housekeeping genes, we

considered the gene distribution across the genome so that a single recombination event would be unlikely to affect more than one locus. For MLSA, genes were amplified using primers designed from the complete genome of *A. citrulli* strain AAC00-1 (NC_008752) with Primer3 v.0.4.0 (http://frodo.wi.mit.edu) (ROZEN; SKALETSKY, 2000). Each reaction mixture contained ~ 20 to 30 ng/μL of DNA template, 1X buffer solution (New England Biolabs Inc., Ipswich, MA), 0.2 mM deoxynucleotide triphosphates mixture (New England Biolabs Inc.), 1 μM of each primer, and 0.25 U of Taq polymerase (New England Biolabs Inc.) for a 25 μL reaction volume. The PCR thermal conditions included an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 90 s. The final extension step was at 72°C for 5 min. The sequences of the primers used in this study are listed in table 5. PCR amplicons were purified with the ExoSap-IT Clean-up system (USB Co., Cleveland, OH) according to the manufacturer's instructions and direct sequencing of the amplicons was performed in the forward and reverse directions at Eurofins Genomics, Huntsville, AL. The seven genes selected for MLSA were sequenced for 20 *A. citrulli* strains, including 18 reference strains (Table 2) and two Brazilian strains representing the PFGE-determined haplotypes B6 (N) and B23. Sequences of the closely related bacterium, *Acidovorax avenae* strain ATCC 19860 (NCBI accession number NC_015138), served as an outgroup. All gene sequences were deposited in NCBI GenBank (Accession numbers: KU984110 to KU984250).

Phylogenetic analysis and single nucleotide polymorphisms. Gene sequences were aligned using ClustalX software (THOMPSON et al., 1997). Phylogenetic trees were generated from the nucleotide sequences using the maximum parsimony method in MEGA6 software (TAMURA et al., 2013). Bootstrap values were derived from 1,000 replicates to validate tree topology. Phylogenetic trees were generated for individual gene sequences, as well as for concatenated sequences and sequence polymorphisms were determined using DnaSP software V5 (LIBRADO; ROZAS, 2009).

Comparative analysis of group I and II *Acidovorax citrulli* genomes. For the comparative analysis, we used the draft genome of seventeen *A. citrulli* strains from several haplotypes (10 from group I; 7 from group II), sequenced by a private company (Figure 5). The draft genomes were obtained by mapping the raw data (short read sequence ~ 96 bp; 30X depth of coverage) to the reference genomes AAC00-1 (NC_008752) and M6 (ECKSHTAIN-LEVI et al., 2016) (GenBank accession number: LKUW00000000) using Geneious software version 8.1.7 (Biomatters Ltd., Auckland, New Zealand; http://www.geneious.com). Additionally, two draft genomes of *A. citrulli* strains from China, tw6 and pslb65 (WANG et al., 2015; WANG; YANG; ZHAO, 2015), from the GenBank database were included (Accession numbers JXDJ00000000 for tw6 and JYHM00000000 for pslb65). According to previous analysis, pslb65 and tw6 are both group I strains. The comparative study was conducted using the MegaBlast program implemented on Geneious (maximum e-value, 0,0001; gap cost, 394 linear; match-mismatch scoring, 1-2; maximum hits, 100).

Seedling virulence assays. The virulence of selected *A. citrulli* strains representing the most abundant haplotypes found in Brazil and other reference strains was evaluated on watermelon (*Citrullus lanatus*), melon (*Cucumis melo*), and squash (*Cucurbita pepo*) seedlings, as previously described (HOPKINS; THOMPSON, 2002; WALCOTT; FESSEHAIE; CASTRO, 2004). Briefly, watermelon cv. 'Crimson Sweet', melon cv. 'Joaquin gold', and squash cv. 'Early Yellow Crookneck' seedlings were grown in 1.4 L plastic pots under greenhouse conditions (28 to 30°C, ~70% relative humidity and 12 h sunlight daily). Seedlings were cultivated in a 3:1 ratio of fine-grade composted pine bark and vermiculite. Two weeks after planting, seedlings were spray-inoculated with cell suspensions of ~1 × 10^6 CFU/mL of group I Brazilian strains, AC 05, AC 23, AC 36, AC 50 and AC 59, and group II strains, AAC 00-1, AAC 213-44, AAC213-46 and AAC 213-47. To generate inoculum, –80°C stock cultures of each strain were cultured on King's B medium at 28°C for 48 h. Subsequently, a single colony of each culture

was transferred to 5 mL of nutrient broth (NB) and incubated at 28°C for 16 h with agitation at 250 RPM in an incubator shaker (Innova, New Brunswick, NJ). Cells in 1 mL of each NB culture were pelleted by centrifugation at 10,000 × g for 2 min, rinsed once with 0.1 M phosphate buffered saline (PBS), and resuspended in 3 mL of PBS. The optical density of the cell suspensions was adjusted to 0.5 at a wavelength of 600 nm (~0.5 × 10^8 CFU/mL) using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY), and 10-fold serial dilutions were performed to generate cell suspensions with ~1 × 10^6 CFU/mL. Using plastic hand-held, spray bottles, cell suspensions of each strain were sprayed until run-off onto ten-day-old seedlings of each cucurbit species. For a negative control, seedlings were inoculated with sterile PBS. Each seedling was incubated in a transparent plastic bag (~100% RH) for 48 h and then the plastic bags were removed and the seedlings were incubated under greenhouse conditions. Seven days after inoculation, seedlings were evaluated for BFB severity according to a previously described 1-to-9 rating scale (HOPKINS; TOMPSON, 2002). This experiment was repeated three times and each experiment included four replicates (individual plants) for each strain-host combination. Each plant was assessed for BFB severity by three independent evaluators. The experiment was arranged in a randomized complete block design. Statistical analysis was performed using the R environment. The R package, multicomp, was used for multiple comparison of groups (HERBERICH; SIKORSKI; HOTHORN, 2010). The R package GGPLOT2 was used to plot the disease severity data.

RESULTS

Strain identification. All Brazilian strains were confirmed as *A. citrulli* by PCR with species-specific primers. However, *A. citrulli* strains representing the two common Brazilian PFGE haplotypes (B23 and B24) were negative when assayed by PCR with the BOXAACF/AACR2 primer set. This was expected as

this primer set does not amplify DNA from some group I *A. citrulli* strains [e.g. haplotypes B3(K), B8(P), B9(R) and B17] (HA et al., 2009).

***Spe*I-PFGE analysis clustered the Brazilian strains into group I.** Out of 74 Brazilian *A. citrulli* strains, 67 were analyzed by PFGE. Among these 67 strains, seven distinct PFGE haplotypes were observed. Six haplotypes, representing 98% of the strains tested, clustered into group I and one strain, AC 60 was determined to be haplotype A3(C) (group II) (Fig. 1). Unfortunately, no data were available on the geographical origin or the host of origin for AC 60. Of the six group I haplotypes observed in the Brazilian population, haplotypes B1(F), B12(X) and B6 (N) were previously identified (WALCOTT; SIKORSKI; HOTHORN, 2004) and three haplotypes (B22, B23 and B24) were previously unreported (Fig. 2). Haplotypes B24 (AC 33, AC 35, AC 36 and AC 37 from watermelon) and B12 (X) (AC 59 from melon) were recovered from southern and northern regions of Brazil, respectively. The greatest *A. citrulli* diversity [haplotypes B1(F), B6(N), B23 and B22] was observed in northeastern Brazil, where haplotype B23 was the most prevalent (85.58% of the strains tested) (Table 1). The other seven Brazilian strains, that were not fingerprinted by PFGE, were assigned to group I or II based on a PCR assay using a primer set (G1/G2) targeting the T3 secreted protein *Aave_2166*, (ZIVANOVIC, 2014) (Fig. 7) and sequence analysis of the housekeeping gene *gyrB* (Fig. 4A).

MLSA confirms that most Brazilian *A. citrulli* strains belong to group I. Based on a previously described MLST scheme for *A. citrulli* (FENG et al., 2009), three housekeeping genes (HKGs) (*gltA, ugpB* and *pilT*) were initially selected for our study. The other HKGs (*gmc, lepA, trpB* and *phaC*) were monomorphic or non-informative (data not shown). In addition to the three HKGs, a putative type III secreted effector gene (*Aave_1548*) (ECKSHTAIN-LEVI et al., 2014) was included, as well as *Aave_3810* (a *luxR* homologue) and *Aave_3062* (*avrRx01*) (JOHNSON; WALCOTT, 2013; LIU et al., 2014). Additional HKGs (*gyrB* and *adk*) were included based on other MLST schemes generally reported

for plant pathogenic bacteria (HARAYAMA; KASAI, 2006). Only two Brazilian strains, representing haplotypes B23 (strain AC 05) and B6(N) (AC 50) were used in MLSA because the Brazilian population was generally lacked genetic diversity. DNA and amino acid sequence data were used for MLSA. Nucleotide sequence data analysis of the HKGs (e.g. *gyrB* and *gltA*) clustered the two Brazilian strains (AC 05 and AC 50) into group I, in agreement with PFGE analysis (Fig. 4A and 4B). The phylogenetic analysis of the putative T3S effector gene, *Aave_1548* (Fig. 6C), the homologue *luxR* gene and the concatenated sequence data (Fig. 4C and 4D) clustered strains AAC 213-50 [haplotype B3(K)] and AAC 213-51 [haplotype B8(P)] as a distinct group, closely related to the group I.

Regarding single nucleotide polymorphisms, the most variable housekeeping gene was *gyrB* (11 SNPs between group I and II), followed by *gltA* (5 SNPs), *adk* and *pilT* (1 SNP each). No amino acid substitutions were detected among the HKGs. Among the virulence-associated genes, *Aave_1548* had 33 SNPs and 16 amino acid substitutions and was the most variable gene. Finally, the putative T3S effector gene, *avrRx01* had a 1-bp deletion at position 558 of the open reading frame in all tested group I strains, resulting in a truncated protein, in agreement with Eckshtain-Levi et al. (2014).

Seedling virulence assays. The aggressiveness of five group I Brazilian strains (AC 05, AC 23, AC 36, AC 50 and AC 59), and four group II strains (AAC00-1, AAC213-44, AAC213-46 and AAC213-47) was compared on melon, watermelon and squash seedlings. We observed high variability in disease severity for the Brazilian strains, especially on melon and watermelon seedlings, with strains AC 05, AC 50 and AC 59 being more aggressive (mean severity ratings of 6, 6.5 and 7, respectively) than strains AC 23 and AC 36 (mean severity ratings of 5.2 and 5, respectively). No significant difference in aggressiveness was observed between group I and II strains on melon and squash (P = 0.13 and 0.97, respectively). However, the Brazilian *A. citrulli* strains were significantly less aggressive than the group II strains on watermelon seedlings ($P < 0.001$) (Fig. 3).

Comparative analysis of *Acidovorax citrulli* genomes. The complete genome sequence of the reference group II AAC00-1 strain (available at NCBI GenBank; NC NC_008752) and group I M6 (ECKSHTAIN-LEVI et al., 2016), which were assembled and annotated in independent projects, were used as reference genomes. The mapped draft genomes of group I *A. citrulli* strains were significantly shorter (~ 400 Kb) in size than the ones belonging to the group II strains (Table 4). Comparative analysis from the genome sequence of AAC00-1 and M6 revealed that AAC00-1 has eight fragments (FA1 to FA8; for fragments of AAC00-1) ranging in size from ~34.9 to ~119.5 kb, and scattered throughout the AAC00-1 genome (ECKSHTAIN-LEVI et al., 2016). To further explore whether this eight fragments reflect universal differences between group I and II strains of *A. citrulli*, we conducted a coverage analysis to determine the percent coverage of the AAC00-1 FA1-FA8 fragments in the sequences of the aforementioned genomes. As controls, we arbitrarily selected nine reference fragments from the AAC00-1 genome (hereafter RFA1 to RFA9), ranging in size from 30.4 to 121.2 kb, and interspersed between fragments FA1 to FA8. The RFAs fragments were highly conserved in all *A. citrulli* strains (Fig. 5). In contrast, a clear distinction between group I and II was observed between group I and II *A. citrulli* strains for fragments FA1 to FA4, which showed an overall high level of coverage in group II strains, and relatively low level of coverage in group I strains. Some exceptions were the fragments FA3, FA4, FA7 and FA8 that showed intermediate to high levels of coverage for some strains (Fig. 5).

DISCUSSION

In Brazil, efforts to manage BFB outbreaks in cucurbit crop fields include the use of disease-free seeds, rotations with non-cucurbit crops and applications of copper-based compounds combined with host plant resistance-inducing chemicals (SALES JUNIOR et al., 2007). Despite this, BFB outbreaks are still common,

particularly in melon producing regions (CONCEIÇÃO et al., 2014). To obtain Brazilian *A. citrulli* strains representing different years, hosts and geographical origin, we collected strains from natural BFB outbreaks in the field and requested older reference strains from the Brazilian culture collections (Culture Collection of Phytobacteriology of the Brazilian Agricultural Research Corporation, Embrapa-Plant Quarantine Unit and Culture collection of the Federal Rural University of Pernambuco, UFRPE). It is important to note that the strains that we collected from the field were mostly recovered from naturally infected fruits in the northeastern region of Brazil, where melon production is heavily concentrated and BFB outbreaks are frequently reported. Since BFB epidemics in other regions of Brazil occur sporadically, we assume that our collection adequately represents the genetic spectrum of *A. citrulli* strains that has occurred in the country. Considering that i) the Brazilian strains were mainly group I, regardless of the year of isolation and host; ii) we found three unique and previously unreported haplotypes in Brazil, and iii) BFB outbreaks occur predominantly on melon rather than watermelon; we hypothesize that group I *A. citrulli* strains are ecologically adapted to Brazil. Since contaminated seedlots represent the main source of inoculum for long distances dissemination, it seems unlikely that only group I strains would be introduced into Brazil on cucurbit seeds. In contrast, it seems more likely that group I strains are better adapted to survive in Brazil.

While group I *A. citrulli* haplotypes, B1(F) and B6(N), were previously reported from Brazil (WALCOTT; FESSEHAIE; CASTRO, 2004), only two strains from melon were analyzed in this study. Of the three new haplotypes reported in the current study, haplotype B23 was the most prevalent, and it occurred from 2001 (AC 51 and AC 52) to 2014 in northeastern Brazil (Table 1). Using rep-PCR, Melo et al. (2014) reported that Brazilian *A. citrulli* strains were closely related to the type strain CFBP 4459, which was designated as group I, although not in accordance with the PFGE-based classification. Similar to the group I strains tested in our study, Melo et al. (2014) reported a significant

reduction in aggressiveness of the *A. citrulli* strains on watermelon seedlings relative to melon. The same strains evaluated by Melo et al. (2014) were included in our study and were placed into groups I (Emb.I97, Emb.E117, Emb.H530, Emb.A11-19, Emb.D348 and Emb.C587) and II (Emb.D349) based on PCR assay for the T3S effector gene, *Aave_2166* (Figure 7) and sequence analysis of the housekeeping gene *gyrB* (Figure 4A). Similar findings on reduced aggressiveness of group I strains on watermelon seedlings were reported by Walcott, Fessehaie and Castro (2004).

Using a database of PFGE fingerprints, we compared a population of *A. citrulli* strains collected in China, a major producer of melon and watermelon seed (n=114 strains, from 2002 to 2013) and Georgia, USA (n=65 Strains, from 1992 to 2012). We observed an uneven distribution of group I (80.7% in China and 6.16% in Georgia) and group II strains (19.3% in China and 93.84% in Georgia). Specifically, haplotypes B5(M) (73.68%) and A3(C) (58.14%) were the most prevalent strains recovered from China and Georgia, respectively. In the state of Georgia in the United States, most BFB outbreaks have occurred in watermelon crops (R. Walcott, *unpublished data*). Our observation of a prevalence of group I *A. citrulli* strains in China is in agreement with other studies (FENG et al., 2009; YAN et al., 2013). While *A. citrulli* strains are disseminated globally with cucurbit seeds, our data suggest that certain haplotypes/groups are predominant in certain geographical regions. More specifically, group I strains appear to occur prevalently in China and Brazil while group II strains are predominant in Georgia, USA.

