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CAROLINA BARBOSA MALAFAIA

FORMAÇÃO DE BIOFILME, ATIVIDADE ANTIBIOFILME DE EXTRATOS VEGETAIS E AVALIAÇÃO DE METODOS DE EXTRAÇÃO DE PROTEINAS EM FITOBACTÉRIAS

Recife, 2016

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Tese de Doutorado apresentada à Coordenação do Programa de Pós-Graduação em Ciências Biológicas da Universidade Federal de Pernambuco, como parte dos requisitos à obtenção do grau de Doutora em Ciências Biológicas.

Profa. Dra. Márcia Vanusa da Silva

Orientadora

Profa. Dra. Maria Tereza dos Santos Correia

Co-orientadora

Prof. Dr. Alexandre José Macedo

Co-orientador

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Data da defesa: 25 de Janeiro de 2016.

Banca examinadora:

_____
Profa. Dra. Márcia Vanusa da Silva
Departamento de Bioquímica – UFPE

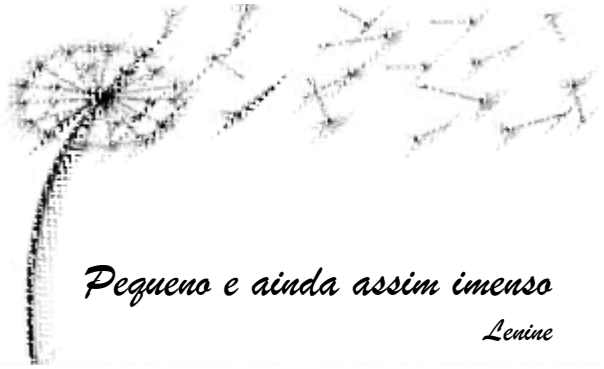
_____
Profa. Dra. Maria Tereza dos Santos Correia
Departamento de Bioquímica – UFPE

_____
Prof. Dr. Thiago Henrique Napoleão
Departamento de Bioquímica – UFPE

_____
Profa. Dra. Elineide Barbosa de Souza
Departamento de Biologia/Microbiologia – UFRPE

_____
Dra Isabel Renata de Souza Arruda
Centro de Tecnologias Estratégicas do Nordeste - CETENE

Recife, 2016



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RESUMO

A formação de biofilme é uma característica importante para as bactérias, por ser uma formação natural e altamente influenciada pelo ambiente circundante, confere aos microrganismos alta tolerância às adversidades e torna-se importante na virulência para patógenos. Sendo assim, apresentamos nesta tese uma investigação da adesão bacteriana e desenvolvimento de biofilme das fitobactérias *Ralstonia solanacearum* (*Rsol*) e *Acidovorax citrulli* (*Acc*), agronomicamente importantes, sobre superfícies hidrofóbicas, foi investigado também o emprego de extratos vegetais de plantas oriundas da Caatinga, na inibição da adesão bacteriana e sua capacidade bactericida contra *R. solanacearum*, e foi determinado o método mais eficiente na preparação amostras proteicas para *R. solanacearum*, *A. citrulli* e *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) para aplicação em estudos futuros de investigação molecular da formação de biofilmes fitopatogênicos. A formação de biofilme por diferentes isolados bacterianos após 24h de incubação em diferentes meios de cultura foi quantificado pelo método de cristal violeta e suas estruturas observadas por microscopia eletrônica de varredura e microscopia confocal. Foram avaliados também 22 extratos aquosos de 16 plantas coletadas na Caatinga quanto a capacidade de inibição da formação de biofilme de *Rsol*. Quanto à eficiência na obtenção de proteínas, foram testados os métodos de Trizol, Fenol, Centrifugação e Lise e avaliados através de eletroforese uni e bidimensional. Quanto a formação de biofilme os resultados obtidos indicam que, nas condições testadas, isolados de *Rsol* se mostrou diferente entre os isolados tanto quantitativa quando morfologicamente onde os isolados B5-5 CGH26 CGH8 e SCN 21 foram moderados ou fortes produtores de biofilme. Já os isolados de *Acc* não foram bons produtores de biofilme, apresentando apenas os isolados Acc1.43 e Acc 1.73 como fortes formadores de biofilme com quantidade e morfologia semelhantes. No *screening* de atividade antibiofilme, dentre os extratos testados apenas ramos de *Harpochilus neesianus* e folhas de *Myroxylon peruiferum* apresentaram atividade antibiofilme superior a 83% e 50%, respectivamente, e *Jacaranda rugosa* apresentou atividade antimicrobiana contra todos os isolados de *Rsol* testados. Quanto à extração de proteínas de alta qualidade o método de Lise foi o mais eficiente para *Rsol* e *Pcc*, apresentando respectivamente 369 ± 4 e 212 ± 3 diferentes spots de proteínas, contudo para *Acc* o método de centrifugação foi o mais indicado com 224 ± 8 spots. De acordo com os resultados deste estudo conclui-se que a formação de biofilme pode ser quantitativa e estruturalmente distinta entre isolados da mesma espécie. O *screening* das propriedades antimicrobianas das plantas fornece base de dados para o desenvolvimento de novos agentes antibacterianos naturais contra fitopatógenos seguros para o meio ambiente e para o desenvolvimento de estudos moleculares da formação de biofilme faz-se necessária uma prévia determinação de métodos para obtenção das macromoléculas a serem analisadas, sendo assim a seleção de métodos de extração é um ponto crucial para obtenção de amostras de qualidade para analises confiáveis.

Palavras chave: *Ralstonia solanacearum*, *Acidovorax citrulli*, *Pectobacterium carotovorum* subsp. *carotovorum*, Biofilme, Caatinga, Proteínas.

ABSTRACT

Biofilm formation is an important feature for bacteria due to its naturally occurring and highly influence by the surrounding environment, giving to the microorganisms high tolerance to adversity and becoming essential in virulence for pathogens. Thus, we present in this thesis an investigation about bacterial adhesion and biofilm development of the phytobacteria *Ralstonia solanacearum* (*Rsol*) and *Acidovorax citrulli* (*Acc*), agronomically important, on hydrophobic surfaces; it was also investigated the use of plant extracts from the Caatinga region through the inhibition of the bacterial adhesion and its bactericidal activity against *R. solanacearum*. The most efficient method to prepare protein samples for *R. solanacearum*, *A. citrulli* and *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) was determined to be applied in future studies of molecular investigation of the formation of pathogenic biofilms. The biofilm formation by different bacterial strains after 24 h incubation in distinct culture media was quantified by the crystal violet method and its structures were observed by scanning electron microscopy and confocal microscopy. There were also evaluated 22 aqueous extracts from 16 plants collected in the Caatinga as its potential of inhibition of *Rsol* biofilm formation. In what concerns the efficiency in obtaining proteins, Trizol, Phenol, centrifugation, and Lyse were the methods evaluated by one- and two-dimensional electrophoresis. The results for biofilm formation demonstrate that, under the tested conditions, *Rsol* strains were different, both quantitatively and morphologically, and the strains namely B5-5, CGH26, CGH8, and SCN 21 were moderate or strong biofilm producers. Regarding the results for *Acc* strains, it is possible to note that they were not good biofilm producers, unless the strains Acc1.43 and Acc1.73 that were considered strong biofilm producers with similar quantity and morphology patterns. In relation to the screening of antibiofilm activity, only branches of *Harpochilus neesianus* and leaves of *Myroxylon peruiferum* presented antibiofilm activity with values higher than 83% and 50%, respectively, and *Jacaranda rugosa* showed activity antimicrobial against all the tested *Rsol* strains. The extraction of high quality proteins was performed most efficiently by the Lysis method for *Rsol* and *Pcc*, respectively with 369 ± 4 and 212 ± 3 different spots of proteins, however the centrifuge method was better for *Acc* with 224 ± 8 spots. According to the results of this study it is possible to conclude that biofilm formation can be quantitatively and structurally distinct from strains of the same species. The screening of the antimicrobial properties of the plants provides data as a basis for the development of new natural antibacterial agents against safe phytopathogens for the environment; in addition, for the development of molecular studies about the biofilm formation it is necessary a preliminary determination of the methods suitable for obtaining the macromolecules to be analyzed, so the selection of extraction methods is a crucial point for obtaining quality samples for reliable analysis.

Keywords: *Ralstonia solanacearum*, *Acidovorax citrulli*, *Pectobacterium carotovorum* subsp. *carotovorum*, Biofilm, Caatinga, Proteins

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Fundamentação Teórica

1. Impactos de fitopatologias na agricultura

A população mundial cresce muito a cada ano e para atender às demandas relacionadas com essa constante expansão, a produção global da agricultura precisa dobrar até 2050; no entanto, as estimativas atuais indicam que a produção de alimentos será muito abaixo do que é necessário para suprir a população (RAY et al., 2013; YULIAR; NION; TOYOTA, 2015). As doenças de plantas, o ataque de insetos e a presença de ervas daninhas nas áreas de plantio diminuem a produção das culturas em todo o mundo em cerca de 36%, e as doenças, sozinhas, têm reduzido o rendimento das culturas em cerca de 14% (AGRIOS, 2005; YULIAR; NION; TOYOTA, 2015). Assim, o controle de doenças de plantas contribui para o aumento da produção de colheitas. Entre as doenças das plantas, as causadas por agentes habitantes de solo são consideradas mais severas do que as doenças transmitidas por sementes ou transportadas pelo ar e são responsáveis por cerca de 20% de perdas na produção anualmente (YULIAR; NION; TOYOTA, 2015).

As fitopatologias são causadas por diversos microrganismos e apresentam extrema importância para o homem devido aos danos causados diretamente às plantas e aos seus produtos, bem como por influenciarem direta ou indiretamente na rentabilidade do empreendimento agrícola (LOPES; ROSSATO, 2013). Essas patologias podem limitar os tipos ou variedades de plantas que podem se desenvolver em determinada área geográfica, como observado em solos contaminados com *Ralstonia solanacearum*, que impedem o plantio de enorme variedade de espécies vegetais incluindo tomate, pimentão, banana e batata (GENIN; DENNY, 2012; PEETERS et al., 2013); reduzem a quantidade e a qualidade dos produtos vegetais, como doenças causadas por *Acidovorax citrulli* causadora da mancha aquosa do melão, doença que dificulta o cultivo de várias espécies da família das Cucurbitáceas (BURDMAN; WALCOTT 2012); e *Xylella fastidiosa*, que tem sido associada a doenças de colheitas economicamente importantes, incluindo citros, uva, ameixa, amêndoa e pêssego (SOUZA et al., 2004; SILVA et al., 2011); além de poderem tornar as plantas venenosas ao homem e animais como ocorre no esporão do centeio e do trigo causado pelo fungo *Claviceps purpurea*, que pode

tornar os produtos vegetais inadequados para consumo pela contaminação com estruturas de frutificação venenosas (PAŽOUTOVÁ et al., 2015).

Doenças de plantas podem levar a custos de controle muito altos como ocorre em algumas fitopatologias, caso observado nas doenças de cereais, cujo custo pode ser alto ou maior que o retorno esperado da cultura (BERGAMIN FILHO; KIMATI, 1995). Para algumas doenças, nenhuma medida de controle efetiva é conhecida, sendo possível a obtenção de colheitas pela combinação de práticas culturais e uso de variedades parcialmente resistentes (LUCAS, 1998). São organismos bastante versáteis, com grande capacidade de adaptação a ambientes diversos. Ao contrário das bactérias patogênicas ao homem e aos animais, as fitobactérias têm uma temperatura ótima de crescimento e multiplicação entre 25 e 30°C pH ótimo em torno do neutro e a maioria são aeróbicas estritas (GOTO, 1992).

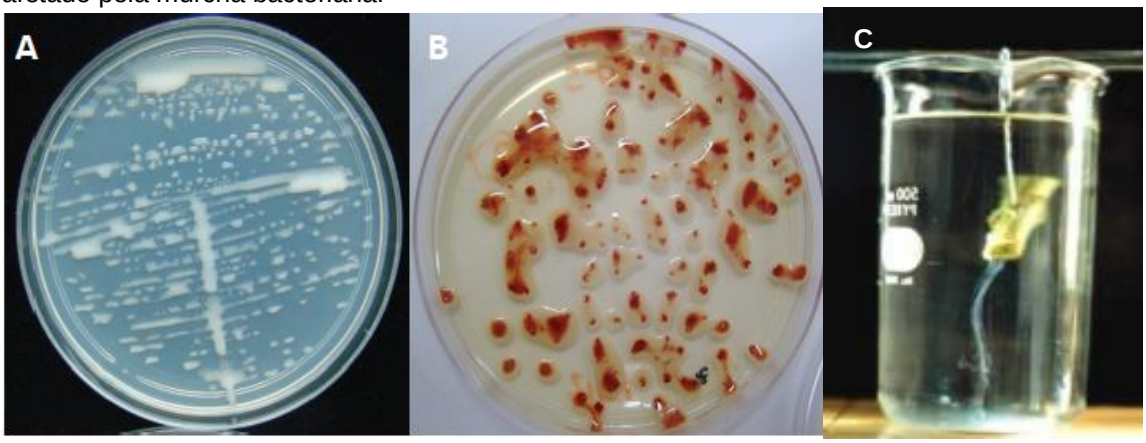
Bactérias são importantes patógenos de plantas, não somente pela alta incidência e severidade em culturas de valor econômico, mas também pela facilidade com que se disseminam e pelas dificuldades encontradas para o controle das enfermidades por elas incitadas. Dentre as bactérias fitopatogênicas de plantas já conhecidos *Ralstonia solanacearum* merece destaque por causar sérios danos a uma grande variedade de hospedeiros (HAYWARD, 1991; RAZA et al., 2016) e *Acidovorax citrulli* por acometer diversas espécies da família Cucurbitaceae de importante valor agro econômico (DUTTA et al., 2014).

1.1 *Ralstonia solanacearum*

A murcha bacteriana é uma doença vascular, causada por *R. solanacearum* (Smith) (Figura 1, A e B) (YABUUCHI et al., 1995). Esse patógeno é um dos mais destrutivos identificados até o momento por induzir sintomas de murcha rápida e fatal em plantas hospedeiras, sendo classificado como o segundo patógeno bacteriano agronomicamente mais importante (HAYWARD, 1994; PEETERS et al., 2013). É uma bactéria Gram negativa, classificada filogeneticamente como pertencente ao reino Procariotae, domínio Bacteria, filo Proteobacteria, classe Betaproteobacteria, ordem Burkholderiales,

família Ralstoniaceae, gênero *Ralstonia*, espécie *R. solanacearum* (Smith), anteriormente chamada de *Bacillus solanacearum*, *Pseudomonas solanacearum* e *Burkhloderia solanacearum* (YABUUCHI et al., 1995).

Figura 1. Colônias de *Ralstonia solanacearum* em meio de Kelman (A) sem e (B) com trifenil tetrazólio; (C) Teste do copo, mostrando exsudação de pus bacteriano em caule de tomateiro afetado pela murcha bacteriana.



Fonte: LOPES; ROSATO, 2013.

Essa bactéria habitante de solo é versátil quanto aos seus hospedeiros. Sabe-se atualmente que infecta mais de 200 espécies vegetais pertencentes a 50 famílias botânicas, contudo, afeta principalmente em espécies da família Solanaceae, que compreende espécies de grande importância econômica mundial como tomate, a batata, o pimentão, a berinjela, o jiló e o tabaco. (LOPES; QUEZADO-DUVAL, 2005; ZULUAGA; PUIGVERT; VALLS, 2013; SAFNI et al., 2014; WU et al., 2015). Está distribuída em todo o mundo, mas principalmente nas regiões tropicais e subtropicais (regiões que detêm a maior parte da produção de alimentos no mundo) causando um forte impacto econômico destrutivo (KHEIRANDISH; HARIGHI, 2015; NISHAT et al., 2015). Perdas de rendimento diretos por *R. solanacearum* variam muito de acordo com o hospedeiro, cultivar, clima, tipo de solo e cultivo padrão. Por exemplo, as perdas de rendimento podem chegar a 90% em cultivos de tomate, de 33 a 90% na batata, de 10 a 30% em tabaco, até 20% no amendoim e uma das culturas mais afetadas é a da banana com percentual de perda de 80 a 100% da produção (ELPHINSTONE, 2005).

O sintoma mais típico da murcha bacteriana é a murcha da planta de

cima para baixo, que é resultante da interrupção parcial ou total do fluxo de água desde as raízes até o topo da planta. Ao infectar a planta através das raízes, a bactéria migra para os vasos do xilema e, uma vez instalada, a bactéria se multiplica aumentando a população de células e a produção de exsudados viscosos de polissacarídeos, que culmina com o bloqueio do fluxo do xilema, impedindo que a água absorvida pelas raízes chegue até a parte aérea da planta, ocorrendo a murcha das folhas. Nos primeiros dias da infecção a murcha se dá começando pelas folhas mais jovens, principalmente nas horas mais quentes do dia, mas a planta pode se recuperar à noite. Contudo, com a evolução da infecção, há o bloqueio total dos vasos o que ocasiona a murcha de forma irreversível levando a planta à morte. A doença se manifesta em qualquer fase do desenvolvimento da planta, embora seja mais comum na frutificação (HAYWARD, 1991; SHEW; LUCAS, 1991; LOPES; ROSSATO, 2013).