In the current study, MLSA grouped *A. citrulli* strains into two distinct groups that agreed with PFGE-based analysis. Comparative studies of bacterial genomes have revealed that bacterial evolution is driven by composite forces acting on the “core” and “flexible” genomes (GIL et al., 2004; SARKAR; GUTTMAN, 2004). The core genome consists of essential genes, such as housekeeping genes, that are less likely to undergo horizontal gene transfer. These genes evolve neutrally and are suitable for measuring the evolutionary history of

clonal lineages. Unlike the core genome, the flexible genome consists of genes that encode proteins involved in adaptation to specific niches, hosts or environments, such as virulence-associated genes. These genes reflect the short-term evolution of a clonal lineage. Analysis of *A. citrulli* housekeeping genes in our study supported the existence of two groups that are distinguishable from the closely related *Acidovorax avenae*, as previously reported (FENG et al., 2009; YAN et al., 2013). This suggests that these two *A. citrulli* groups diverged long ago. Further evidence for evolutionary forces that have driven the groups apart is the presence of genes encoding the VapBC-like Toxin-Antitoxi system in group II strains and their absence in group I strains (SHAVIT et al., 2015). Nevertheless, a more detailed analysis of the evolutionary history of *A. citrulli* is needed to determine how these two groups evolved.

In 2007, the Joint Genome Institute released the sequence of strain AAC00-1 (GenBank accession NC_008752), considered by the *A. citrulli* research community as the group II model strain of this bacterium. *A. citrulli* M6 was isolated in Israel in 2002 from a symptomatic melon fruit (BURDMAN et al., 2005) and in recent years became the model group I strain for fundamental investigation of BFB. Using this strain, were identified pathogenicity and virulence determinants of *A. citrulli*, including type III secretion (BAHAR; BURDMAN, 2010), type IV pili (BAHAR; BURDMAN, 2010; BAHAR; KRITZMAN; BURDMAN, 2009) and polar flagella (BAHAR et al., 2008). The M6 was also used to characterize phenotypic variation in *A. citrulli* strains (SHRESTHA et al., 2013) and to develop PCR-based seed heath testing assays (BAHAR et al., 2008). Based on comparative analysis, the differences between the genome sequences reaffirm the hypothesis that these two group of organisms diverged during their evolutionary history and that these fragments or a significant part of them were gradually acquired by several horizontal gene transfer events by ancestral group I strains, leading to a separation of the two groups and their subsequent adaptation to different hosts in the *Cucurbitaceae* family. We cannot exclude the possibility that

some of the FA fragments are unstable in group II genomes, and some may have been lost, partially or entirely, by some group II strains. The relative coverage of some FA fragments (e.g., FA6 to FA8) in group I genomes and the low coverage of FA5 in few group II haplotypes, suggest that both acquisition and loss events may have occurred.

This study showed that BFB outbreaks in Brazil are predominantly caused by group I *A. citrulli* strains, regardless of the host or region. We also identified three unique haplotypes of *A. citrulli* from northeastern Brazil. By analyzing a historical PFGE database we observed that even though *A. citrulli* is globally disseminated with cucurbit crop seeds, there was also a prevalence of group I strains in China, while BFB outbreaks in Georgia, USA were associated predominantly with group II strains. Finally, we showed a remarkable difference between the genome sequences of *A. citrulli* group I and II strains, reinforcing the divergent evolution hypothesis. The genome sequences of the group II *A. citrulli* strain, AAC00-1 and other group I strains (pslb65 and TW6; Genbank accession numbers JYHM01000000 and JXDJ01000000 respectively) will allow a more detailed investigation of molecular host-pathogen interactions, which should ultimately improve BFB management by identifying new targets for resistance breeding efforts.

REFERENCES

ASSIS, S. M. P. et al. Mancha aquosa do melão causada por *Acidovorax avenae* subsp. *citrulli*, no Estado do Rio Grande do Norte. **Fitopatologia Brasileira**, Brasília, v. 24, n. 2, p. 191, 1999.

BAHAR, O.; BURDMAN, S. Bacterial fruit blotch: a threat to the cucurbit industry. **Israel Journal of Plant Sciences**, Tel Aviv, v. 58, n. 1, p. 19-31, Apr. 2010.

BAHAR, O. et al. New subspecies-specific polymerase chain reaction-based assay for the detection of *Acidovorax avenae* subsp. *citrulli*. **Plant Pathology**, Honolulu, v. 57, n. 4, p. 754-763, Aug. 2008.

BAHAR, O.; KRITZMAN, G.; BURDMAN, S. Bacterial fruit blotch of melon: screens for disease tolerance and role of seed transmission in pathogenicity. **European Journal of Plant Pathology**, Dordrecht, v. 123, n. 1, p. 71-83, July 2009.

BURDMAN, S.; WALCOTT, R. Pathogen profile *Acidovorax citrulli*: generating basic and applied knowledge to tackle a global threat to the cucurbit industry. **Molecular Plant Pathology**, London, v. 13, n. 8, p. 805-815, Oct. 2012.

CAVALCANTI, M. T. et al. Growth of *Acidovorax avenae* subsp. *citrulli* under different variable temperature, pH, sodium chloride concentrations and carbon sources. **Ciência Rural**, Santa Maria, v. 35, n. 6, p. 1313-1318, dez. 2015.

CONCEIÇÃO, C. S. et al. Combined effect of yeast and silicon on the control of bacterial fruit blotch in melon. **Scientia Horticulturae**, Amsterdam, v. 174, n. 1, p. 164-170, July 2014.

DUTTA, B. et al. Location of *Acidovorax citrulli* in infested watermelon seeds is influenced by the pathway of bacterial invasion. **Phytopathology**, Saint Paul, v. 102, n. 5, p. 461-468, May 2012.

ECKSHTAIN-LEVI, N. et al. Comparative analysis of type III secreted effector genes reflects divergence of *Acidovorax citrulli* strains into three distinct lineages. **Phytopathology**, Saint Paul, v. 104, n. 11, p. 1152-1162, Nov. 2014.

ECKSHTAIN-LEVI, N. et al. Insights from the genome sequence of *Acidovorax citrulli* M6, a group I strain of the causal agent of bacterial fruit blotch of cucurbits. **Frontiers in Microbiology**, Lausanne, v. 7, n. 7, p. 1-16, Apr. 2016.

FENG, J. et al. Multilocus sequence typing reveals two evolutionary lineages of *Acidovorax avenae* subsp. *citrulli*. **Phytopathology**, Saint Paul, v. 99, n. 8, p. 913-920, Aug. 2009.

GIL, R. et al. Determination of the core of a minimal bacterial gene set. **Microbiology and Molecular Biology Reviews**, New York, v. 68, n. 3, p. 518-537, Sept. 2004.

HA, Y. et al. Simultaneous detection of *Acidovorax avenae* subsp. *citrulli* and *Didymella bryoniae* in cucurbit seedlots using magnetic capture hybridization and real-time polymerase chain reaction. **Phytopathology**, Saint Paul, v. 99, n. 6, p. 666-678, June 2009.

HALFELD-VIEIRA, B. A.; NECHET, K. L. Mancha-aquosa da melancia em Roraima. **Fitopatologia Brasileira**, Brasília, v. 32, n. 3, p. 26, jun. 2007.

HARAYAMA, S.; KASAI, H. Bacterial phylogeny reconstruction from molecular sequences. In: STACKEBRANDT, E. (Ed.). **Molecular identification, systematics, and population structure of prokaryotes**. Berlin: Springer-Verlag, 2006. p. 106-138.

HERBERICH, E.; SIKORSKI, J.; HOTHORN, T. A robust procedure for comparing multiple means under heteroscedasticity in unbalanced designs. **PLoS One**, San Francisco, v. 5, n. 3, p. e9788, Mar. 2010.

HOPKINS, D. L.; THOMPSON, C. M. Evaluation of *Citrullus* sp. germplasm for resistance to *Acidovorax avenae* subsp. *citrulli*. **Plant Disease**, Quebec, v. 86, n. 1, p. 61-64, Jan. 2002.

JOHNSON, K. L.; WALCOTT, R. R. Quorum sensing contributes to seed-to-seedling transmission of *Acidovorax citrulli* on watermelon. **Journal of Phytopathology**, Berlin, v. 161, n. 8, p. 562-573, Aug. 2013.

KING, E. O.; WARD, M. K.; RANEY, D. E. Two simple media for the demonstration of pyocyanin and fluorescin. **Journal of Laboratory and Clinical Medicine**, New York, v. 44, n. 2, p. 301-307, Aug. 1954.

LIBRADO, P.; ROZAS, J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. **Bioinformatics**, Oxford, v. 25, n. 11, p. 1451-1452, Apr. 2009.

LIU, H. et al. Domain Dissection of avrRxo1 for suppressor, avirulence and cytotoxicity functions. **PLoS ONE**, San Francisco, v. 9, n. 12, p. e113875, Dec. 2014.

MACAGNAN, D. et al. Mancha bacteriana da melancia: uma nova bacteriose no Estado de Minas Gerais. **Summa Phytopathologica**, Jaguariúna, v. 29, n. 3, p. 286-287, nov. 2003.

MARIANO, R. L. R. et al. Ocorrência da mancha-aquosa do melão na Bahia. **Fitopatologia Brasileira**, Brasília, v. 29, n. 5, p. 147-148, ago. 2004.

MARIANO, R. L. R.; SILVEIRA, E. B. Mancha-aquosa: importante bacteriose do meloeiro no Brasil. **Anais da Academia Pernambucana de Ciência Agronômica**, Recife, v. 1, n. 1, p. 79-88, jan. 2004.

MELO, L. A. et al. Comparing *Acidovorax citrulli* strains from melon and watermelon□: Phenotypic characteristics, pathogenicity and genetic diversity. **Tropical Plant Pathology**, Brasília, v. 39, n. 2, p. 154-162, Mar. 2014.

O'BRIEN, R. G.; MARTIN, H. L. Bacterial blotch of melons caused by strains of *Acidovorax avenae* subsp. *citrulli*. **Australian Journal of Experimental Agriculture**, Melbourne, v. 39, p. 479-485, 1999.

OLIVEIRA, J. C. et al. Caracterização de isolados de *Acidovorax avenae* subsp. *citrulli*. **Fitopatologia Brasileira**, Brasília, v. 32, n. 6, p. 480-487, dez. 2007.

ROBBS, C. F. et al. Mancha bacteriana da melancia no estado de São Paulo, causada por *Pseudomonas pseudoalcaligenes* subsp. *citrulli*. **Fitopatologia Brasileira**, Brasília, v. 16, n. 2, p. 48, 1991. Abstract.

ROZEN, S.; SKALETSKY, H. J. Primer3 on the WWW for general users and for biologist programmers. In: KRAWETZ, S.; MISENER, S. (Ed.). **Bioinformatics methods and protocols:** methods in molecular biology. Totowa: Humana, 2000. p. 365-386.

SALES JUNIOR, R. et al. Eficiência de Acibenzolar-S-Methyl e Oxicloreto de Cobre no controle de *Acidovorax avenae* subsp . *citrulli* , agente causal da "Mancha-Aquosa" no meloeiro. **Revista de Biologia e Ciências da Terra**, Campina Grande, v. 7, n. 1, p. 66-70, jul. 2007.

SANTOS, A. A.; VIANA, F. M. **Mancha-aquosa do melão**. Fortaleza: EMBRAPA-SPI, 2000. 12 p.

SARKAR, S.; GUTTMAN, D. S. Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. **Applied and Environmental Microbiology**, Washington, v. 40, n. 4, p. 1999-2012, Apr. 2004.

SCHAAD, N. W. et al. *Pseudomonas pseudoalcaligenes* subsp. *citrulli* subsp. nov. **International Journal of Systematic Bacteriology**, Reading, v. 28, p. 117-125, 1978.

SCHAAD, N. W. et al. Reclassification of subspecies of *Acidovorax avenae* as *A. avenae* (Manns 1905) emend.; *A. cattleyae* (Pavarino, 1911) comb. nov.; *A. citrulli* Schaad et al.; 1978) comb. nov.; and proposal of *A. oryzae* sp. nov. **Systematic and Applied Microbiology**, Stuttgart, v. 31, n. 8, p. 434-446, Dec. 2008.

SHAAD, N. W.; JONES, J. B.; CHUN, W. **Laboratory guide for identification of plant pathogenic bacteria**. 3rd ed. Saint Paul: APS, 2001. 373 p.

SHAVIT, R. et al. The vapB-vapC operon of *Acidovorax citrulli* functions as a bona-fide toxin-antitoxin module. **Frontiers in Microbiology**, Lausanne, v. 6, p. 1-16, 2015.

SHRESTHA, R. K. et al. Phenotypic variation in the plant pathogenic bacterium *Acidovorax citrulli*. **PLoS ONE**, San Francisco, v. 8, n. 9, p. e73189-16, Sept. 2013.

TAMURA K. et al. MEGA6: molecular evolutionary genetics analysis version 6.0. **Molecular Biology and Evolution**, Chicago, v. 30, n. 12, p. 2725-2729, Oct. 2013.

THOMPSON, J. D. et al. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. **Nucleic Acids Research**, Oxford, v. 24, p. 4876-4882, 1997.

UENO, B.; COUTO, M. E. O.; UESUGUI, C. H. Ocorrência de Mancha-aquosa em melão no Estado do Rio Grande do Sul. **Fitopatologia Brasileira**, Brasília, v. 28, n. 3, p. S246, ago. 2003. Resumo.

WALCOTT, R. R. et al. Investigating intraspecific variation of *Acidovorax avenae* subsp. *citrulli* using DNA fingerprinting and whole cell fatty acid analysis. **Phytopathology**, Saint Paul, v. 90, n. 2, p. 191-196, Feb. 2000.

WALCOTT, R. R.; FESSEHAIE, A.; CASTRO, A. C. Differences in pathogenicity between two genetically distinct groups of *Acidovorax avenae* subsp. *citrulli* on cucurbit Hosts. **Journal of Phytopathology**, Berlin, v. 152, n. 5, p. 277-

285, May 2004.

WALCOTT, R. R.; GITAITIS, R. D. Detection of *Acidovorax avenae* subsp. *citrulli* in watermelon seed using immunomagnetic separation and the polymerase chain reaction. **Plant Disease**, Quebec, v. 84, n. 4, p. 470-474, Apr. 2000.

WALL, G. C.; SANTOS, V. M. A new bacterial disease on watermelon in the Mariana Islands. **Phytopathology**, Saint Paul, v. 78, p. 1605, 1988.

WANG, T. et al. Genome Sequence of *Acidovorax citrulli* group 1 strain pslb65 causing bacterial fruit blotch of melons. **Genome Announcements**, Washington, v. 3, n. 2, p. e00327-15, Apr. 2015.

WANG, T.; YANG, Y.; ZHAO, T. Genome sequence of a copper-resistant strain of *Acidovorax citrulli* causing bacterial fruit blotch of melons. **Genome Announcements**, Washington, v. 3, n. 2, p. e00310-15, Apr. 2015.

WEBB, R. E.; GOTH, R. W. A seedborne bacterium isolated from watermelon. **Plant Disease Reporter**, Washington, v. 49, p. 818-821, 1965.

WILLEMS, A. et al. Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov.; comb. nov.; *Acidovorax avenae* subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax konjaci*. **International Journal of Systematic Bacteriology**, Reading, v. 42, p. 107-119, 1992.

YAN, S. et al. Genetic diversity analysis of *Acidovorax citrulli* in China. **European Journal of Plant Pathology**, Dordrecht, v. 136, n. 1, p. 171-181, May 2013.

ZIVANOVIC, M. **Characterization of *Acidovorax citrulli* virulence on watermelon and melon**. 2014. 73 p. Thesis (Ph.D. in Plant Pathology) - The University of Georgia, Athens, 2014.