A murcha bacteriana é favorecida por alta temperatura e alta umidade do solo e plantios sucessivos realizados no mesmo terreno podem agravar a incidência da doença uma vez que em geral, a partir do terceiro plantio, os solos se tornam tão contaminados pela bactéria que levam ao abandono da cultura (MAROUELLI; LOPES; SILVA, 2005).

Outros patógenos podem causar sintomas de murcha e escurecimento vascular similares aos observados na murcha bacteriana, como é o caso de *Verticillium* spp. e *Fusarium oxysporum* f. sp. *lycopersici*. Em campo, uma maneira fácil, rápida e corriqueiramente utilizada para diagnosticar corretamente a murcha bacteriana, é avaliação da presença de exsudação por meio do teste do copo (Figura 1 C). Este teste é feito cortando-se uma pequena porção (cerca de 5 cm) da parte mais inferior do caule da planta doente e colocando-a ligeiramente submersa em frasco transparente com água limpa. A presença de um filete leitoso saindo do tecido em direção ao fundo do copo indica a presença da *R. solanacearum* (LOPES; ROSSATO, 2013).

As dificuldades no controle desse patógeno estão associadas com a sua capacidade de crescer endofiticamente, ser transportado através da água e sua relação de sobrevivência com ervas daninhas e sobrevivência por longos períodos no solo (WANG; LIN, 2005; YULIAR; NION; TOYOTA, 2015; NISHAT

et al., 2015); além da ampla distribuição geográfica e ampla gama de hospedeiros incomuns deste patógeno. Métodos de controle como a rotação de culturas com plantas não hospedeiras devem ser feitos ao longo de vários anos (KHEIRANDISH; HARIGHI, 2015). O saneamento do campo, controle químico da doença atualmente utilizado e o plantio de variedades resistentes são geralmente ineficazes. A técnica de fumigação do solo tem ligeiro ou nenhum efeito. Diante disto, a investigação de combate a este patógeno faz-se necessária, a fim de desenvolver estratégias mais eficientes no combate ou controle dessa doença.

1.2 *Acidovorax citrulli*

Acidovorax citrulli (SCHAAD et al. 1978), agente causal da mancha aquosa (ou mancha bacteriana do fruto) foi descrita primeiramente nos Estados Unidos em 1965 afetando cultivos de melancia (*Citrullus lanatus*) (WEBB; GOTH, 1965) e em 1996 em melão (*Cucumis melo*) (ISAKEIT et al., 1997) e já foi relatada em várias regiões produtoras nas Ilhas Marianas (WALL; SANTOS, 1988), na Índia (RANE; LATIN, 1990) e na Austrália (O'BRIEN; MARTIN, 1999). Foi identificada pela primeira vez no Brasil, no estado do Rio Grande do Norte, região nordeste do Brasil, em 1997 (ASSIS et al., 1999) estendendo-se posteriormente para os estados do Ceará e de Pernambuco. Por sua alta severidade, é atualmente considerada uma das mais sérias ameaças à cultura do melão (SALES JÚNIOR; MENEZES, 2001).

Essa bactéria é Gram-negativa, aeróbica, que forma colônias lisas e esbranquiçadas em meio King-B (Figura 2) (MELO et al., 2004). É classificada filogeneticamente como pertencente ao reino Procariote, domínio Bacteria, filo Proteobacteria, classe Betaproteobacteria, ordem Burkholderiales, família Comamonadaceae, gênero: *Acidovorax*, Espécie: *A. citrulli* (BAHAR; DE LA FUENTE; BURDMAN, 2010).

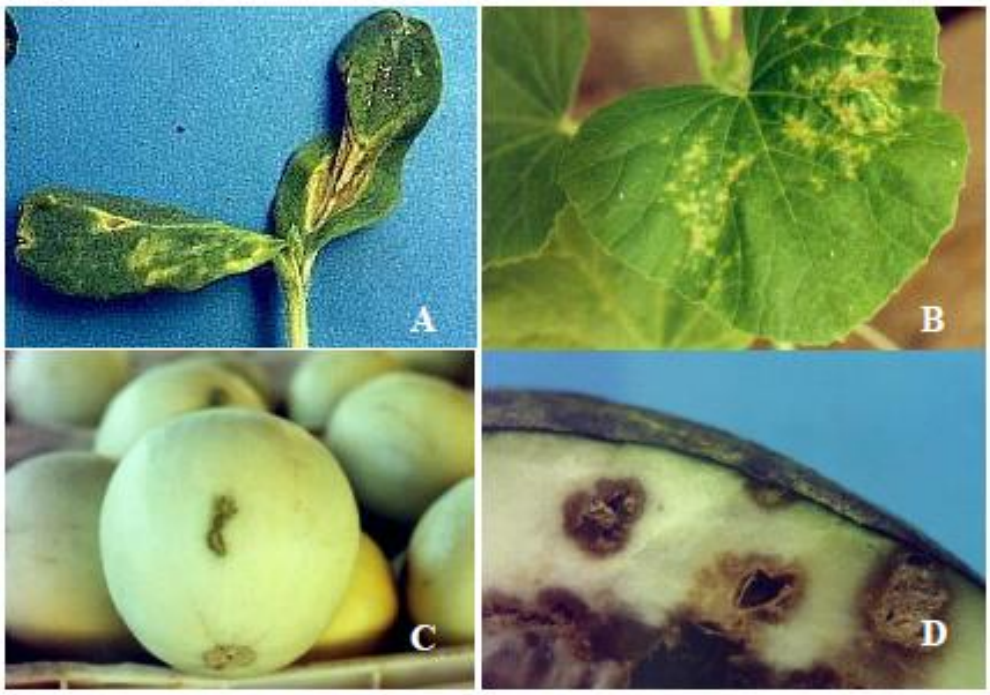
Figura 2. Colônias de *Acidovorax citrulli* em meio de King-B.

Fonte: WALCOTT, 2005.

Este fitopatógeno é de ocorrência limitada a espécies da família Cucurbitaceae (LATIN; HOPKINS, 1995; SHRESTHA, et al., 2013), relatado primeiramente infectando plantas cultivadas: melancia (*Citrullus lanatus* L.) (RANE; LATIN, 1992; DUTTA et al., 2014), melão (*Cucumis melo* L.) (ISAKEIT et al.,1997; ALVES et al., 2010), pepino (*Cucumis sativus* L.) (MARTIN; O'BRIEN, 1999, NASCIMENTO; MARIANO; SILVA, 2004) e abóbora (*Curcubita pepo* L.) (LANGSTON JUNIOR et al., 1999).

Os sintomas da mancha-aquosa podem se manifestar em qualquer fase de desenvolvimento e em todos os tecidos, exceto na rais, da planta hospedeira, sendo mais comuns e facilmente visualizados nos frutos (Figura 3). Plântulas oriundas de sementes infectadas apresentam grandes manchas encharcadas de coloração verde-escura (SANTOS; VIANA, 2000) e marrons no hipocótilo, cotilédones e folhas adultas e, às vezes, necrose no hipocótilo, podendo resultar em colapso ou tombamento e morte das mudas após alguns dias (HOPKINS; CUCUZZA; WATTERSON, 1996; SANTOS; VIANA, 2000). Lesões são frequentemente observadas ao longo das nervuras ou nas margens da folha (O'BRIEN; MARTIN, 1999) (Figura 4 A e B). Dependendo das condições climáticas e da cultivar, as manchas podem crescer e coalescer, e a necrose estender-se por quase a totalidade da área foliar (SALES JÚNIOR; MENEZES, 2001). Mesmo quando há infecção na folha, o efeito sobre o desenvolvimento da planta é praticamente imperceptível, contudo esta passa a servir como reservatório da bactéria para infecção dos frutos (ISAKEIT, 1999).

Figura 3. Sintomas da mancha-aquosa do melão. (A) em folhas cotiledonares, (B) em folhas definitivas, em (C) frutos de melão Orange e (D) frutos de melão pele de sapo.



Fonte: SILVA, 2002.

Os sintomas mais notáveis da doença estão nos frutos maduros antes da colheita, embora a infecção somente ocorra durante a floração e formação do fruto. Na casca dos frutos, ocorrem manchas de coloração verde-oliva, variando de 1 a 5 mm de diâmetro (SALES JÚNIOR; MENEZES, 2001), com ou sem halo, as quais progridem rapidamente, tornando-se aquosas, marrom-claras, atingindo grandes áreas e podem ocorrer rachaduras no centro das lesões. A infestação e o desenvolvimento da doença requerem alta umidade e temperatura (SANTOS; VIANA, 2000).