TABLES AND FIGURES

Table 1 Brazilian *Acidovorax citrulli* strains used in this study including the host of origin, geographical origin, the date collected, the source and the *SpeI*-digested pulse field gel electrophoresis haplotype and group.

Strains (other designation)	Host	Origin[c]	Date collected	Source[b]	Group	PFGE haplotype[a]
AC 24 (Aac 5.3)	Melon	RN	2001	UFRPE	I	B1(F)
AC 50 (Aac 1.12)	Melon	RN	2001	UFRPE	I	B6(N)
AC 59 (AC 604)	Melon	RR	2009	Embrapa RR	I	B12(X)
AC 36 (AGR 009.1-P)	Watermelon	RS	2013	Agronomica	I	B24
AC 35 (AGR 011.1-P)	Watermelon	RS	2013	Agronomica	I	B24
AC 33 (AGR 012.1-P)	Watermelon	RS	2013	Agronomica	I	B24
AC 37 (AGR 008.1-P)	Watermelon	RS	2013	Agronomica	I	B24
AC 05, 01, 02, 03, 04, 06, 07, 08, 09, 10, 11	Melon	RN	2014	UFLA	I	B23
AC 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22	Melon	RN	2014	UFLA	I	B23

(*Continued*…)

Table 1 (*Continued* …)

Strains (other designation)	Host	Origin[c]	Data collected	Source[b]	Group	PFGE haplotype[a]
AC 27, 28, 29, 30, 31, 32, 34, 38, 39, 40, 41	Melon	RN	2014	UFLA	I	B23
AC 42, 43, 44, 45, 46, 47, 48, 49, 53, 54, 55	Melon	RN	2014	UFLA	I	B23
AC 56, 57, 58, 61, 62, 63, 64, 65, 66, 67, 68	Melon	RN	2014	UFLA	I	B23
AC 51 (Aac 180)	Watermelon	PE	2001	UFRPE	I	B23
AC 52 (Aac R2)	Cantaloupe	RN	2001	UFRPE	I	B23
AC 25(Aac 5.16)	Melon	RN	2001	UFRPE	I	B22
AC 23 (Aac 1.45)	Melon	RN	2001	UFRPE	I	B22
AC 60 (Aac 11)	Unknown	Unknown	Unknown	UFLA	II	A3(C)
Emb.I97	Melon	RS	Unknown	Embrapa	I	ND
Emb.E117 (UFV Ac 14)	Watermelon	MG	2004	Embrapa	I	ND
Emb.H530	Melon	RS	Unknown	Embrapa	I	ND
Emb.A11-19	Melon	RN	2000	Embrapa	I	ND
Emb.D348 (CNPH Aac Maisa 2)	Melon	RN	2003	Embrapa	I	ND
Emb.C587 (Aac 1.31)	Melon	RN	2002	Embrapa	I	ND
Emb.D349 (CNPH Aac 1213)	Melon	Unknown	2003	Embrapa	II	ND

[a] Letters in parenthesis represent previously reported haplotypes (WALCOTT et al., 2000) while letter/number combinations represent current haplotype designations.
[b] Culture Collection of Phytobacteriology of: Embrapa= The Brazilian Agricultural Research Corporation-Plant Quarentine unit; UFRPE=Federal Rural University of Pernambuco; UFLA=Federal University of Lavras; Agronomica= Phytosanitary Diagnostic Laboratory; Embrapa RR= The Brazilian Agricultural Research Corporation-Roraima unit;
[c] Brazilian state. ND = not determined.

Table 2 Representative *Acidovorax citrulli* strains used for multilocus sequence analysis and virulence assays.

Strain (other designation)	Host	Geographical origin	Date collected	Source	Group	PFGE haplotype[a]
AAC 00-1	Watermelon	Georgia, USA	1990	R. Walcott	II	A1(A)
AAC 213-44	Unknown	Unknown	Unknown	R. Walcott	II	A3(C)
AAC 213-46	Unknown	Unknown	Unknown	R. Walcott	II	A11(W)
AAC 213-47	Unknown	Thailand	Unknown	R. Walcott	II	A2(B)
AAC 213-41	Unknown	Unknown	Unknown	R. Walcott	II	A2(B)
AAC 213-42	Unknown	Unknown	Unknown	R. Walcott	II	A4(D)
AAC 213-48	Unknown	Unknown	Unknown	R. Walcott	II	A5(E)
AAC 213-45	Unknown	Unknown	Unknown	R. Walcott	II	A28
AAC 213-49	Unknown	Unknown	Unknown	R. Walcott	II	A26
ATCC 29625 (AAC92-300)	Watermelon	Georgia, USA	1969	ATCC[b]	I	B3(K)
M6	Melon	Israel	2002	S. Burdman	I	B21
AAC 213-50	Unknown	Unknown	Unknown	R. Walcott	I	B3(K)
AAC 213-51	Pumpkin	China	Unknown	R. Walcott	I	B8(P)

(*Continued ….*)

Table 2 (*Continued*…)

Strain (other designation)	Host	Geographical origin	Date collected	Source	Group	PFGE haplotype[a]
AAC 213-52	Unknown	Unknown	Unknown	R. Walcott	I	B13(Y)
AAC 213-53	Melon	Brazil	2002	R. Walcott	I	B13(Y)
AAC 213-54	Unknown	Unknown	Unknown	R. Walcott	I	B12(X)
AAC 213-55	Unknown	Unknown	Unknown	R. Walcott	I	B9(R)
AAC 213-58	Unknown	Unknown	Unknown	R. Walcott	I	B5(M)
AAC 213-59	Melon	China	2009	R. Walcott	I	B5(M)
AAC 213-60	Unknown	Unknown	Unknown	R. Walcott	I	B5(M)
AAC 213-61	Melon	Mexico	2002	R. Walcott	I	B5(M)

[a] Letters in parenthesis represent previously reported haplotypes (WALCOTT et al., 2000; WALCOTT; FESSEHAIE; CASTRO, 2004) while letter/number combinations represent current haplotype designations.
[b]ATCC=American Type Culture Collection.

Table 3 Single nucleotide polymorphisms (SNPs) and amino acid substitutions on seven genes of *Acidovorax citrulli* used for multilocus sequence analysis.

Gene name[a]	Biological function	Length of the open reading frame (nt)	SNPs and amino acid substitutions between groups I/II, nt (aa)
adk	Cellular energy	657	1 (0)
gyrB	DNA replication	2,634	11 (0)
pilT	Twitching motility	1,044	1 (0)
Aave_1548	T3 secreted effector	1,458	33 (16)
gltA	Citrate cycle	1,311	5 (0)
avrRx01	T3 secreted effector	1,245	16 (6)
luxR	Transcriptional regulator	729	3 (2)

[a]Genes named according to AAC 00-1 annotation in NCBI
ND, not determined

Table 4 Genome sizes of representative *Acidovorax citrulli* strains mapped to the references group I (M6) and II (AAC00-1) genomes.

Acidovorax citruli strains	Group	Length (Mb)[a]
AAC 00-1 (Reference)	II	5.35
AAC 213-44	II	5.32
AAC 213-46	II	5.34
AAC 213-47	II	5.33
AAC 213-41	II	5.24
AAC 213-42	II	5.35
AAC 213-48	II	5.28
AAC 213-45	II	5.33
AAC 213-49	II	5.26
M6 (Reference)	I	4,85
AAC 213-50	I	4.86
AAC 213-51	I	4.89
AAC 213-52	I	4.83
AAC 213-53	I	4.86
AAC 213-54	I	4.76
AAC 213-55	I	4.76
AAC 213-58	I	4.83
AAC 213-59	I	4.83
AAC 213-60	I	4.86
AAC 213-61	I	4.88

a Length of the sequence mapped to the reference genomes

Table 5 List of oligonucleotides primers used in this study

Gene [a]	Primer name	Sequence	Size (bp)
adk	2561- 51F	5` CGCGTTCATCTGCCAGAAAT 3`	510
	2561- 560R	5` TTCGCCCAGGTGGAGTAGTA 3`	
gyrB	0003- 834F	5` CATCGTCGTCACCATCCACT 3`	742
	0003- 1575R	5` GCGTGATGATGCTGCTCTTG 3`	
pilT	0637- 31F	5` GTGAAGAACAAGGCCTCCGA 3`	1000
	0637- 1030R	5` TCTCGGGAATCTTGGCCTTG 3`	
Aave_1548	1548- 50F	5` TTTCCATTCGGCAACACGC 3`	870
	1548- 919R	5` AAGAAATCTTCGCGGCTTGC 3`	
gltA	2199- 8F	5` TGGCAGACAACAAAGCAACG 3`	1224
	2199- 1231R	5` GGTCGCCGATCATTTCGTTG 3`	
avrRx01	3062- 18F	5` CCGGCGAAATTCAGCGAATT 3`	741
	3062- 758R	5` AGCTTGATGTACGCGTCGAT 3`	
luxR	3810- 57F	5` GTTGGCCTGCTTTGAACAGG 3`	661
	3810- 717 R	5` TATGAGACCCAGGTTGGCTG 3`	

[a] Partial sequence

FIGURES

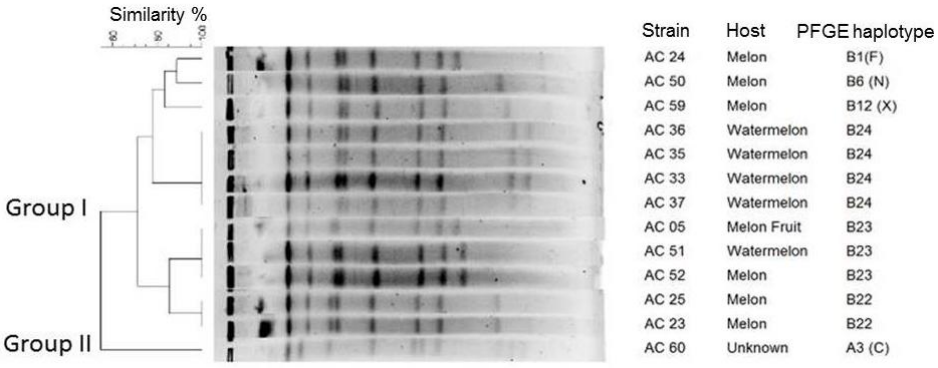

Figure 1 Dendogram indicating the relationship among haplotypes of representative *Acidovorax citrulli* strains form Brazil based on *SpeI* digestion and pulsed field gel electrophoresis. Distance matrix generated by Dice`s coeficient of similarity and dendogram generated by UPGMA.

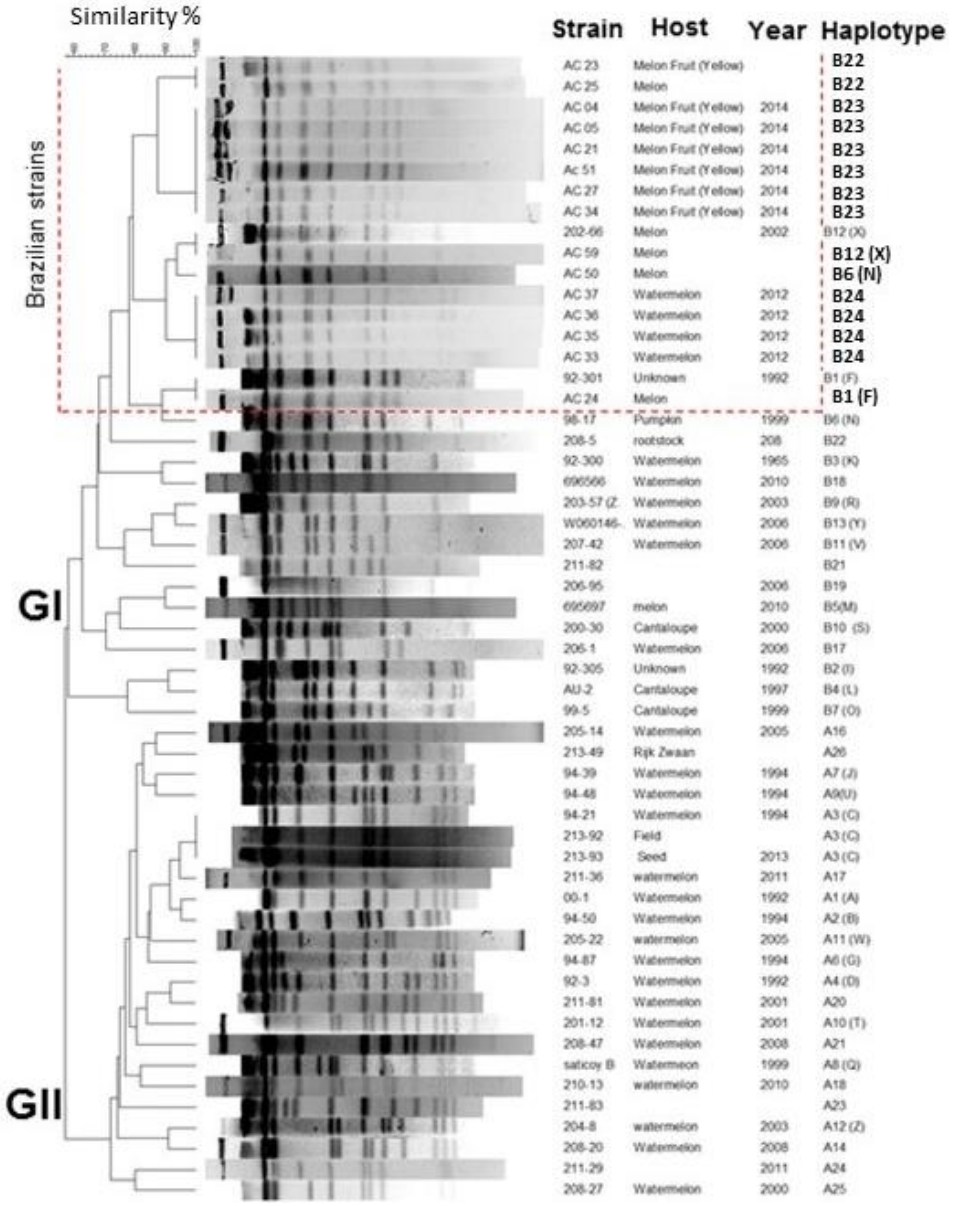

Figure 2 Dendogram showing the relationship between reference and Brazilian *Acidovorax citrulli* strains based on *SpeI* genomic DNA digestion and pulsed field gel electrphoresis. Distance matrix generated by Dice`s coeficient of similarity and dendogram generated by UPGMA.

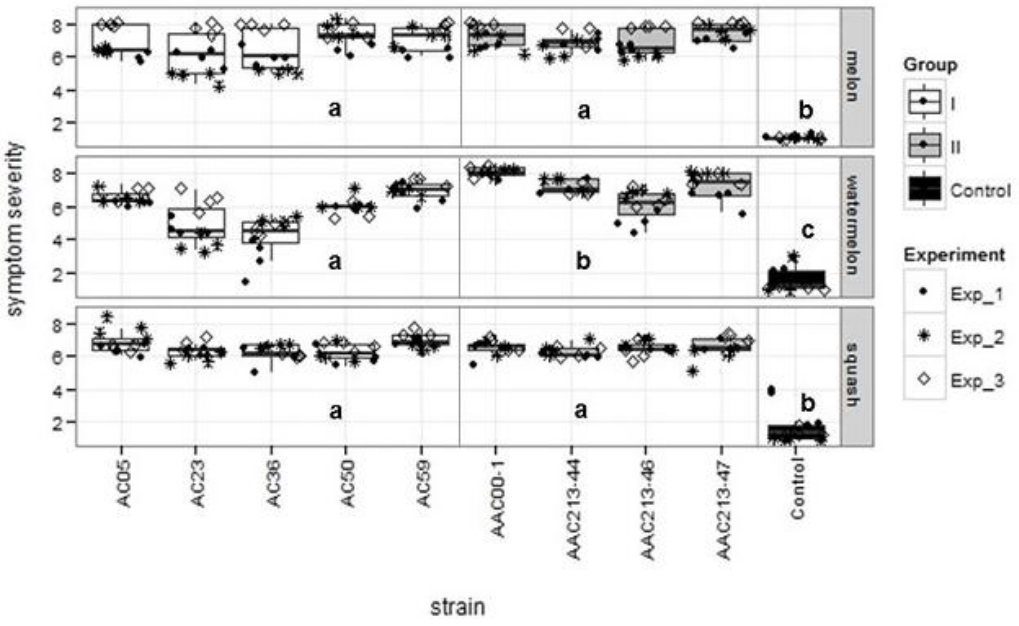

Figure 3 Symptom severity induced by selected *Acidovorax citrulli* strains on different cucurbit (watermelon cv. Crimson Sweet, melon cv. Joaquin Gold and squash cv. Early crookneck Yellow) seedlings. The experiment was repeated three times. Different letters indicate significant differences ($P < 0.05$) among groups (I, II and Control) in each host plant.