A necrose ou simples lesão na casca não reflete o dano que ocorre na polpa imediatamente abaixo, ou seja, a parte interna já pode estar bastante comprometida, mesmo quando essa lesão, externamente, se mostra com apenas de 0,5 a 2,0 cm de diâmetro (O'BRIEN; MARTIN, 1999). Na fase mais avançada da doença, os frutos são rapidamente destruídos como resultado da ação de microrganismos secundários que penetram através das rachaduras (COSTA et al., 2001). Após a colheita, a severidade dos sintomas da mancha-aquosa não aumenta drasticamente (RUSHING et al., 1997). A bactéria pode sobreviver de forma assintomática de forma epifítica nas plantas hospedeiras,

particularmente quando não estiver presente a microbiota antagônica ou competitiva em nutrientes (ROBBS, 1991).

As formas mais comuns de disseminação a longas distâncias desse patógeno se dão por utilização de sementes contaminadas (O'BRIEN; MARTIN, 1999; MARIANO; SILVEIRA, 2004; SHRESTHA, et al., 2013) e pelo transplantio de mudas contaminadas (HOPKINS et al., 1992). A disseminação da doença dentro da área de plantio entre plântulas ou plantas vizinhas e das folhas para os frutos ocorre através de respingos de água de chuva e/ou irrigação, solos previamente infestados, transmissão através de insetos, utensílios agrícolas, operários de campo (SANTOS; VIANA, 2000) e aerossóis (HOPKINS et al., 1992). A infestação pode ocorrer também durante a polinização podendo resultar em sementes infestadas, embora os frutos permaneçam assintomáticos (WALCOTT; GITAITIS; CASTRO, 2003). *Acidovorax citrulli* sobrevive durante a entressafra em restos de cultura, em hospedeiros silvestres e cultivados (NASCIMENTO; MARIANO; SILVA, 2004), em sementes (SANTOS; VIANA, 2000) e no solo por algumas semanas (ISAKEIT, 1999). É importante que se conheça a dinâmica populacional de *A. citrulli* sobrevivendo nesses locais para que medidas eficientes de controle possam ser estabelecidas, impedindo a disseminação do patógeno.

2. Biofilme

Por muitos anos acreditou-se que bactérias, ao contrário de organismos multicelulares, existiam como indivíduos autossuficientes e mantinham um estilo de vida livre estritamente unicelular e que as massas agregadas encontradas em infecções eram consideradas apenas a soma desses indivíduos (COSTERTON et al., 2003). Tal percepção de bactérias unicelulares foi estabelecida por estudos de culturas puras que levaram à identificação de agentes patogênicos e o desenvolvimento de tratamentos com antibióticos (LI; TIAN, 2012). Contudo, o crescimento planctônico de culturas puras raramente ocorre em ambientes naturais e grande parte dos microrganismos vive em agregados de células aderidas, crescendo sobre uma superfície, seja ela biótica ou abiótica, e imersos em uma matriz extracelular amorfa conhecida

como biofilme (SAUER, 2003; BOGINO et al., 2013; MASÁK et al., 2014).

Biofilmes apresentam um elevado grau de organização, onde as bactérias formam comunidades estruturadas, coordenadas, funcionais e respondem a um gradiente de difusão de nutrientes e resíduos, modulando seu metabolismo em função da sua posição dentro do biofilme (DUNNE JUNIOR, 2002; RAMEY et al., 2004). Acredita-se que a formação de biofilme é uma característica universal das bactérias (LÓPEZ; VLAMAKIS; KOLTER, 2011) e representa o tipo de crescimento bacteriano predominante na natureza (O'TOOLE; KAPLAN; KOLTER, 2000). Estima-se que 80% da biomassa microbiana do planeta encontram-se no estado de biofilme (RICHARDS; MELANDER, 2009). As células planctônicas (bactérias de vida livre) são importantes para a rápida proliferação e dispersão dos microrganismos para novos ambientes, enquanto que as células sésseis nos biofilmes caracterizam a cronicidade (TRENTIN; GIORDANI; MACEDO, 2013).

O biofilme é geralmente constituído por até 95% de água, de 2 a 5% de células microbianas e de 3 a 6% de matriz, e esta corresponde de 50 a 90% da massa seca total do biofilme (SUTHERLAND, 2001; MARTÍNEZ; VADYVALOO, 2014). A matriz é uma estrutura complexa produzida pelos próprios organismos constituintes, na qual as células do biofilme estão incorporadas, composta por uma variedade de substâncias poliméricas extracelulares (EPS – *extracellular polymeric substances*) como exopolissacarídeos, glicoproteínas e glicolípidos; proteínas e DNA extracelular (e-DNA) que formam a arquitetura tridimensional do biofilme e são responsáveis pela adesão às superfícies e pela coesão no biofilme. Os exopolissacarídeos são geralmente de poli-N-acetilglicosamina, porém algumas bactérias são capazes de sintetizar diferentes polissacarídeos que conferem propriedades fisiológicas distintas à matriz (FLEMMING; NEU; WOZNIAK, 2007; LARSEN et al., 2007; MARTÍNEZ; VADYVALOO, 2014).

As proteínas presentes desempenham importantes papéis para a formação de biofilmes, incluindo as proteínas estruturais, tais como adesinas e outras proteínas de superfície celular associadas como fimbrias, pili e flagelos, e proteínas especificamente envolvidas na adesão célula-a-célula que facilitam a aderência do biofilme à superfície (BERK et al., 2012; MARTÍNEZ;

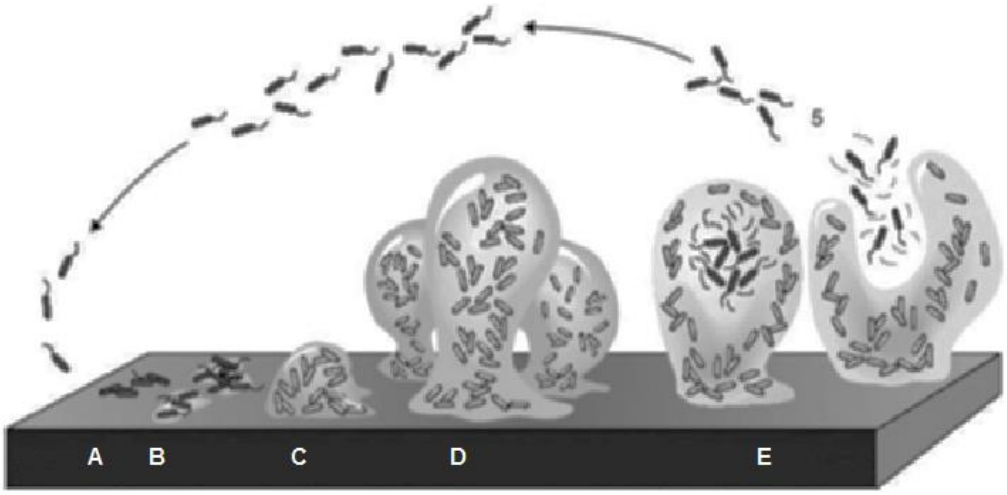
VADYVALOO, 2014). A composição da matriz pode ser encontrada em quantidades variáveis de acordo com a espécie ou consórcio de espécies que estejam compondo o biofilme e essa garante que a estrutura do biofilme seja altamente resistente aos agentes antimicrobianos (BRANDA et al., 2005; BÖCKELMANN et al. 2006, FLEMMING; WINGENDER, 2010; MARTÍNEZ; VADYVALOO, 2014).

As EPS determinam as condições imediatas de vida das células do biofilme que vivem neste microambiente, afetando a porosidade, densidade populacional, disponibilidade de água, carga, propriedades de sorção, hidrofobicidade e estabilidade mecânica (FLEMMING; NEU; WOZNIAK, 2007). Esta matriz interage com o ambiente, por exemplo, anexando o biofilme às superfícies, fornecendo nutrientes para os organismos no biofilme através de suas propriedades de sorção, que permite sequestro de substâncias dissolvidas e particuladas do ambiente (JOUBERT; WOLFAARDT; BOTHA, 2006). Permite também a troca de material genético, mantendo um pool genético grande e acessível e facilitando a transferência horizontal de genes, devido às células serem mantidas em estreita proximidade e não estarem totalmente imobilizadas (HAUSNER; WUERTZ, 1999; FLEMMING; NEU; WOZNIAK, 2007).

Diversos processos estão envolvidos na formação de biofilme sobre uma superfície biótica ou abiótica, iniciando-se pela adesão na superfície, proliferação bacteriana dentro de micro colônias e expansão, formando estruturas altamente organizadas. O modelo mais aceito atualmente divide a formação do biofilme em cinco diferentes etapas (Figura 4). A adsorção de substâncias orgânicas dissolvidas no meio forma um filme condicionante (KATSIKOGIANNI; MISSIRLIS, 2004) que promove a atração de microrganismos para a superfície condicionada. Em seguida, ocorre o processo de adesão primária (ou adesão reversível) dos microrganismos à superfície (Figura 4 A), o qual é o primeiro estágio na formação (onde as células podem ser facilmente removidas por simples processos de lavagem) que, de forma geral, corresponde ao processo de interação entre bactérias e superfícies. Em superfícies bióticas esta etapa é acompanhada por interações moleculares mediadas por ligações específicas do tipo receptor-ligante, enquanto que a

adesão em superfícies abióticas ocorre mediada por interações físico-químicas não específicas (DUNNE JUNIOR, 2002; TRENTIN; GIORDANI; MACEDO, 2013).