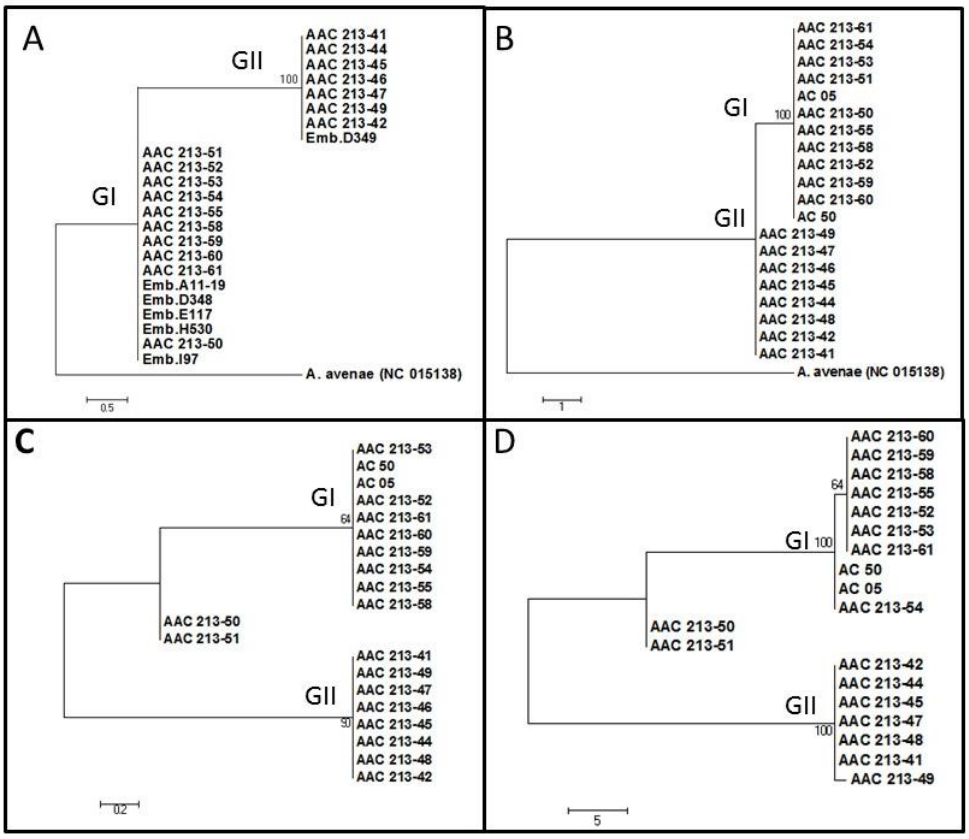

Figure 4 Phylogenetic trees generated from the nucleotide sequences of representative genes of *Acidovorax citrulli* by the maximum parsimony method with 1000 replicates bootstrap test. **GI** = group I and **GII** = group II. **A**, Tree based on *gyrB* sequence data. **B**, Tree based on *gltA* sequence data. **C**, Tree based on *luxR* sequence data. **D**, Tree based on sequences of all 7 genes combined.

G	strain	H	AAC00-1 FA fragments								AAC00-1 RFA fragments								
			1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	9
II	AAC001	A1	100	100	100	100	100	100	100	100	100	100	100	100	100	99.8	100	100	100
	AAC213-47	A2	89.2	93.3	100	100	100	100	100	100	100	100	100	100	100	100	100	99.6	100
	AAC213-44	A3	88.1	100	100	100	100	100	95	99	100	100	100	100	100	100	100	100	100
	AAC213-42	A4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	99.9	100
	AAC213-48	A5	100	100	100	100	1.6	100	95	97	100	100	100	100	100	100	100	100	100
	AAC213-46	A11	100	100	100	100	100	100	100	100	100	95.7	100	100	100	100	100	100	100
	AAC213-49	A26	100	100	100	100	6.6	75	100	100	100	100	100	100	100	100	100	100	100
	AAC213-45	A28	89.2	100	100	100	100	95.7	100	100	100	100	100	100	100	100	100	100	100
I	AAC213-50	B3	6.3	5.3	18.1	27.7	1.9	6.6	12.2	19	90.8	100	100	88.7	100	100	100	99.8	95
	AAC213-61	B5	6.3	5.3	1.6	12.3	1.9	2.3	92.1	84.8	100	100	100	100	99	99.8	99.8	99.8	95
	AAC213-60	B5	6.3	9.2	1.6	12.3	1.9	2.3	25.7	54.2	100	100	100	100	100	100	99.8	99.8	95
	AAC213-58	B5	6.3	5.3	1.6	12.3	1.9	6.6	36.7	54.2	100	100	100	100	100	100	99.8	99.8	95
	AAC213-59	B5	7.9	5.3	1.6	12.3	1.9	6.6	25.7	54.2	100	100	100	100	100	100	99.8	99.8	95
	AAC213-51	B8	6.3	5.3	18.1	29.6	1.9	73.3	9.1	10.5	100	100	100	88.7	100	95	100	99.8	95
	AAC213-55	B9	6.3	8.8	1.6	12.3	1.9	6.6	0	3	100	100	100	100	100	100	99.8	99.8	95
	AAC213-54	B12	6.3	5.5	1.6	12.3	1.9	2.3	0	0	100	100	100	100	100	100	100	99.8	95
	AAC213-53	B13	6.3	5.3	1.6	10.7	1.9	2.3	25.8	54.2	100	100	100	82.3	100	100	99.8	99.8	95
	AAC213-52	B13	6.3	5.3	1.6	12.3	1.9	6.6	38.7	54.2	100	100	100	100	100	100	100	99.8	95
	M6	B21	16.4	21.7	2.1	20.3	4.6	6.8	0.9	3.1	100	98.4	100	100	99	100	100	97.2	99
China	pslb65	N.D	15.1	21.8	2.1	20.3	5	6.8	87.1	90.6	100	99.9	100	100	98.8	99.4	100	96.6	99.6
	tw6	N.D	20.9	21.8	71.5	54.8	6.4	8.5	0.9	3.2	100	100	100	100	98.3	99.2	96.6	94.8	99.5

Figure 5 Coverage percentage of *Acidovorax citrulli* strain AAC00-1 FA fragments 1 to 8 and RFA fragments 1 to 9 in the draft genomes of several group I and II *A. citrulli* strains. The fragments were used as queries in MegaBlast analysis (Geneious 8.1.7) against the strain contigs and the total coverage was calculated. The percentage of coverage is indicated for each fragment/strain. To emphasize the picture, a color scale was used that correlates with the level of coverage. From low to high coverage: dark red, light red, orange, yellow, light green, dark green. G, group; H, PFGE-based haplotype (N.D., not determined).

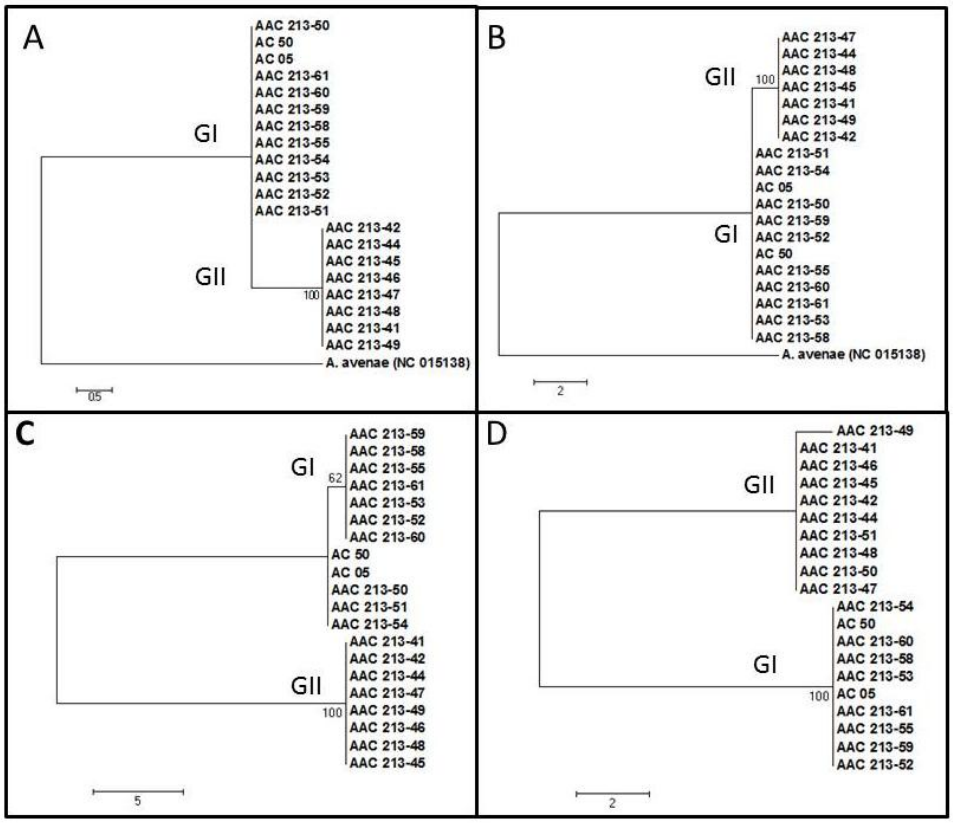

Figure 6 Phylogenetic trees generated from the nucleotide sequences of representative MLSA genes of *Acidovorax citrulli* by the maximum parsimony method with 1,000 replicates bootstrap test. GI=group I and GII=group II. A, Tree based on *adk* sequence data. B, Tree based on *pilT* sequence data. C, Tree based on *Aave_1548* sequence data. D, Tree based on *avrRx01* sequence data.

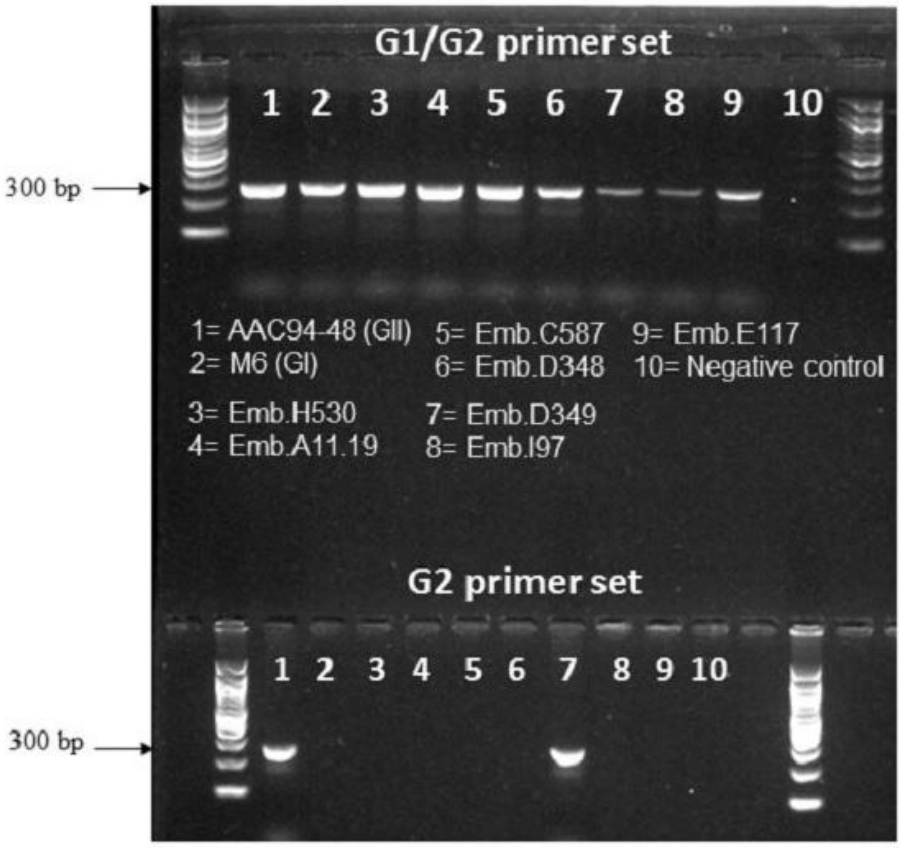

Figure 7 PCR-based assay for identification of group I (G1/G2 primer set) and of group II *Acidovorax citrulli* strains (G2 primer set). Numbers 1 and 2 represent the positive controls of group II and I respectively. Numbers 3 to 9 represent the Brazilian *A. citrulli* strains, and number 10 is the negative control

CHAPTER III

The influence of temperature on *in vitro* growth and survival of *Acidovorax citrulli*

ABSTRACT

Acidovorax citrulli, the causal agent of bacterial fruit blotch of cucurbits (BFB), has threaten the watermelon and melon production worldwide. This bacterial species is differentiated into two genetically and pathogenically distinct groups of strains. Group I strains, including the American Type Culture Collection (ATCC) type strain, were mainly isolated from non-watermelon cucurbit hosts, while group II strains were mainly recovered from watermelon. In Brazil, BFB outbreaks have occurred predominantly on melon producing fields and have been mainly associated with group I *A. citrulli* strains. Despite its economic importance, and the efforts employed to combat BFB in the cucurbit industry, the basic aspects of *A. citrulli* ecology have been rarely addressed. To better understand how the genetically distinct groups of *A. citrulli* are affected by environmental changes, we investigated the effect of temperature on *in vitro* growth and survival of *A. citrulli* group I and II strains on filter paper. Among the 15 distinct haplotypes of each different group (group I and II), we observed a differential effect of temperature on *in vitro* growth of representative group I and II *A. citrulli* strains. Specifically, out of 18 group II strains tested, all grew at 40°C and 41°C. On the other hand, only three group I strains (haplotypes B8(P), B3(K) and B15) out of 15 grew at 40°C. Three strains representing haplotype B8(P) were the only group I strains that grew at 41°C. The population densities of the representative group I and II *A. citrulli* strains (M6 and AAC18-ST respectively) were slightly affected when desiccated on filter paper discs and kept at 25°C for 4 weeks. The M6 population was reduced from 3.36×10^6 to 4.46×10^5 CFU/mm^2 and AAC8-1ST from 1.08×10^7 to 8×10^6 CFU/mm^2. At 41°C, the group I strain M6 had its population density severely reduced (from 10^7 to 10^3 CFU/mm^2) within 4 weeks post inoculation. Within the same period of time the population density recovered of group II strain AAC18-ST declined from 10^7 to 10^6 CFU/mm^2. In summary, our results showed that temperature was limiting for *A. citrulli* group I strains on either nutrient-rich or -depleted conditions. This study provides the first evidence to suggest that temperature might be a driver in the ecological adaptation of *A. citrulli* populations. Hence, this information will contribute to the development of more effective strategies to manage this threatening disease.

Keywords: Bacterial fruit blotch**.** *Cucumis melo*. Population density. Heat. Genetic variability.

INTRODUCTION

Bacterial fruit blotch (BFB) is an economically important disease of melon and watermelon worldwide (BURDMAN; WALCOTT, 2012). In Brazil, BFB has been a major problem to the melon production in Northeastern region, since watermelon outbreaks are sporadic (CONCEIÇÃO et al., 2014; SALES JUNIOR et al., 2007). The causal organism is a Gram-negative, nonfluorescent, rod-shaped, oxidase-positive bacterium, named *Acidovorax citrulli* (syn. *Acidovorax avenae* subsp. *citrulli*) (SCHAAD et al., 2008; WILLEMS et al., 1992). The bacterial species is differentiated into two genetically and pathogenically distinct groups of strains (BURDMAN et al., 2005; WALCOTT; FESSEHAIE; CASTRO, 2004). Group I isolates, including the American Type Culture Collection (ATCC) type strain were mainly recovered from non-watermelon cucurbit hosts, such as cantaloupe melon (*Cucumis melo var. cantaloupensis*), cucumber (*Cucumis sativus*), honeydew melon (*C. melo var. indorus*), squash and pumpkin (*Cucurbita pepo*, *Cucurbita maxima* and *Cucurbita moschata*) whereas group II isolates were mainly recovered from watermelon (BURDMAN et al., 2005; DUTTA et al., 2012; WALCOTT; FESSEHAIE; CASTRO, 2004). While group I isolates were moderately aggressive on a range of cucurbit hosts, group II isolates were highly aggressive on watermelon but moderately aggressive on non-watermelon hosts (WALCOTT et al., 2000; WALCOTT; FESSEHAIE; CASTRO, 2004).

Contaminated cucurbit seeds are the main source of inoculum for long distances dissemination (LATIN; HOPKINS, 1995). Even low levels of *A. citrulli*-infected seedlots in transplant houses can initiate BFB outbreaks under favorable conditions of temperature and relative humidity (GITAITIS; WALCOTT, 2007). In the absence of effective chemical control options or resistant cultivars, BFB management relies heavily on exclusion of *A. citrulli* from cucurbit production systems (RANE; LATIN, 1992). At present, the most common strategies are exclusion by seed healthy testing, BFB management in seed production fields, and

certification of transplants in greenhouses (GITAITIS; WALCOTT, 2007). Despite the considerable efforts to exclude *A. citrulli* from agricultural systems, BFB is still quite common on transplant production facilities and melon production fields in Brazil (CONCEIÇÃO et al., 2014; OLIVEIRA et al., 2007).

The cucurbitaceous weed citron melon (*Citrullus lanattus* var. *citroides*), common in southeastern USA, has been reported as a weed host of *A. citrulli*. The bacterium was reported to cause BFB symptoms on citron melon fruits. The strains isolated from symptomatic fruits were pathogenic on watermelon seedlings, suggesting a potential role of the weed in the spread of *A. citrulli* to watermelon in the field (ISAKEIT; BLACK; JONES, 1998). The long-term survival of *A. citrulli* in citron melon seeds was investigated by Dutta et al. (2014). The results indicated that *A. citrulli* can survive/overwinter in citron melon seeds at 4°C and 50% RH for at least 7 years. In northeastern Brazil, *Acidovorax citrulli* has been reported to survive in cucurbitaceous weeds (*Luffa cylindrical* and *Momordica charantia* L.) as alternative hosts in melon producing fields (NASCIMENTO; MARIANO; SILVA, 2004).

Destruction of weeds and volunteers is an important step in BFB management (GITAITIS; WALCOTT, 2007). Furthermore, it is also important to know the factors affecting long-term survival of phytopathogenic bacteria, including host genotype, environmental conditions (temperature and relative humidity) and sensitivity to desiccation. Studies investigating the basic aspects of *A. citrulli* ecology are still lacking. Therefore, the objective of this research was to investigate the effects of temperature on *in vitro* growth and survival of the two genetically distinct groups of *A. citrulli*.

MATERIALS AND METHODS

Bacterial strains. *Acidovorax citrulli* strains used in this study are listed in table 1. The strains were routinely grown on nutrient agar plates (NA, Difco) at

28°C for 48h. For cells suspensions, single colonies were transferred to 5 mL of Nutrient Broth (NB, Himedia) and incubated at 28°C for 18h under constant agitation (270 RPM) in an incubator shaker (Innova, New Brunswick, NJ). The optical density of the cell suspensions was adjusted to 0.5 at a wavelength of 600 nm (~0.5×10^8 CFU/mL) using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY). For the survival assay, the constitutively kanamycin-resistant strains M6 (plasmid pGT-trp) (CHALUPOWICZ et al., 2015) and AAC 8-1ST (plasmid pAG408) (WALCOTT; GITAITIS; CASTRO, 2003) were grown on NA plates amended with kanamycin (100 µg/mL).

Effect of temperature on *in vitro* growth of *A. citrulli* strains. *Acidovorax citrulli* strains representing 15 group I and 18 group II haplotypes (Table 1) were used to determine the influence of temperature on *in vitro* growth of *A. citrulli* groups. Strains were grown in 5 mL of Luria Bertani broth (LB) at 28°C for 16 h with agitation at 250 RPM in an incubator shaker. Cells suspensions were adjusted spectrophotometrically to a concentration of 10^4 CFU/mL in 0.1 M PBS. Cells suspensions of each strain (40 µL) were transferred into a sterile Honeycomb microplate containing 360 µL of LB/well. Each strain was replicated in ten wells. Bacterial growth was measured at OD600 using a Bioscreen microplate reader (Bioscreen-C Automated Growth Curve Analysis System-USA). Cultures were grown at 40°C, 41°C and 45°C with agitation at 270 RPM. Optical density was measured every 30 min for 39 h. This experiment was conducted twice and statistical analysis was performed using the R environment. One-way analysis of variance (ANOVA) was used to analyze the mean values of final optical densities from each *A. citrulli* group at different temperatures.

Influence of temperature on survival of *Acidovorax citrulli* strains on filter paper. Since tolerance to desiccation and temperature are important factors for bacterial survival, we investigated the influence of temperature on survival of two genetically distinct *A. citrulli* strains (M6 and AAC 8-1ST) on filter paper. Cells suspension were generated as described above and were mixed (1:1) to a

peptone-gelatin solution according to Takatsu (1980). Discs (50 mm diameter) of sterile filter paper (Whatman Inc., Sanford, ME) were incubated in each suspension for 10 hours followed by air-drying in a laminar flow chamber at ~25°C for 12 h. After drying, each piece of filter paper was incubated in a partially open Petri dish in a glass desiccator cabinet containing silica gel for 1 day prior to incubation at different temperatures (25 and 41°C). After 0 (filter paper processed immediately after inoculation), 1, 2, 3, and 4 weeks post inoculation (WPI), samples of filter paper (~6 mm^2) were removed and macerated using a sterile glass rod in a sterile 1.5 ml microcentrifuge tube containing 1 ml of 0.1M PBS. Macerates were vortexed for 30 s (Vortex Genie2; Fisher Scientific International, Pittsburgh, PA) and bacterial populations were estimated by ten-fold serial dilution in PBS followed by spread plating 100 µl of aliquots onto NA plates amended with kanamycin (100 µg/mL). Bacterial colonies were counted after 24 to 72 h of incubation. In addition, confirmation of the identity of the bacterial colonies was conducted by PCR assay using *A. citrulli*-specific primers as previously described (BAHAR et al., 2008). This experiment was conducted twice and each treatment had three replicates per experiment. Bacterial populations (CFU/mm^2) were log10-transformed prior to analysis. Data were pooled to determine mean bacterial populations after preliminary statistical analysis showed no significant ($P > 0.05$).

RESULTS

***Acidovorax citrulli* group II strains have higher growth temperature maxima.** Among the 15 group I and 18 group II strains tested (each strain representing a distinct PFGE haplotype), we observed a significant reduction in growth of the group I strains at 40°C ($P = 0.003$) and 41°C ($P < 0.001$) compared to the group II strains (Fig. 2). Interestingly, some group I strains [AAC 207-41, haplotype B15; AAC 92-300, haplotype B3(K); AAC 208-12, IA 58-116 and IA58- 1, haplotype B8(P)] grew at 40°C. At 41°C, the only group I strains that

grew were the representatives of haplotype B8(P) (AAC 208-12, IA 58-116 and IA 58-1). In contrast, all the group II strains tested grew at 40°C and 41°C (Table 1). No *A. citrulli* strain grew at 45°C (Fig. 2).

Survival of *Acidovorax citrulli* group I strain is affected by temperature. The survival of *A. citrulli* strains on filter paper was differentially affected by temperature. At 0 weeks post inoculation (WPI) the population of the group II *A. citrulli* strain (AAC8-1ST) and the group I (M6) ranged from approximately 10^6 to 10^7 CFU/mm^2 (Fig. 3 and 4). Subsequently, the paper discs were kept at 41 and 25°C and the population dynamics of the group I and II strains were accessed weekly. The survival of strain M6 at 41°C was significantly affected. The M6 population dropped from 10^7 to 10^5 CFU/mm^2 within 2 WPI, while the AAC8-1ST population from 10^7 to 10^6 at the same period of time (Fig. 3). From 2 to 4 WPI the M6 population continued to decrease and reached ~ 10^3 CFU/mm^2. On the other hand, the AAC8-1ST population size remained practically the same and was almost twice larger than M6 (Fig. 3). Finally, the population density of *A. citrulli* strains at 25°C were just slightly affected within the 4 WPI. The M6 population was reduced from 3.36 x 10^6 to 4.46 x 10^5 CFU/mm^2 and AAC8-1ST from 1.08 x 10^7 to 8 x 10^6 CFU/mm^2.

DISCUSSION

Acidovorax citrulli is an economically important pathogen of cucurbitaceous crops worldwide. Despite advances in recent years, the effectiveness of seed treatments and pathogen exclusion by seed health testing remains limited in minimizing losses caused by BFB. Evidence for the existence of at least two genetically distinct groups within *A. citrulli* has been widely reported (ECKSHTAIN-LEVI et al., 2014; FENG et al., 2009; WALCOTT; FESSEHAIE; CASTRO, 2004; YAN et al., 2013). These two groups differ on their host preference association, substrate utilization profiles and genomic DNA sequence

(ECKSHTAIN-LEVI et al., 2014; SHAVIT et al., 2015; WALCOTT et al., 2000). Based on previous studies on genetic diversity of *A. citrulli* populations worldwide, suggesting that group I and II may be adapted for different ecological niches, we hypothesized that these groups may differ in their maximal growth temperatures. Growth at 41°C is a common characteristic of genera (*Comomonas*, *Hydrogenophaga* and *Variovorax*) of the *Comomonadaceae* family. Accordingly, growth at 41°C differentiates phytopathogenic *Acidovorax* species from other plant pathogens, such as *Pseudomonas*, *Ralstonia* and *Burkholderia* (SCHAAD et al., 2001). The influence of temperature, pH, sodium chloride concentration and carbon sources on *A. citrulli* growth was previously investigated by Cavalcanti et al. (2005); however, a limited number of strains was used in this study. More specifically, only four strains from melon were analyzed which does not represent the genetic variability of *A. citrulli*. While growth at 41°C was previously reported to be a feature of *A. citrulli*, in the current study we observed difference in the ability of groups I and II *A. citrulli* strains to grow at this temperature. Specifically, all group II strains grew at 41°C while most group I strains did not. Interestingly, a representative strain from the group I haplotype B8(P) (IA58-1) that grew at 41°C, was recovered from plant introductions at the USDA North Central Plant Introduction Station Ames, Iowa, USA, and is closely related to the type strain, ATCC 29625 (one PFGE polymorphism) (WALCOTT; FESSEHAIE; CASTRO, 2004). However, ATCC 29625 did not grow at 41°C. These *A. citrulli* strains were previously reported to be atypical strains based on substrate utilization profiles, induction of BFB symptoms on watermelon fruits or HR on tobacco and might represent a distinct subgroup among the group I population (WALCOTT et al., 2000; WALCOTT; FESSEHAIE; CASTRO, 2004). Similarly, the sequence analysis of the virulence-associated genes (putative T3S effector gene *Aave_1548* and the *luxR* homologue gene) showed that the representative strains of haplotypes B8(P) (AAC 213-51) and B3(K) (AAC 213-50) are a distinct subgroup of group I (*unpublished data*).

Although economically important, the basic aspects of *A. citrulli* ecology have been poorly understood. As a highly specialized seedborne bacterium, *A. citrulli* can survive associated with citron melon (*Citrullus lanatus* var. *citroides*) seeds for at least 7 years during the storage conditions (4°C and 50 % of relative humidity) (DUTTA et al., 2014). Storage of watermelon and melon seeds at low temperature (5°C or below) also contributed to *A. citrulli* survival for 34 and 40 years respectively (BLOCK; SHEPHERD, 2008). Another strategy for the survival of *A. citrulli* in the field is the association with non-host or volunteer plants between season. Potential alternative hosts of *A. citrulli* were investigated on cucurbitaceous weeds in Northeastern Brazil (NASCIMENTO; MARIANO; SILVA, 2004). According to the authors, the naturally occurring weeds *Cucumis myriocarpus*, *Mumurdica charantia* and *Luffa aegyptiaca* can held *A. citrulli* in local melon fields from season to season. Therefore, the destruction of cucurbitaceous weeds is an important strategy for the integrated management of BFB in melon fields.

The longevity of primary inoculum is essential for the success of bacterial diseases and depends upon the ability of plant pathogenic bacteria to escape or endure adverse environmental conditions (SCHUSTER; COYNE, 1974). Among the environmental factors that impact microbial invasion and host colonization, temperature is one of the most important.

In nature, vital metabolic activities, such as protein synthesis and enzymatic reactions are generally temperature-sensitive. Temperature fluctuates both, daily and seasonally and is considered as one of the key determinants for disease epidemics (AGRIOS, 2005), nutrient assimilation and growth of microorganisms (HUERTA; MILLING; ALLEN, 2015; KOCKS et al., 1998). Regarding the plant-microbe interactions, the production of *Pseudomonas syringae* phytotoxin coronatine is induced at 18°C and repressed at 28°C (WEI; SNEATH; BEER, 1992). Similarly, plant immunity is also linked with ambient temperature changes. Cheng et al. (2013) showed that effector-triggered immunity (ETI)

signaling in *Arabidopsis* is preferentially activated at relatively low temperatures (10 - 23°C), whereas pattern-triggered immunity (PTI) signaling is activated at moderately elevated temperatures (23 - 33°C). The temperature preference for ETI and PTI signaling activation was coincident with the temperature effect on bacterial physiology; the elevated temperatures inhibit bacterial effector secretion but promote bacterial proliferation.

Analyzing the effect of different temperatures on *A. citrulli* severity on artificially-inoculated melon fruits, Silveira et al. (2004) observed larger external lesions on melon fruits when kept at 35°C, while deeper lesions at 30°C. At 40°C, a significant reduction on external diameter and depth of lesions were observed. These findings suggest that the pathogen`s virulence may have been affected by the reduction of cellular metabolism at 40°C. Interestingly, the strain used in this study was originally isolated from melon in northeastern Brazil.

Distinct ecological characteristics between closely related bacteria often involves change in biochemical activities that results from gene gain, gene loss, or mutations that change the biochemical activities of proteins, resulting in ecological differentiation without genetic isolation (RETCHLESS; LAWRENCE, 2012). It is possible that group I and II *A. citrulli* strains have gained or lost genes during their evolutionary history. Examples of such genes include the putative type three secreted effector gene *Aave_2708* (homologue to outer protein J of *Xanthomonas campestris* pv. *vesicatoria*) (ECKSHTAIN-LEVI et al., 2014) and the *vapB-vapC* operon, which encodes a stable toxin (VapC), and a labile antitoxin (VapB) (toxin-antitoxin system**)** (SHAVIT et al., 2015), that are present in group II *A. citrulli* strains but absent in group I strains. These differences might reflect in the competitive fitness or adaptation to a specific ecological niche or host.

In closing, we report for the first time the influence of genetic variability on capacity of *A. citrulli* strains to differentially endure changes in temperature. The temperature showed to be of paramount importance for *A. citrulli* ecology, as a limiting factor regardless of the environment`s nutrient (rich or depleted)

condition. This information can definitely lead to the development of a more effective strategy of plant breeding and cultivar trials for BFB management. Since the genome sequence of group I and II *A. citrulli* strains are already available on public databases, including AAC00-1 and M6, the molecular basis of the phenotype presented by these strains (Fig. 1) could be further investigated through mutagenesis studies.