Figura 4. Esquema para modelo mais aceito dos estágios de desenvolvimento do biofilme bacteriano. (A) Adesão reversível; (B) Adesão irreversível; (C) Início da maturação do biofilme; (D) Biofilme totalmente maduro com arquitetura complexa; (E) Desestruturação do biofilme e dispersão das células que darão início a novos biofilmes.



Fonte: SAUER, 2003.

Após ocorrer a adesão inicial estabelece-se uma ancoragem mais firme entre a célula microbiana e a superfície, chamada adesão irreversível (Figura 4 B), mediada principalmente por interações físico-químicas específicas (ligações covalentes, iônicas e pontes de hidrogênio), ou através de pontes estabelecidas por estruturas extracelulares específicas dos microrganismos (apêndices celulares filamentosos ou polímeros extracelulares). Em seguida inicia-se a primeira fase da maturação do biofilme (Figura 4 C), que é principalmente caracterizada por interações célula-célula e a formação de componentes de superfície importantes que contribuem para a estrutura do biofilme (MARTÍNEZ; VADYVALOO, 2014). Essa fase é o início do desenvolvimento de sua arquitetura, onde as células já firmemente aderidas começam a multiplicar-se sobre as inicialmente aderidas, e começam a produzir a matriz de EPS formando assim, micro colônias que se desenvolvem dando origem ao biofilme (BRECX; THEILADE; ATTSTRÖM, 1983). Nesta fase ocorre também transporte de nutrientes da fase líquida para a interface líquido-biofilme, bem como no interior do filme microbiano. O biofilme totalmente maduro (Figura 4 E) apresenta alta densidade celular, e arquitetura de forma complexa, que

consiste em micro colônias em forma de torre denominadas de cogumelos, incorporados na matriz (LAVERTY; GORMAN; GILMORE, 2013). Apresentam estruturas como canais que permeiam todo o biofilme e funcionam como um sistema de circulação, permitindo a troca de nutrientes, sinais químicos, enzimas, circulação de água e a eliminação de metabólitos potencialmente tóxicos, e exibem cooperatividade metabólica (BOGINO et al., 2013).

Por fim, ocorre a fase de desestruturação do biofilme e dispersão das células (Figura 4 D). Nesta etapa essas comunidades sésseis podem dar origem a células planctônicas que podem rapidamente se dispersar para colonizar novas superfícies (THORMANN et al., 2006). As células podem se dispersar de forma ativa, através de processos mediados pela própria bactéria, ou de forma passiva por forças externas, como tensão do fluido e abrasão (KAPLAN, 2010). Na maioria das vezes o estado nutricional do ambiente dita o comportamento de bactérias, incluindo a dispersão de biofilme, que pode resultar da diminuição ou aumento de nutrientes no ambiente (MARTÍNEZ; VADYVALOO, 2014). A dispersão do biofilme é importante para a sobrevivência das espécies (WOOD; HONG; MA, 2011), bem como para a transmissão de patógenos dos reservatórios ambientais para hospedeiros, transmissão entre hospedeiros e para a disseminação da infecção dentro de um hospedeiro (KAPLAN, 2010).

Em comparação com a forma de vida planctônica, a capacidade dos microrganismos de se anexar em superfícies e se associarem em biofilmes fornece uma vantagem evolutiva constituindo uma forma de proteção ao seu desenvolvimento, que permite o aumento da sobrevivência por favorecer relações simbióticas e permitindo a sobrevivência em ambientes hostis (LAVERTY; GORMAN; GILMORE, 2013). As células que os compõe apresentam expressão gênica, metabolismo, fisiologia e comportamento diferentes das células planctônicas geneticamente correspondentes (SIMÕES; SIMÕES; VIEIRA, 2010; KAY et al., 2011).

Os biofilmes mostram extraordinária resistência aos biocidas convencionais, uma vez que suportam concentrações de 10 a 1000 vezes maiores de antibióticos que as necessárias para matar bactérias planctônicas geneticamente equivalentes, tornando-os muito difíceis de serem erradicados

em hospedeiros vivos. Esse estilo de vida também protege as bactérias contra predadores, competição interespecífica, suportam diminuição de nutrientes, mudanças de pH e temperatura, radiação e, em infecções, resistem às respostas de defesa do hospedeiro (SAUER, 2003). Os mecanismos envolvidos na resistência das células do biofilme aos agentes antimicrobianos são complexos e apenas parcialmente conhecidos. A formação destas comunidades sésseis e sua resistência inerente aos antimicrobianos estão na raiz de muitas infecções bacterianas persistentes e crônicas. Os biofilmes têm importância especial em ambientes médicos, agrícola e industrial, apresentando benefícios e prejuízos (SAUER, 2003; RAMEY et al., 2004).

Diversos mecanismos têm sido propostos para explicar a maior resistência dos biofilmes a antibióticos, entre eles a menor taxa de crescimento microbiano (EVANS BROWN; GILBERT, 1990) resultante da compressão espacial que impede a divisão celular (WATNICK; KOLTER, 2000; STEWART; FRANKLIN, 2008) e/ou da difusão limitada de nutrientes, vitaminas e cofatores (LÓPEZ; VLAMAKIS; KOLTER, 2011); a impenetrabilidade do biofilme, indução de mecanismos de resistência (DONLAN; COSTERTON, 2002; STEWART; MUKHERJEE; GHANNOUM, 2004) e ainda a expressão de sistemas antitoxina que bloqueiam os antibióticos (LEWIS, 2005). A difusão limitada de moléculas antimicrobianas no biofilme tem sido relacionada à interação direta do antibiótico com proteínas e polissacarídeos da matriz (DONLAN, 2000; MAH; O´TOOLE, 2001) e à barreira física formada pela matriz (HALL-STOODLEY; STOODLEY, 2009). Além disso, o microambiente do biofilme facilita a transferência de material genético (elementos genéticos extra cromossômicos, como plasmídeos) entre as bactérias estimulando a variabilidade e mutações adaptativas como a transferência de resistência (SUH; RAMAKRISHNAN; PALMER, 2010), uma vez que as taxas de conjugação entre células do biofilme são maiores que em células planctônicas (HAUSNER; WUERTZ, 1999).

Plantas podem ser colonizadas por bactérias em todos os seus tecidos e a maioria das associações planta-bactéria dependem das interações fisiológicas entre a bactéria e os tecidos vegetais e, os biofilmes formados refletem a natureza de seus locais de colonização (RAMEY et al., 2004). A formação de biofilme em plantas está associada a respostas simbióticas e

patogênicas (BOGINO et al., 2013). Quando encontradas em associação com plantas, certas bactérias promotoras de crescimento, como as rizobactérias, não só induzem o crescimento das plantas, mas também as protegem de patógenos habitantes de solo em um processo conhecido como controle biológico. Em contraste, outras rizobactérias em uma matriz do biofilme podem causar patogênese em plantas. Embora pesquisas sugiram que a formação de biofilme em plantas está associada com o controle biológico e de resposta patogênica, pouco se sabe sobre como as plantas regulam esta associação (RUDRAPPA; BIEDRZYCKI; BAIS, 2008; BOGINO et al., 2013).

O desenvolvimento do biofilme contribui para a virulência de muitas espécies de bactérias fitopatogênicas através de vários mecanismos, incluindo o bloqueio dos vasos do xilema, resistência aumentada aos compostos antimicrobianos da planta, e/ou reforço da colonização em habitats específicos (MANSFIELD et al., 2012). Os processos de auto agregação e desenvolvimento de biofilme são relevantes para colonização e sobrevivência da bactéria na planta hospedeira.

Alguns dos patógenos bacterianos, agro economicamente importantes, como *Ralstonia solanacearum*, *Xanthomonas oryzae* pv. *oryzae*, *X*. *campestris* pv. *campestris*, *Erwinia amylovora*, *Pantoea stewartii* subsp. *stewartii*, *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. *actinidiae* e *Xylella fastidiosa* (patógenos vasculares) causam sintomas de doença por inibição das funções do xilema (MANSFIELD et al., 2012; BAE et al., 2015). Eles começam a infectar plantas através de raízes, folhas ou flores, dependendo de agentes patogênicos e, finalmente, atingem esse tecido. Uma vez alcançado tal tecido, produzem quantidades elevadas de exopolissacarídeos, e formam biofilme nas paredes do tecido. Como resultado, bloqueiam fisicamente o movimento da água através dos tecidos do xilema, causando sintomas de murcha (SCHUETZ; SMITH; ELLIS, 2013; BAE et al., 2015). Desta forma, compreender a complexidade e a diversidade dos biofilmes formados durante as interações planta-micróbio poderá beneficiar e contribuir com os esforços para elucidar a forma de controle de biofilmes associados aos vegetais.

Uma abordagem multidisciplinar tem sido empregada no tratamento e

controle de biofilmes, devido à valorização crescente do papel que eles desempenham na medicina moderna (TRENTIN; GIORDANI; MACEDO, 2013). Recentemente, tem havido um crescimento no interesse de estudos com biofilmes de fitopatógenos devido aos grandes impactos agro econômicos por eles causados. As estratégias para o combate de biofilmes podem, basicamente, ser divididas em dois segmentos: a inibição da formação de biofilmes e a erradicação ou tratamento de biofilmes já formados. Considerando o modo como pode ser alcançada a inibição da formação de biofilmes, duas grandes abordagens podem ser feitas: através da inibição do crescimento bacteriano, pelo uso de compostos bactericidas ou bacteriostáticos ou, através do bloqueio da adesão bacteriana e, consequentemente, da formação de biofilme por uma via que não envolve a morte bacteriana (terapias anti-virulência), explorando novos mecanismos de ação de novos compostos, visando dificultar o desenvolvimento da resistência microbiana (CLATWORTHY; PIERSON; HUNG, 2007; RASKO; SPERANDIO, 2010; TRENTIN; GIORDANI; MACEDO, 2013).

3. Biomoléculas vegetais no controle de fitopatologias

A convivência com patógenos já presentes em muitas áreas agricultáveis torna-se um ônus obrigatório dentro da agricultura moderna e, o tratamento de fitopatologias tem sido baseado no emprego de agroquímicos, que são em muitos casos, associados a outras práticas de controle de doenças como rotação de culturas e emprego de controle biológico, físico e genético, para que se tornem eficientes e economicamente viáveis a fim de garantir alta produtividade e qualidade de produção (FLORES et al., 2004). Essa prática é observada com maior intensidade nos países economicamente mais desenvolvidos, onde a agricultura é tecnologicamente mais avançada, acompanhando o crescimento do interesse público pela quantidade e qualidade de insumos agrícolas (AGRIOS, 2005).

O controle químico de fitopatologias atualmente é feito através de vários tipos de produtos, incluindo os pesticidas, cujo grupo mais importante para o controle de doenças de plantas é o dos fungicidas e bactericidas, com

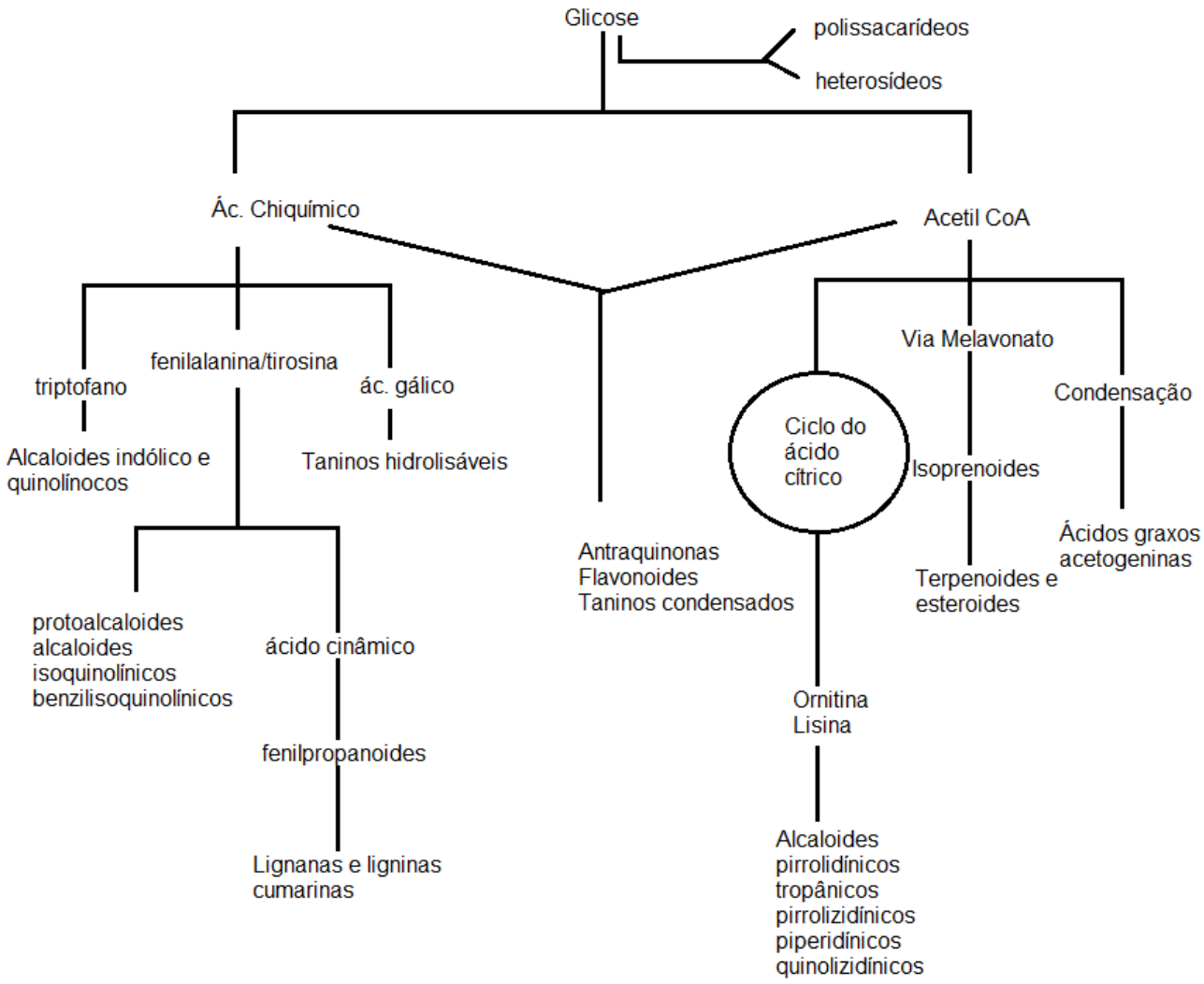
propriedades químicas e biológicas muito variáveis, podendo envolver vários princípios de controle em função da natureza do produto, da época, da metodologia de aplicação e do estágio de desenvolvimento epidemiológico da doença (GHINI; KIMATI, 2000). Contudo, o incremento nos custos, a perda da eficiência desses produtos ao longo dos anos e os problemas ambientais advindos destas práticas, apontam a necessidade da busca de alternativas para o controle de fitopatógenos, dentre as quais a busca por moléculas oriundas do metabolismo secundário de plantas, que apresentam atividade biológica contra fitopatógenos. Sua utilização no combate a doenças de plantas apresenta grande apelo econômico e, principalmente, ecológico (CASTRO, 2010).

As plantas produzem ampla diversidade de compostos orgânicos chamados metabólitos secundários, que têm função ligada à ecologia da planta, isto é, sua relação com o meio ambiente (KAYS, 1991; TAIZ; ZEIGER, 2013). Essas substâncias são produzidas em resposta a estímulos ambientais bastante variáveis, de natureza física, química ou biológica, que podem alterar a composição química dos vegetais (BARREIRO; MANSUR, 2001). A produção desses componentes tem como função proteger a planta contra herbívoros, desempenham funções importantes em interações planta-patógeno, bem como para beneficiá-la na competição com outros vegetais. Além disso, favorecem a atração de polinizadores, de animais dispersores de sementes, e o controle de microrganismos simbiontes. Acrescidos a estes fatores bióticos, a produção de metabólitos secundários também protege o vegetal de influências externas, como temperatura, umidade, proteção contra raios UV e deficiência de nutrientes minerais (ALVES, 2001; PERES, 2004; SIMÕES et al., 2010). Princípios ativos presentes no extrato bruto de plantas, sintetizados no metabolismo secundário, apresentam ação biológica diretamente contra patógenos ou na indução de resistência de plantas, devido características elicitoras (SCHWAN-ESTRADA et al., 2003).

Os metabólitos secundários podem ser divididos em três grupos principais: compostos fenólicos, terpenoides e alcaloides (Figura 5), utilizados na defesa contra estresses bióticos e abióticos (TAIZ; ZEIGER, 2013). Os compostos fenólicos são derivados do ácido chiquímico e ácido mevalônico, os

terpenos são produzidos a partir do ácido mevalônico (no citoplasma) ou do piruvato e 3-fosfoglicerato (no cloroplasto) e os alcaloides são provenientes de aminoácidos aromáticos (triptofano e tirosina), os quais são derivados do ácido chiquímico e de aminoácidos alifáticos (ornitina, lisina). Flavonoides, taninos e ligninas fazem parte dos compostos fenólicos; óleos essenciais, saponinas, carotenoides e a maioria dos fitoreguladorres são terpenos; nicotina, cafeína e vincristina são alguns exemplos de alcaloides (ALVES, 2001; PERES, 2004).