REFERENCES

AGRIOS, G. **Plant pathology**. 5th ed. San Diego: Academic, 2005. 922 p.

BAHAR, O. et al. New subspecies-specific polymerase chain reaction-based assay for the detection of *Acidovorax avenae* subsp. *citrulli*. **Plant Pathology**, Honolulu, v. 57, n. 4, p. 754-763, Aug. 2008.

BLOCK, C. C.; SHEPHERD, L. M. Long-term survival and seed transmission of A*cidovorax avenae* subsp. *citrulli* in melon and watermelon seed. **Plant Health Progress**, Saint Paul, Dec. 2008. Available from: <https://www.plantmanagementnetwork.org/pub/php/brief/2008/melon/>. Accessed: Sept. 15, 2015.

BURDMAN, S. et al. Molecular, physiological, and host-range characterization of *Acidovorax avenae* subsp . *citrulli* isolates from Watermelon and Melon in Israel. **Plant Disease**, Quebec, v. 89, n. 12, p. 1339-1347, Dec. 2005.

BURDMAN, S.; WALCOTT, R. Pathogen profile *Acidovorax citrulli*: generating basic and applied knowledge to tackle a global threat to the cucurbit industry. **Molecular Plant Pathology**, London, v. 13, n. 8, p. 805-815, Oct. 2012.

CAVALCANTI, M. T. et al. Growth of *Acidovorax avenae* subsp. *citrulli* under different variable temperature, pH, sodium chloride concentrations and carbon sources. **Ciência Rural**, Santa Maria, v. 35, n. 6, p. 1313-1318, dez. 2005.

CHALUPOWICZ, L. et al. Cotyledons are the main source of secondary spread of *Acidovorax citrulli* in melon nurseries. **Plant Pathology**, Honolulu, v. 64, n. 3, p. 528-536, June 2015.

CHENG, C. et al. Plant immune response to pathogens differs with changing temperatures. **Nature Communications**, London, v. 4, p. 2530, 2013.

CONCEIÇÃO, C. S. et al. Combined effect of yeast and silicon on the control of bacterial fruit blotch in melon. **Scientia Horticulturae**, Amsterdam, v. 174, n. 1, p. 164-170, July 2014.

DUTTA, B. et al. Location of *Acidovorax citrulli* in infested watermelon seeds is influenced by the pathway of bacterial invasion. **Phytopathology**, Saint Paul, v. 102, p. 461-468, 2012.

DUTTA, B. et al. Long-term survival of *Acidovorax citrulli* in citron melon (*Citrullus lanatus* var. *citroides*) seeds. **Plant Pathology**, Honolulu, v. 63, n. 5, p.

1130-1137, Jan. 2014.

ECKSHTAIN-LEVI, N. et al. Comparative analysis of type III secreted effector genes reflects divergence of *Acidovorax citrulli* strains into three distinct lineages. **Phytopathology**, Saint Paul, v. 104, n. 11, p. 1152-1162, Nov. 2014.

FENG, J. et al. Multilocus sequence typing reveals two evolutionary lineages of *Acidovorax avenae* subsp. *citrulli*. **Phytopathology**, Saint Paul, v. 99, n. 8, p. 913-920, Aug. 2009.

GITAITIS, R.; WALCOTT, R. The epidemiology and management of seedborne bacterial diseases. **Annual Review of Phytopathology**, Palo Alto, v. 45, p. 371-397, 2007.

HUERTA, A. I.; MILLING, A.; ALLEN, C. Tropical strains of *Ralstonia solanacearum* outcompete race 3 biovar 2 strains at lowland tropical temperatures. **Applied and Environmental Microbiology**, Washington, v. 81, n. 10, p. 3542-3551, May 2015.

ISAKEIT, T.; BLACK, M. C.; JONES, J. B. Natural Infection of citronmelon with *Acidovorax avenae* subsp. *citrulli*. **Plant Disease**, Quebec, v. 82, n. 3, p. 351, Mar. 1998.

JOHNSON, K. L. et al. Efficacy of a nonpathogenic *Acidovorax citrulli* strain as a biocontrol seed treatment for bacterial fruit blotch of cucurbits. **Plant Disease**, Quebec, v. 95, n. 6, p. 697-704, June 2011.

KOCKS, C. G. et al. Survival and Extinction of *Xanthomonas campestris* pv *campestris* in soil. **European Journal of Plant Pathology**, Dordrecht, v. 104, p. 911-923, 1998.

LATIN, R. X.; HOPKINS, C. L. Bacterial fruit blotch of watermelon, the hypothetical exam question becomes reality. **Plant Disease**, Quebec, v. 79, p. 761-765, 1995.

NASCIMENTO, A. R. P.; MARIANO, R. L. R.; SILVA, E. I. Hospedeiros alternativos de *Acidovorax avenae* subsp. *citrulli*. **Horticultura Brasileira**, Brasília, v. 22, n. 3, p. 345-349, set. 2004.

OLIVEIRA, J. C. et al. Caracterização de isolados de *Acidovorax avenae* subsp. *citrulli*. **Fitopatologia Brasileira**, Brasília, v. 32, n. 6, p. 480-487, dez. 2007.

RANE, K.; LATIN, R. Bacterial fruit blotch of watermelon: association of the

pathogen with seed. **Plant Disease**, Quebec, v. 76, p. 509-512, 1992.

RETCHLESS, A. C.; LAWRENCE, J. G. Ecological adaptation in bacteria: speciation driven by codon selection. **Molecular Biology and Evolution**, Oxford, v. 29, n. 12, p. 3669-3683, June 2012.

SALES JUNIOR, R. et al. Eficiência de Acibenzolar-S-Methyl e Oxicloreto de Cobre no controle de *Acidovorax avenae* subsp . *citrulli* , agente causal da "Mancha-Aquosa" no meloeiro. **Revista de Biologia e Ciências da Terra**, Campina Grande, v. 7, n. 1, p. 66-70, jul. 2007.

SCHAAD, N. W. et al. Reclassification of subspecies of *Acidovorax avenae* as *A. avenae* (Manns 1905) emend.; *A. cattleyae* (Pavarino, 1911) comb. nov.; *A. citrulli* Schaad et al.; 1978) comb. nov.; and proposal of *A. oryzae* sp. nov. **Systematic and Applied Microbiology**, Stuttgart, v. 31, n. 8, p. 434-446, Dec. 2008.

SCHUSTER, M. L.; COYNE, D. P. Survival mechanisms of phytopathogenic bacteria. **Annual Review of Phytopathology**, Palo Alto, v. 12, p. 199-221, 1974.

SHAVIT, R. et al. The vapB-vapC operon of *Acidovorax citrulli* functions as a bona-fide toxin-antitoxin module. **Frontiers in Microbiology**, Lausanne, v. 6, p. 1-12, Jan. 2015.

SILVEIRA, E. B. et al. Influencia da temperatura, umidade, concentracao de inoculo de Acidovorax avenae subsp. citrulli e idade do fruto no desenvolvimento da mancha-aquosa em melão. **Fitopatologia Brasileira**, Brasília, v. 29, n. 1, p. 34-38, fev. 2004.

TAKATSU, A. Preservação das bactérias fitopatogênicas pelo método de dessecação. **Fitopatologia Brasileira**, Brasília, v. 5, p. 461, 1980. Resumo.

WALCOTT, R. R. et al. Investigating intraspecific variation of *Acidovorax avenae* subsp. *citrulli* using DNA fingerprinting and whole cell fatty acid analysis. **Phytopathology**, Saint Paul, v. 90, n. 2, p. 191-196, Feb. 2000.

WALCOTT, R. R.; FESSEHAIE, A.; CASTRO, A. C. Differences in pathogenicity between two genetically distinct groups of *Acidovorax avenae* subsp. *citrulli* on cucurbit Hosts. **Journal of Phytopathology**, Berlin, v. 152, n. 5, p. 277-285, May 2004.

WALCOTT, R. R.; GITAITIS, R. D.; CASTRO, A. C. Role of blossoms in watermelon seed infestation by *Acidovorax avenae* subsp. *citrulli*. **Phytopathology**, Saint Paul, v. 93, n. 5, p. 528-534, May 2003.

WEI, Z. M.; SNEATH, B. J.; BEER, S. V. Expression of *Erwinia amylovora* hrp genes in response to environmental stimuli. **Journal of Bacteriology**, Washington, v. 174, p. 1875-1882, 1992.

WILLEMS, A. et al. Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov.; comb. nov.; *Acidovorax avenae* subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax konjaci*. **International Journal of Systematic Bacteriology**, Reading, v. 42, p. 107-119, 1992.

YAN, S. et al. Genetic diversity analysis of *Acidovorax citrulli* in China. **European Journal of Plant Pathology**, Dordrecht, v. 136, n. 1, p. 171-181, May 2013.

Table 1 Effect of temperature on *in vitro* growth of group I and II *Acidovorax citrulli* strains. The ability of strains representing different *SpeI*-digested pulse field gel electrophoresis haplotypes to grow at 40° and 41°C is shown.

Group	Strain	PFGE-haplotype	Growth [a]	
			at 40°C	at 41°C
I	AC 24	B1(F)	-	-
	AC 36	B25	-	-
	AC 50	B12(X)	-	-
	M6	B21	-	-
	AAC 207-41	B15	+	-
	AAC 207-42	B11(V)	-	-
	AAC 98-17	B6(N)	-	-
	AAC 92-300	B3(K)	+	-
	AAC 92-305	B2(I)	-	-
	AAC 213-56	B9(R)	-	-
	AAC 206-105	B5(M)	-	-
	AAC 200-30	B10(S)	-	-
	AAC 206-2	B16	-	-
	AAC 208-12	B8(P)	+	+
	IA 58-116	B8(P)	+	+
	IA 58-1	B8(P)	+	+
II	AAC 00-1	A1(A)	+	+
	AAC 94-21	A3(C)	+	+
	AAC 211-36	A17	+	+
	AAC 205-14	A16	+	+
	AAC 208-47	A21	+	+
	AAC 206-79	A22	+	+
	AAC 94-36	A2(B)	+	+
	AAC 92-3	A4(D)	+	+

(*Continued….)*

Table 1 (*Continued*...)

Group	Strain	PFGE-haplotype	Growth [a]	
			at 40°C	at 41°C
II	AAC 94-87	A6(G)	+	+
	SATICOY B	A8(Q)	+	+
	AAC 94-48	A9(U)	+	+
	AAC 201-12	A10(T)	+	+
	AAC 204-5	A12(Z)	+	+
	AAC 208-20	A14	+	+
	AAC 210-12	A18	+	+
	AAC 211-83	A23	+	+
	AAC 208-27	A25	+	+
	AAC 213-45	A28	+	+
	AAC 8-1ST	A9(U)	+	+

[a] (OD600 > 0,350)

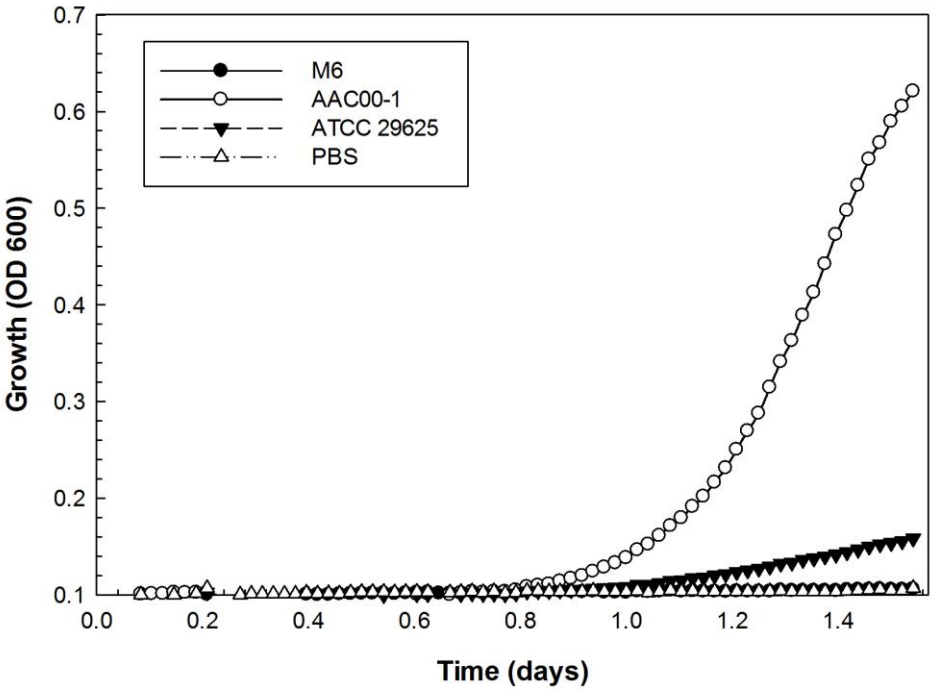

Figure 1 Population growth of representative *Acidovorax citrulli* strains (M6= group I, AAC00-1= group II and ATCC 29625= group I, type strain) in Luria Bertani broth at 41°C. 0.1 M phosphate buffer saline buffer (PBS) was used as a negative control. Each mesurement represents the mean of ten thecnical replicates. The experiment was repeated twice.

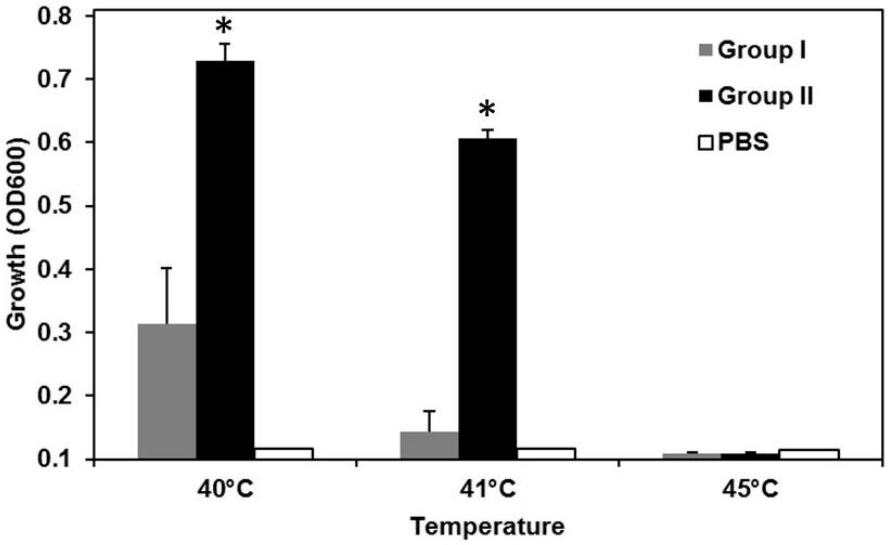

Figure 2 Effect of temperature on growth of group I and II *Acidovorax citrulli* strains. Representative strains from 15 group I and 18 group II haplotypes were grown in Luria Bertani broth for 39 h. Bars represent the mean value of the final optical density of each group. Each strain had ten technical replicates and the experiment was repeated twice. Asterisks represent significant difference between groups I and II at 40°C and 41°C (P = 0.003 and < 0.001 respectively). PBS buffer was inoculated as a negative control.

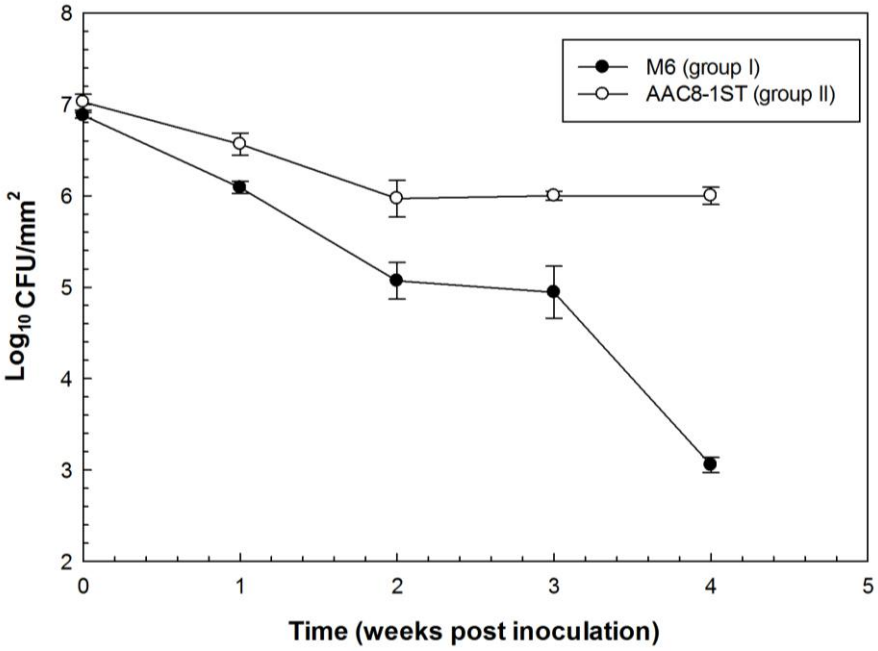

Figure 3 Survival of group I and II *Acidovorax citrulli* strains on filter paper at 41°C. Each data point represents the mean $\log_{10}$ CFU/mm^2 value estimated by dilution plating on NA medium. Bars represent standard errors of the means (n = 6).

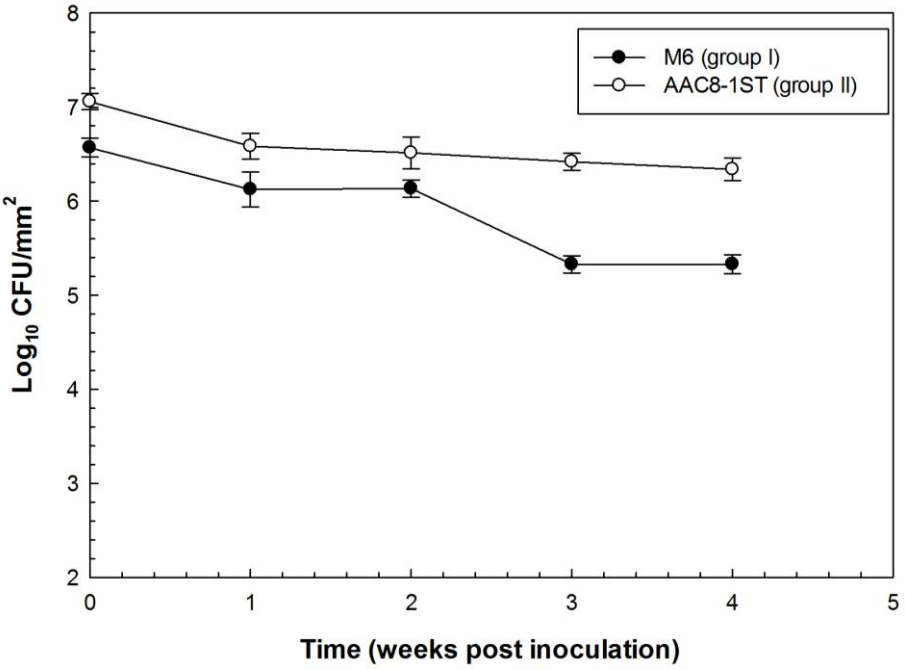

Figure 4 Survival of group I and II *Acidovorax citrulli* strains on filter paper at 25°C. Each data point represents the mean $\log_{10}$ CFU/mm^2 value estimated by dilution plating on NA medium. Bars represent standard errors of the means (n = 6).

CHAPTER IV

Protocol for *Acidovorax citrulli* detection on melon seeds

ABSTRACT

Bacterial fruit blotch (BFB) of cucurbits is an economically important disease of melon and watermelon crops worldwide. This destructive disease has the seed as its main source of long distances inoculum dissemination. In order to improve the detection of *Acidovorax citrulli* on cucurbitaceous seeds, we developed a new primer set that amplifies the DNA sequence of different Brazilian haplotypes and compared the efficiency of Bio-PCR, IC-PCR and qPCR on artificially and naturally infested seed lots. In contrast to the results obtained from PCR using previously reported primers for BFB TaqMan assay, the new primer set described here was able to detect the newly reported *A. citrulli* haplotypes (B22 and B23) as well as the type strain (ATCC 29625; syn. IBSBF 1851). Through PCR, as expected, a low concentration of cells suspension was detected. Direct PCR from the seed washes failed to detect *A. citrulli*, due to the presence of inhibitory compounds from seeds. Bio-PCR was able to detect *A. citrulli* in seed lots (n=1000 seeds) with 1 % of infection and higher. Single tube immunocapture PCR (IC-PCR) was more sensitive than Bio-PCR and was able to detect *A. citrulli* in seed lots with at least 0,1% of infection. Moreover, IC-PCR was 24 hours faster than Bio-PCR. Finally, similar to IC-PCR, quantitative real time PCR (qPCR) showed to be sensitive on *A. citrulli* detection (seed lots with minimum of 0,1% of infection) and showed to be suitable for high-throughput testing of seed samples.

Keywords: *Cucumis melo*. PCR-based assay. Bacterial fruit blotch. Seed health testing.

INTRODUCTION

Bacterial fruit blotch of cucurbits (BFB) is a seed-transmitted disease, caused by the Gram- negative bacterium *Acidovorax citrulli* (SCHAAD et al., 2008). Bacterial fruit blotch has been a serious threat to cucurbit seed and fruit industry. The destructive potential of BFB was fully realized during the late 1980`s, following severe outbreaks in watermelon fields in the United States that led to high yield losses of up to 100% (LATIN; HOPKINS, 1995; RANE; LATIN, 1992). Since then, the disease has spread worldwide and been reported in many other cucurbits, including pumpkin, squash and cucumber (BURDMAN; WALCOTT, 2012). One reason for its rapid spread was the increased trade of contaminated seed lots. Although weeds, contaminated plant debris and volunteer seedlings can be sources of *A. citrulli* inoculum, the most significant inoculum source is infested seeds (FENG et al., 2013; RANE; LATIN, 1992).

To date, comprehensive BFB management guidelines rely heavily on seed health testing (BAHAR et al., 2008; HA et al., 2009; WALCOTT et al., 2006). Currently, seedling grow-out (SGO) and polymerase chain reaction (PCR)-based tests are the most widely used seed assay for *A. citrulli* detection and is recognized as the United States Department of Agriculture National Seed Health System (USDA-NSHS) standard (www.seedhealth.org) (HA et al., 2009; SCHAAD et al., 1995). Technically, SGO is simple but requires large areas of greenhouse space and labor. Additionally, in large greenhouses, it is difficult to maintain temperature and relative humidity conditions, which may lead to disease escape. Another drawback is the need for experienced test evaluators to recognize BFB symptoms. In many cases, subsequent confirmatory tests including pathogen isolation and pathogenicity tests are necessary, making the SGO expensive to conduct.

Over the past decade, many PCR-based seed health assays have been reported (FENG et al., 2013). Since the presence of seed compounds may inhibit PCR, leading to false-negative results, several pre-PCR methods have been

developed including DNA purification and selective enrichment of target organisms (BIO-PCR) (SCHAAD et al., 1995) and immunomagnetic separation and PCR (IMS-PCR) (WALCOTT; GITAITIS, 2000). The sensitivity, time and expenditure varies among these methods and are important factors that have to be considered when developing a successful PCR strategy for detection of a target pathogen.

Due to the genetic variability of Brazilian *A. citrulli* population, the widely-used qPCR primers developed for rapid identification of *A. citrulli* (HA et al., 2009) failed to produce positive reactions for some Brazilian haplotypes, leading to false-negative results. In addition, most PCR-based protocols have not been evaluated for its cost-efficiency in routine detection of *A. citrulli*. Thus, the objective of this research was to design an *A. citrulli*-specific primer set that could be able to detect the different Brazilian haplotypes by qPCR and establish an effective protocol for the routine detection of *A. citrulli* on seeds.

MATERIALS AND METHODS

Bacterial strains. The *A.citrulli* strains representing the different Brazilian haplotypes and references used in this study are listed in table 1. Bacterial strains were routinely grown on nutrient broth (NB, Difco Laboratories, Detroit, MI, USA) or NA (NB containing 15 g/l agar) at 28°C for 48 h. For cells suspensions, single colonies were transferred to 5 mL of Nutrient Broth (NB, Himedia) and incubated at 28°C for 18h under constant agitation (270 RPM) in an incubator shaker (Innova, New Brunswick, NJ). The optical density of the cell suspensions was initially adjusted to 0.5 at a wavelength of 600 nm (~0.5×10^8 CFU/mL) using a spectrophotometer (Spectronic 20; Bausch and Lomb, Rochester, NY). Bacterial genomic DNA was obtained using the ArchivePure DNA cell/Tissue kit (5 PRIME, Gaithersburg, MD, USA).

Contaminated melon seed lots. Seed lots were consisted of samples of 1000 seeds. For seed lots with different levels of infestation, vacuum-infiltrated (artificially infected) or naturally infected seeds harvested from BFB outbreaks were combined with previously confirmed BFB-free seeds. Artificially infected seeds were prepared according to Araujo, Mariano e Michereff (2005). Four different levels of seed lot infestation were used (0, 0.1, 1 and 10%), each level consisting of three replicates. Seeds were excised at the bottom to facilitate the endosperm exposure to the 0.1 M phosphate-buffered saline (PBS) solution. Seed samples were agitated at 140 RPM for 4 h at room temperature (~ 27° C) in Erlenmeyer flasks containing 250 mL of sterile PBS. Subsequently, aliquots of 1 mL of seed washes (eight replicates) were taken from each flask, centrifuged at 10,000 x g for 2 minutes, the supernatant decanted and the pellet resuspended in 0.1 M of PBS. Ten-fold serial dilutions were generated (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) prior plating on semi-selective media for Bio-PCR assay. For IC-PCR seed washes were directly used.

Biological enrichment and polymerase chain reaction (BIO-PCR). For the biological enrichment, the King`s medium B (KING; WARD; RANEY, 1954) was used for spread-plating of serial diluted seed extracts or cell suspensions. The plates were incubated at 28°C for 48 h and typical *A. citrulli* colonies were confirmed by PCR with specific primers (Table 2). Turbid Cells suspensions were boiled in 1 mL of ultra-pure water for 10 min, followed by 5 min chilling in ice. An aliquot of 1 µL of each cells suspension was used as PCR template. The PCR thermal conditions were an initial step of 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec., 60°C for 30 sec. and 72°C for 30 sec. The final elongation step was performed at 72°C for 5 min. After electrophoresis, agarose gels were visualized and digital images were captured in tagged image file format (TIF) under ultraviolet transillumination with an Eagle Eye II Still Video System (Stratagene, La Jolla, CA, USA).

Single tube immunocapture PCR (IC-PCR). Tube preparations were performed according to previously described methods (PENG et al., 2002). Briefly, *A. citrulli*-specific capture antibody (Agdia, Inc., Indiana, USA) was diluted to 2.5 µg/mL with coating buffer (15mM Na_2CO_3, 35 mM $NaHCO_3$, in 1 L of sterile distilled water, pH 9.6) solution. An aliquot of 50 µL of diluted antibody was added into a 0.2 mL polypropylene PCR tube and incubated at 4°C for 4 h. Subsequently, the tubes were rinsed four times with 100 µL of sterile phosphate buffer saline (PBS) containing 0.05% Tween-20 to remove the non-bounded antibodies. Fifty µL of bacterial cell suspensions or seed wash was added into the antibody coated PCR tubes. After 4 h of incubation at 28°C, the PCR tubes were rinsed with 100 µL of sterile distilled-water, and after removing the solution, PCR reaction was directly performed. PCR reactions included 2,5 µL of 10× reaction buffer (25 mM $MgCl_2$), 0.4 µL of Taq DNA Polymerase (5 U/µL), 1 µL of each primer (25 µM), 1.5 µL of 2.5 mM dNTP mixture and ddH_2O was added to make the final reaction volume of 50 µL.

Quantitative real time PCR (qPCR). The qPCR reactions were performed in the Eco Real-Time PCR system (Illumina, Inc., California, USA). The reactions had a final volume of 20 µL containing 10 µL of Fast EvaGreen® Master Mix, 1 µl of each primer, 1µL of template (genomic DNA or seed wash) and 7 µL of sterile ultra-pure water. Thermal conditions were set as an initial denaturation step of 95ºC for 5 min. followed by 40 cycles of 95ºC for 5 sec. and 61ºC for 45 sec.

PCR primer design. Primers were designed and produced from a conserved 360- base-pair (bp) DNA sequence for the 16S ribosomal RNA (rRNA) gene from a strain of *A. citrulli*. This fragment was purified using the Wizard Genomic DNA purification kit (Promega Corporation) and sent off to sequencing. Sequence data were edited manually, and the identity of the sequence was inferred by locating the taxonspecific primers that were used to generate the amplicon.

Based on this sequence data, forward and reverse primers (ACITF61 and ACITR158) were designed (Table 2). These sequences amplified a 98-bp segment.

Table 1 *Acidovorax citrulli* strains

Strains	Origin	Source[a]	Group	PFGE haplotype[b]
AGR 011.1-P	RS	Agronomica	I	B24
Aac 5.16	RN	UFRPE	I	B22
Aac 180	PE	UFRPE	I	B23
IBSBF 1851	Georgia, USA	IBSBF	I	B3(K)
AAC 94-48	Georgia, USA	R. Walcott	II	A9(U)

[a] Culture Collection of Phytobacteriology of: UFRPE= Universidade Federal Rural de Pernambuco; Agronomica= Laboratorio de Diagnostico Fitossanitario; IBSBF= Insituto Biologico.

[b] Letters in parenthesis represent previously reported haplotypes (WALCOTT et al., 2000; WALCOTT; FESSEHAIE; CASTRO, 2004), while letter/number combinations represent current haplotype designations.

Table 2. Primers used in this study

Primer name	Sequence (5` - 3`)	Source	Size (bp)
ACITF61	CATCTGTCTTACATCACCGCC	This study	98
ACITR158	CTAACTACGTGCCAGCAGCC		
AACF	GCGTATGAGTCCCGA AGA AAT	Ha et al. (2009)	90
AACR2	GCA TGCCTTGTATTCAGCTAT		

RESULTS

Sensitivity and specificity of *A. citrulli* primers. As expected, PCR amplification with the *A. citrulli*-specific primers developed in this study produced the predicted 98-bp fragment with DNA template or cell suspensions. In contrast to the widely-used primer set AACF/AACR2, which failed to detect some of the Brazilian *A. citrulli* strains, the ACIT primer set was able to detect the Brazilian haplotypes B22 (Aac 5.16), B23 (Aac 180) and B24 (AGR 011.1-P), that represent the major genetic spectrum of *A. citrulli* in Brazil (Fig. 1). The limit of detection of *A. citrulli* cell suspensions by the ACIT primer set was as low as 3,4 CFU/mL on PCR assay (Fig 2).

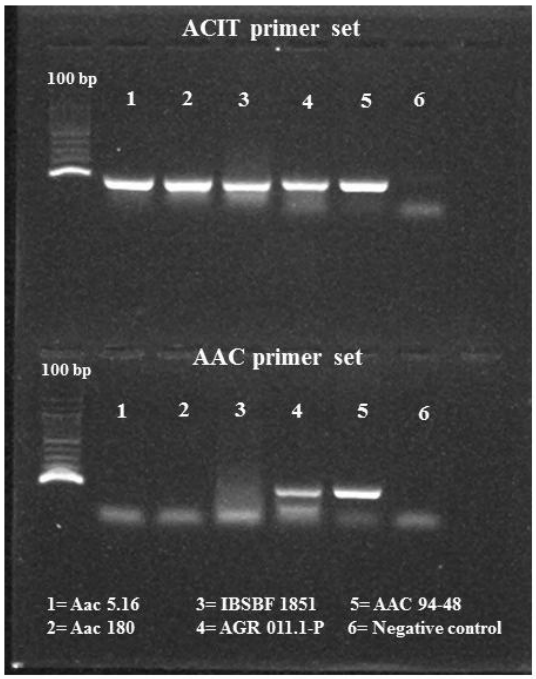

Figure 1 Polymerase chain reaction for detection of *Acidovorax citrulli* population representing different haplotypes.