Figura 5. Esquema simplificado da principal rota de biossíntese de metabólitos-secundários.



Fonte: SIMOES et al. 1999 (adaptado).

A Caatinga, única formação vegetacional exclusivamente brasileira, ocupa 60% do território da região Nordeste (GIL, 2002), compreendendo uma área de aproximadamente 826.411 km^2 (MMA/IBAMA, 2010), que abrange os estados do Piauí, Ceará, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas, Sergipe, Bahia e Minas Gerais (SILVA; ALBUQUERQUE, 2005). A Caatinga tem como característica o potencial hídrico reduzido no solo, com acentuado período de estação seca (entre sete e dez meses). Sua flora nativa apresenta então características anatômicas, morfológicas e funcionais especializadas para a sobrevivência destas plantas a estas condições de clima e solo (DRUMOND et al., 2000).

A Caatinga é um ecossistema rico em recursos genéticos, com alta biodiversidade quando comparada a outras regiões semiáridas no mundo, apresentando uma variada cobertura vegetal, em grande parte determinada pelo clima, relevo e embasamento geológico. A Caatinga vem se destacando como área promissora na investigação de novas biomoléculas com atividade biológica, devido às espécies vegetais estarem adaptadas às condições extremas de estresse ambiental, característicos das regiões semiáridas, como baixa pluviosidade, temperaturas elevadas e altas taxas de evapotranspiração e incidência de luz UV (CARRARA, 1996). Embora seja uma das áreas mais ameaçadas do mundo, há poucos estudos sobre o potencial biotecnológico da Caatinga (ARCOVERDE et al., 2014). A expansão das ações voltadas para a bioprospecção de metabólitos secundários oriundos de plantas da Caatinga é extremamente importante, uma vez que as plantas tropicais têm sido fonte de materiais para a descoberta de novos medicamentos. Logo, se torna potencial na busca de novas alternativas para a agricultura (SOUZA; FELFILI, 2006; ARCOVERDE et al., 2014).

Estudos recentes sobre produtos naturais com ação antiformação de biofilme, como extratos aquosos obtidos de diversas plantas medicinais da Caatinga brasileira (TRENTIN et al., 2011), têm apresentado resultados promissores. Taninos purificados de plantas medicinais representam uma importante classe de compostos bioativos e impedem a formação de biofilme de *P. aeruginosa* por inibirem o crescimento bacteriano (efeito bacteriostático) (TRENTIN et al., 2013). Outros estudos que investigaram a ação de metabólitos

secundários da classe dos óleos essenciais mostraram que eles agem na superfície celular bacteriana causando, principalmente o comprometimento da estrutura e função da membrana plasmática, assim como coagulação do citoplasma (BURT, 2004; BAKKALI et al., 2008). Trabalhos com *Vinca minor* L. indicaram atividade de metabólitos secundários contra uma variedade de bactérias Gram positivas (GRUNJIĆ et al., 2015).

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Objetivos

1. Objetivo Geral

O presente trabalho teve por objetivo caracterizar estruturalmente o biofilme formado por fitobactérias de importância agronômica, avaliar o potencial antibiofilme e antibacteriano de extratos aquosos de plantas da Caatinga, além de investigar métodos de preparo de amostras de proteínas que mais se adequem a analises proteômica para as bactérias estudadas.

2. Objetivos Específicos

- ✓ Investigar a formação de biofilme por diferentes isolados de *Ralstonia solanacearum* e *Acidovorax citrulli*;
- ✓ Classificar os isolados quando a intensidade de formação de biofilme em Não formador, fraco, moderado ou forte formador de biofilme;
- ✓ Caracterizar morfologicamente os biofilmes dos isolados classificado como moderado ou forte formador de biofilme, através de microscopia eletrônica de varredura e microscopia confocal de varredura à laser;
- ✓ Obter extratos aquosos de plantas coletadas no vale do Catimbau – Caatinga
- ✓ Investigar a potencial atividade antibiofilme e antimicrobiana contra isolados de *R. solanacearum* formadores de biofilme;
- ✓ Realiza o fracionamento orgânico de pelo menos um extrato com atividades contra todos os isolados de *R. solanacearum* testados;
- ✓ Testar diferentes metodológicas de extração de proteínas de *R. solanacearum*, *A. citrulli* e *Pectobacterium carotovorum* subsp. *carotovorum*;
- ✓ Definir através de 2D-PAGE a metodologia de extração de proteínas mais adequada para a análise proteômica a ser aplicada em futuros estudos das proteínas envolvidas na formação de biofilme em fitopatógenos.

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Biofilm formation by phytopathogenic bacteria on hydrophobic surfaces

Carolina Barbosa Malafaia[a*], Myrzânia de Lira Guerra[b], Alexandre José Macedo[c], Elineide Barbosa de Souza[b], Maria Tereza dos Santos Correia[a], Márcia Vanusa Silva[a]

[a] Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife, Pernambuco, Brasil.

[b] Departamento de Biologia, Universidade Federal Rural de Pernambuco, Recife, Pernambuco, Brasil.

[c] Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brasil.

[*]Corresponding author: Carolina Barbosa Malafaia. Email: carol08malafaia@hotmail.com Tel.: +55 81 2126 8547; fax: +55 81 2126 8576.

Abstract

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

Keywords: *Acidovorax citrulli* subsp. *citrulli*; *Ralstonia solanacearum*; SEM; CLSM; abiotic surface.

1. Introduction

In nature microorganisms usually live as adhered cells aggregates, growing on a surface and immersed in an amorphous extracellular matrix known as biofilm (1, 2). The biofilm matrix is a complex structure composed by a variety of extracellular polymeric substances, such as exopolysaccharides, proteins and extracellular DNA. This structure allows the biofilm to be highly resistant to antimicrobial agents (3), conferring special importance in medical, agricultural and industrial environments (1).

The biofilm formation is extremely important for the survival of bacteria, besides contributing for the phytopathogens virulence via several mechanisms, including the block of xylem vessels, the increase of bacterial resistance to antimicrobial compounds and/or the increase of habitats colonization (2, 4). It is known that microbial populations use cellular links to adhere to solid supports, surfaces and particles where they grow and survive in natural state. This process is the first stage in the biofilm formation and occurs primarily via nonspecific physicochemical interactions and then by molecular interactions mediated by specific binding of the receptor-ligand type (5, 6). The microbial colonization is regulated dependently of the population density by *quorum sensing* signalling (7) that, combined with bacterial surface components, extracellular compounds and environmental signals, are required for biofilm development (2).

Several phytopathogenic Gram-negative bacteria are causing significant economic damage to global agriculture, since their control methods are still extremely inefficient. *Ralstonia solanacearum*, the cause of bacterial wilt, is a pathogen derived from soil source that penetrates into the xylem vessels through the root (8), where it multiplies and produces polysaccharides which increase the

xylem viscosity, causing clogging and consequent wilting and death of the plant. This bacterium has about 450 host species, belonging to over 54 botanical families (9, 10), especially species of the Solanaceae family (11). *Acidovorax citrulli* is the causal agent of bacterial fruit blotch, a disease that leads to decay fruit and often occurs in plants of Cucurbitaceae family (12), especially melon (*Cucumis melo*), watermelon (*Citrullus lanatus*) (13) and pumpkin (*Cucurbita maxima*) (14).

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

2. Material and Methods

2.1. Bacterial Isolates

A total of 13 isolates were used in this study, six of *R. solanacearum* of melon (10) and seven of *A. citrulli* of pepper (15). These isolates were obtained by Culture Collection of the Phytobacteriology Laboratory of the Agronomic Department of the Universidade Federal Rural de Pernambuco (UFRPE), Brazil, where they were isolated, identified and biochemically characterized. The isolate identity was confirmed by PCR of the 16S region, conducted at the Laboratory of Molecular Biology of the Biochemistry Department of Universidade Federal de Pernambuco (UFPE), Brazil.

2.2. Media

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

2.3. Quantification of Biofilm Formation

Biofilm production quantification in microplates was based on the adapted method described by Trentin et al. (16). In this study, sterile polystyrene 96 well microplates (Costar 3599) were purchased from Corning Inc. (USA). The isolates were cultured in NYDA medium (NYD added 18 g/L agar) for 24 h at 28 °C and a bacterial suspension in saline (0.9 % NaCl), corresponding to McFarland scale (3×10^8 CFU/mL), was used in the tests. One hundred and forty microliters of medium (NYD, TZC or YDC), 40 µL of bacterial suspension and 20 µL of distilled water were used for the biofilm formation. Rifampicin (32 µM) was used as antibiotic control. The plates were aerobically incubated for 24 h at 28 °C. The experiment was made at least in triplicate for each isolate.