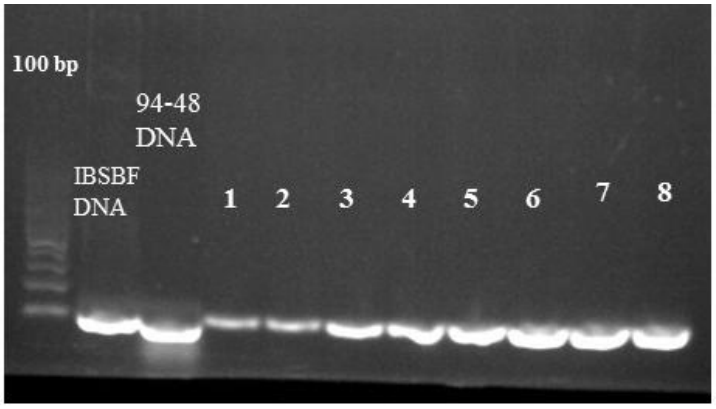

Figure 2 Limit of detection of *Acidovorax citrulli* cell suspensions by PCR with ACIT primer set. Numbers 1 to 8: 1: 3,4 x 10^0; 2: 3,4 x 10^1; 3: 3,4 x 10^2; 4: 3,4 x 10^3; 5: 3,4 x 10^4; 6: 3,4 x 10^5; 7: 3,4 x 10^6 e 8: 3,4 x 10^7 CFU/mL.

Biological enrichment prior to PCR detection of *Acidovorax citrulli* (BIO-PCR). In order to validate the use of BIO-PCR on *A. citrulli* detection, melon seed lots with different levels of infection were prepared. Primarily, the BIO-PCR was tested with seed lots obtained from artificially inoculated seeds. The enrichment method was able to detect the artificially infected seed lots at the minimum level of 1% of infected seeds in a proportion of 6,66 x 10^1 CFU/ mL (mean of 4 replicates). When naturally infected seed lots were used, the Bio-PCR method was able to detect at least one cell at the minimum level of 1% (frequency of detection of 10 subsamples out of 12) (Table 3). PCR reactions directly from the seed extracts without plating failed to detect *A. citrulli* on both naturally and artificially infected seed lots (data not shown).

Single tube immunocapture PCR (IC-PCR). The IC-PCR was used as an enrichment method in which the capture antibody-coated tubes are incubated with seed extracts following the PCR with specific primers. The IC-PCR showed to be sensitive to detect bacterial cells suspensions at an inoculum level of 3,4 x

10 CFU/ mL. When naturally infested seed lots were tested, the limit of *A. citrulli* detection was increased in comparison to Bio-PCR. The IC-PCR could detect *A. citrulli* in seed lots starting from 0,1% of infestation level (frequency of detection of 2 out of 4 subsamples). The frequency of detection was increased at higher levels of seed lot infection (Fig. 3).

Table 3. Biological enrichment following PCR of seed lots with different infection levels.

Seed infection levels (%)	Bio-PCR	
	Artificially infected[a]	Naturally infected[b]
0	0	(0/12)
0,1	0	(0/12)
1	6,66 x 10	(10/12)
10	1,41 x 10^3	(12/12)

[a] Mean of three replicates; numbers in CFU/ mL
[b] Frequency of detection.; total of 12 subsamples

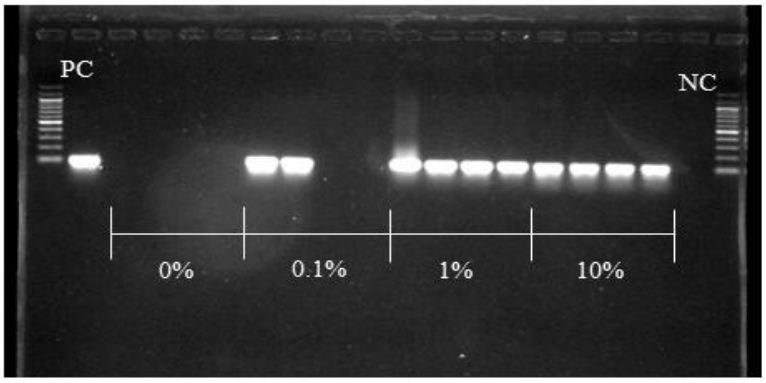

Figure 3 Single tube immunocapture PCR (IC-PCR) for detection of *Acidovorax. citrulli* on melon seed lots with different levels of naturally infested seeds. PC= positive control (cell suspension); NC= Negative control (0,1M PBS).

Quantitative real time PCR (qPCR). The primer set developed in this study was primarily sought to be used in a fast and reliable qPCR assay for rapid identification of *A. citrulli*. The ACIT primer set was able to detect *A. ctrulli* on infested seed lots with at least 0,1% of infection (Table 4). The Ct values ranged from 14 to 22 cycles on average, indicating strong positive reactions in detecting target nucleic acids.

Table 4 qPCR detection of *A. citrulli* on melon seed lots

Seed infection levels (%)	qPCR	
	Artificially infected[a]	Naturally infected
0	0*	0
0,1	19,3	22,1
1	15,2	19,4
10	14.5	17, 3

[a] Ct values= cycle threshold that is required for detection of the fluorescent signal;
*Mean of five replicates

DISCUSSION

Chemical control of BFB has been ineffective (RANE; LATIN, 1992) and the most feasible disease-management strategy has been to eliminate infested seed lots prior to planting. Implementation of this measure requires rapid, reliable and sensitive seed-detection assays. The broadly used seedling grow-out and the commercially available enzyme linked immunosorbent assay (ELISA) are limited by the greenhouse space, inconsistency of results and time requirements.

Molecular-based detection assays play an important role on seed health testing (FENG et al., 2013; SCHAAD et al., 1995). Polymerase chain reaction,

particularly, have been of essential use on routine detection of plant pathogenic bacteria, due to its sensitivity and time requirements to perform the analysis. However, low pathogen population and inhibitory compounds can severely limit the application of PCR for seed health testing (SCHAAD et al., 1995; WALCOTT et al., 2006). In addition, due to the genetic diversity of *A. citrulli* populations worlwide, some PCR-based protocols fail to detect some *A. citrulli* haplotypes (HA et al., 2009).

In our study we designed a primer set, flanking a 98-bp fragment that is suitable to be used in a qPCR assay to be employed in routine detection of *A. citrulli* on cucurbitaceous seeds. The ACIT primer was able to amplify the DNA from the newly reported haplotypes (B22, B23 and B24) for *A. citrulli* as well as from the type strain (IBSBF 1851; syn. ATCC 29625), unlike the TaqMan PCR assay previously developed (HA et al., 2009). It is important to note that false-negatives are critical on seed health testing, because tolerance for *A. citrulli* in commercial cucurbit seed lots is zero (BURDMAN; WALCOTT, 2012), and are subject to strict market regulations.

A strategy to overcome the low pathogen population and the inhibitory compounds that limit the PCR-based assays, is the enrichment of target bacterial population on semi selective agar or liquid medium prior to PCR. The Bio-PCR, is a technique widely used for this purpose (SONG et al., 2004; SCHAAD et al., 2007). After a 15 to 24 h enrichment step, the method could detect the target bacteria as few as 10^1 CFU/mL (SONG et al., 2003, 2004). Our findings showed that Bio-PCR enrichment method has a satisfactory limit of detection and considerable low cost. However, it should take at least 3 days to complete the whole procedures for detecting *A. citrulli* and additional confirmatory tests must be performed in order to correctly identify the pathogen.

An alternative enrichment method used in this study was the IC-PCR. This method relies on the pathogen`s enrichment by a specific capture antibody prior to PCR (PENG et al., 2002). One of the advantages of this method over Bio-PCR is

time, within 2 days the results were obtained. Besides the increase on limit of detection, IC-PCR also showed high repeatability, being suitable to be used in routine detection. A similar technique is IMS-PCR, which involves immunomagnetic separation and PCR (WALCOTT; GITAITIS, 2000). By this technique, antibodies, covalently attached to magnetic beads, bind target cells by specific antibody-antigen interactions during incubation with cells suspensions. The immunomagnetic bead (IMB)-bacteria complexes are held by magnetic force while non-target cells and inhibitory compounds are rinsed away. Template DNA can be released from IMB-bound target cells by boiling for 15 min. However, a drawback for this method is the startup cost of the equipment and the need of personnel training.

Considering the genetic variability of *A. citrulli* strains worldwide, methods targeting genomic DNA sequences are not completely secure and may no longer work for a specific group of strains, due to genetic exchanges between isolates over time (horizontal gene transfer, base insertion, or deletion). For this reason, the use of new target sequence is needed. In this study we described methods for seed health testing that could be used as a routine procedure for *A. citrulli* detection, due to their simplicity, cost and the potential use for high-throughput testing of seed samples compared to other standard assays. In addition, we describe a new primer set that can be used for rapid identification of Brazilian *A. citrulli* strains minimizing the risk of false-negative results.

REFERENCES

ARAÚJO, D. V.; MARIANO, R. L. R.; MICHEREFF, S. J. Métodos de inoculação de *Acidovorax avenae* subsp. *citrulli* em melão. **Summa Phytopathologica**, Jaguariúna, v. 31, n. 1, p. 69-73, jan. 2005.

BAHAR, O. et al. New subspecies-specific polymerase chain reaction-based assay for the detection of *Acidovorax avenae* subsp. *citrulli*. **Plant Pathology**, Honolulu, v. 57, n. 4, p. 754-763, Aug. 2008.

BURDMAN, S.; WALCOTT, R. Pathogen profile *Acidovorax citrulli*: generating basic and applied knowledge to tackle a global threat to the cucurbit industry. **Molecular Plant Pathology**, London, v. 13, n. 8, p. 805-815, Oct. 2012.

FENG, J. J. et al. Advances in detection of *Acidovorax citrulli*, the causal agent of bacterial fruit blotch of cucurbits. **Seed Science and Technology**, Bassersdorf, v. 41, n. 1, p. 1-15, Apr. 2013.

HA, Y. et al. Simultaneous detection of *Acidovorax avenae* subsp. *citrulli* and *Didymella bryoniae* in cucurbit seedlots using magnetic capture hybridization and real-time polymerase chain reaction. **Phytopathology**, Saint Paul, v. 99, n. 6, p. 666-678, June 2009.

KING, E. O.; WARD, M. K.; RANEY, D. E. Two simple media for the demonstration of pyocyanin and fluorescin. **Journal of Laboratory and Clinical Medicine**, New York, v. 44, n. 2, p. 301-307, Aug. 1954.

LATIN, R. X.; HOPKINS, C. L. Bacterial fruit blotch of watermelon, the hypothetical exam question becomes reality. **Plant Disease**, Quebec, v. 79, p. 761-765, 1995.

PENG, X. et al. Rapid detection of Shigella species in environmental sewage by an immunocapture PCR with universal primers. **Applied and Environmental Microbiology**, Washington, v. 68, n. 5, p. 2580-2583, May 2002.

RANE, K.; LATIN, R. Bacterial fruit blotch of watermelon: association of the pathogen with seed. **Plant Disease**, Quebec, v. 76, p. 509-512, 1992.

SCHAAD N. W.; BERTHIER-SCHAAD, Y.; KNORR, D. A high throughput membrane BIO-PCR technique for ultra-sensitive detection of *Pseudomonas syringae* pv.*phaseolicola*. **Plant Pathology**, Honolulu, v. 56, n. 1, p. 1-8, Feb. 2007.

SCHAAD, N. W. et al. A combined biological and enzymatic amplification (BIO-PCR) technique to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed extracts. **Phytopathology**, Saint Paul, v. 85, p. 243-248, 1995.

SCHAAD, N. W. et al. Reclassification of subspecies of *Acidovorax avenae* as *A. avenae* (Manns 1905) emend.; *A. cattleyae* (Pavarino, 1911) comb. nov.; *A. citrulli* Schaad et al.; 1978) comb. nov.; and proposal of *A. oryzae* sp. nov. **Systematic and Applied Microbiology**, Stuttgart, v. 31, n. 8, p. 434-446, Dec. 2008.

SONG, W. et al. Detection of *Acidovorax avenae* subsp. *avenae* in rice seeds using BIO-PCR. **Journal of Phytopathology**, Berlin, v. 152, n. 12, p. 667-676, Dec. 2004.

SONG, W. Y. et al. Use of PCR for rapid identification of *Acidovorax avenae*. In: IACOBELLIS, N. S. et al. (Ed.). ***Pseudomonas syringae* and related pathogens**. Dordrecht: Kluwer Academic, 2003. p. 531-544.

WALCOTT, R. R. et al. Progress towards a commercial PCR-based seed assay for *Acidovorax avenae* subsp. *citrulli*. **Seed Science and Technology**, Bassersdorf, v. 34, n. 1, p. 101-116, Apr. 2006.

WALCOTT, R. R.; FESSEHAIE, A.; CASTRO, A. C. Differences in pathogenicity between two genetically distinct groups of *Acidovorax avenae* subsp. *citrulli* on cucurbit Hosts. **Journal of Phytopathology**, Berlin, v. 152, n. 5, p. 277-285, May 2004.

WALCOTT, R. R.; GITAITIS, R. D. Detection of *Acidovorax avenae* subsp. *citrulli* in watermelon seed using immunomagnetic separation and the polymerase chain reaction. **Plant Disease**, Quebec, v. 84, n. 4, p. 470-474, Apr. 2000.

GENERAL CONCLUSIONS

In northeastern Brazil, where the weather is typically hot, melon fields are extensively planted year-round, due to the high demand for its export and domestic consumption. Watermelon and melon are cultivated concomitantly, however, the watermelon acreage is generally smaller during the rainy season of the year (from May to August), when the weather conditions are more conducive for BFB outbreaks. In contrast, most growers usually keep melon fields during this period of the year, in order to supply the domestic and international demand. As a result, a major portion of watermelon production generally escapes BFB epidemics, while melon fields are frequently attacked during this season. This recurring situation, along with the genetic variability amongst *A. citrulli* populations, may have promoted outbreaks/a better establishment of group I *A. citrulli* strains in Brazilian melon fields. Agricultural practices providing a large monoculture may dramatically change the selection pressure, allowing bacterial populations to rapidly adapt to a single host species, given the dynamic nature of bacterial genomes. Additionally, although not addressed in this study, it is important to mention the potential role of volunteer seedlings or cucurbitaceous weeds on BFB epidemiology in Brazil. These sources might contribute to *A. citrulli* inoculum, which under conducive conditions would incite BFB outbreaks on subsequent crops.

Particularly, this study showed that BFB outbreaks in Brazil are predominantly caused by group I *A. citrulli* strains, regardless of the host or region. We also identified three unique haplotypes of *A. citrulli* from northeastern Brazil. These findings advance our understanding and should provide a basis for more effective BFB management in Brazil, more specifically in northeastern region, where the disease seems to be endemic on melon. By analyzing a historical PFGE database we observed that even though *A. citrulli* is globally disseminated with cucurbit crop seeds, there was a prevalence of group I strains in China, while BFB

outbreaks in Georgia, USA were associated predominantly with group II strains. Finally, we showed a remarkable physiological variation in growth temperature maxima between group I and II *A. citrulli* strains that must be further investigated and, developed a new primer set that reacts positively with different Brazilian *A. citrulli* haplotypes, minimizing the risks of false-negatives on the routine PCR-based assays. The genome sequences of the group II *A. citrulli* strain, AAC00-1 and other group I strains (M6, pslb65 and TW6; Genbank accession numbers LKUW00000000, JYHM01000000 and JXDJ01000000 respectively) and the constant innovation in DNA sequencing technology, will allow a more detailed investigation of population genomics and molecular host-pathogen interactions, which should ultimately improve BFB management by identifying new targets for resistance breeding efforts.