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

the optical density (OD) was measured at 570 nm (Spectramax M2e multimode Microplate Reader, Molecular Devices, USA).

Thus, biofilm formation was evaluated by OD cut (ODc), following the classification of Stepanovic et al. (17), which consists of three standard deviations above the mean OD of the negative control, where: OD ≤ ODc - no biofilm producer, ODc < OD ≤ (2 x ODc) – weak biofilm producer, (2 x ODc) < OD ≤ (4 x ODc) - moderate biofilm producer and (4 x ODc) < OD - strong biofilm producer. Biofilms formed by isolates that presented as moderate or strong producers were observed in Scanning Electron Microscopy and Confocal Laser Scanning Microscopy

2.4. Scanning Electron Microscopy (SEM)

Biofilms were cultured in 96 well plate microplates (as described in item 2.3) with a Permanox™ slide (NalgeNunc International, USA) in each well. After 24 h incubation at 28 °C, the samples were fixed in glutaraldehyde (2.5 %) for 2.5 h, washed with cacodylate buffer (100 mM, pH 7.2) and dehydrated in increasing concentrations of acetone. The Permanox™ slides were dried by CO_2 critical point technique (CPD 030 Balzers, Liechtenstein), fixed on aluminum bases, covered with platinum film and examined in a scanning electron microscope (JEOL JSM-5800).

2.5. Confocal Laser Scanning Microscopy (CLSM)

Bacteria biofilms were cultured in CEELview™ glass bottom dish plates (35/10 mm, 4 compartments) under the same conditions of item 2.3 for 24 h at 28 °C and after this period the free bacteria were washed with PBS buffer (10

mM). The Live/Dead BacLight Kit (Invitrogen) was used to visualize the presence of live and dead bacteria, in green and red respectively, of according to the specifications of the manufacturer, and Concanavalin A tetramethylrhodamine conjugate (Invitrogen) was used to evidence the formation of microbial matrix (18). The experiment was performed in duplicate and visualized in confocal microscope (Olympus Fluoview 1000 - FV1000) with excitation light at 473 nm for green and 559 nm for red under objective of 60×.

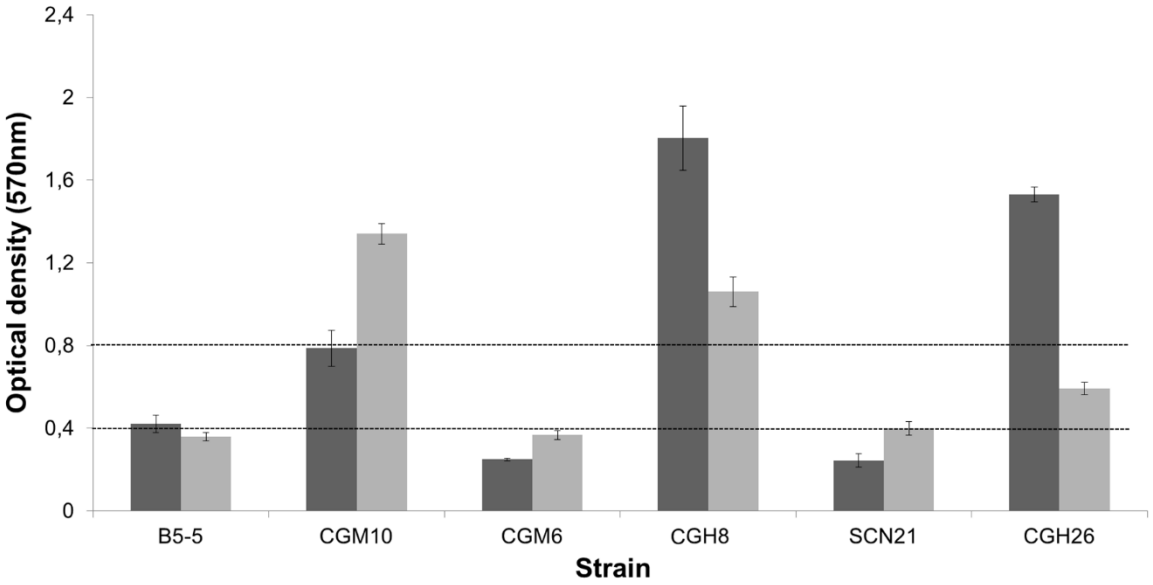
3. Results

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

SEM analysis showed biofilm structures produced by the different isolates in NYD medium (Fig. 2). It was possible to observe that the isolates of *R. solanacearum* were morphologically distinct from each other. B5-5 showed a filamentous pattern biofilm, where the bacteria were joined together forming long

filaments of attached cells (Fig. 2 panel 1). In contrast, CGH26 isolate (Fig. 2 panel 4) showed a dense and uniform biofilm with increased formation of multilayers and a matrix around of bacteria. The CGM10 and CGH8 isolates (Fig. 2 panels 2 and 3, respectively) possess a very similar pattern of biofilm formation between them, with the formation of a monolayer biofilm, which bacterial aggregates isolated were formed after 24h. *A. citrulli* showed structurally, dense, uniform and multilayered biofilms, more similar between the isolates. The images obtained by SEM were in accordance with the OD 570 nm biofilm quantification.

The biofilm was analyzed by CLSM after 24 hours using concanavalin A marking matrix and the staining Live (green)/Dead (red) to the bacteria (Fig. 3). It was observed the matrix formation in CGM10, CGH8, CGH26 and Aac1.43; contrary, it was not possible to detect the presence of biofilm matrix in B5-5 and Aac1.73 isolates. Live/Dead staining pattern showed a heterogeneous distribution among isolates. One can observe that B5-5 had the same filamentous pattern obtained by SEM, but with a great amount of viable cells. The other isolates showed considerable amount of adhered cells. The CGH8 showed an equal Live/Dead cells proportion while the CGH26 showed basically viable cells. Further, Aac1.43 and Aac1.73 isolates showed few adherent cells, but only Aac1.73 had a viable cell monolayer adhesion. These results were discordant from those obtained by SEM.



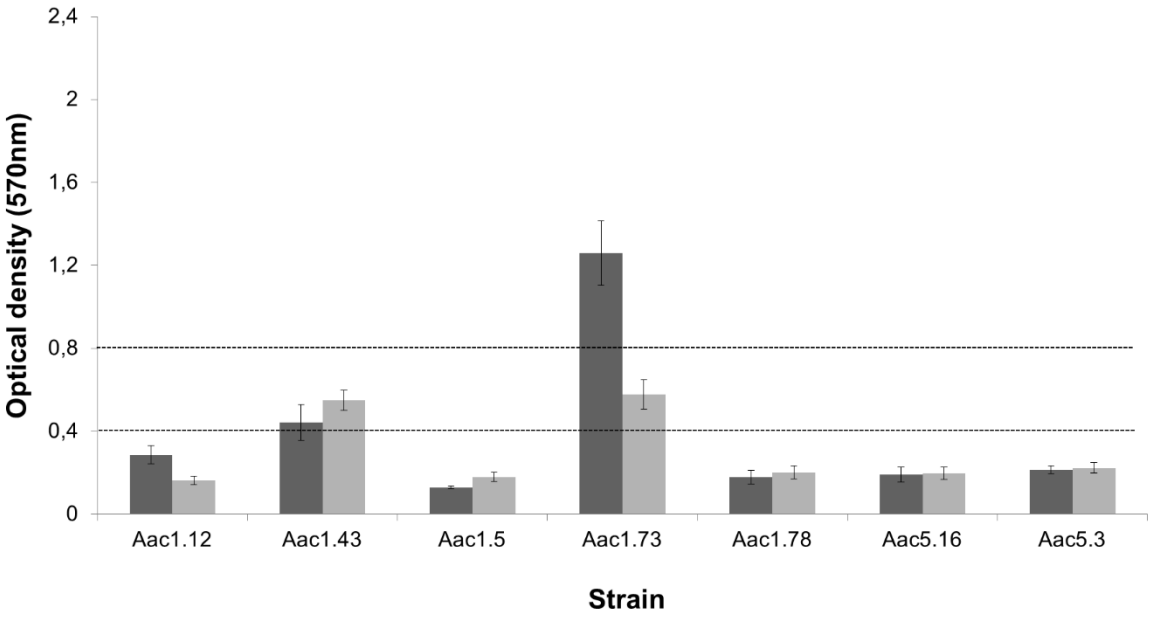


Fig. 1 Pure culture biofilm formation assay in ■ NYD media and ■ TZC media for *Ralstonia solanacearum* (A) and ■ YDC media for *Acidovorax citrulli* (B). Isolates below the dotted lines did not produced or were weak biofilm producers, between the dotted lines were moderate biofilm producers and above the dotted lines were considered strong biofilm producers. The bars represent the mean values (n = 24).

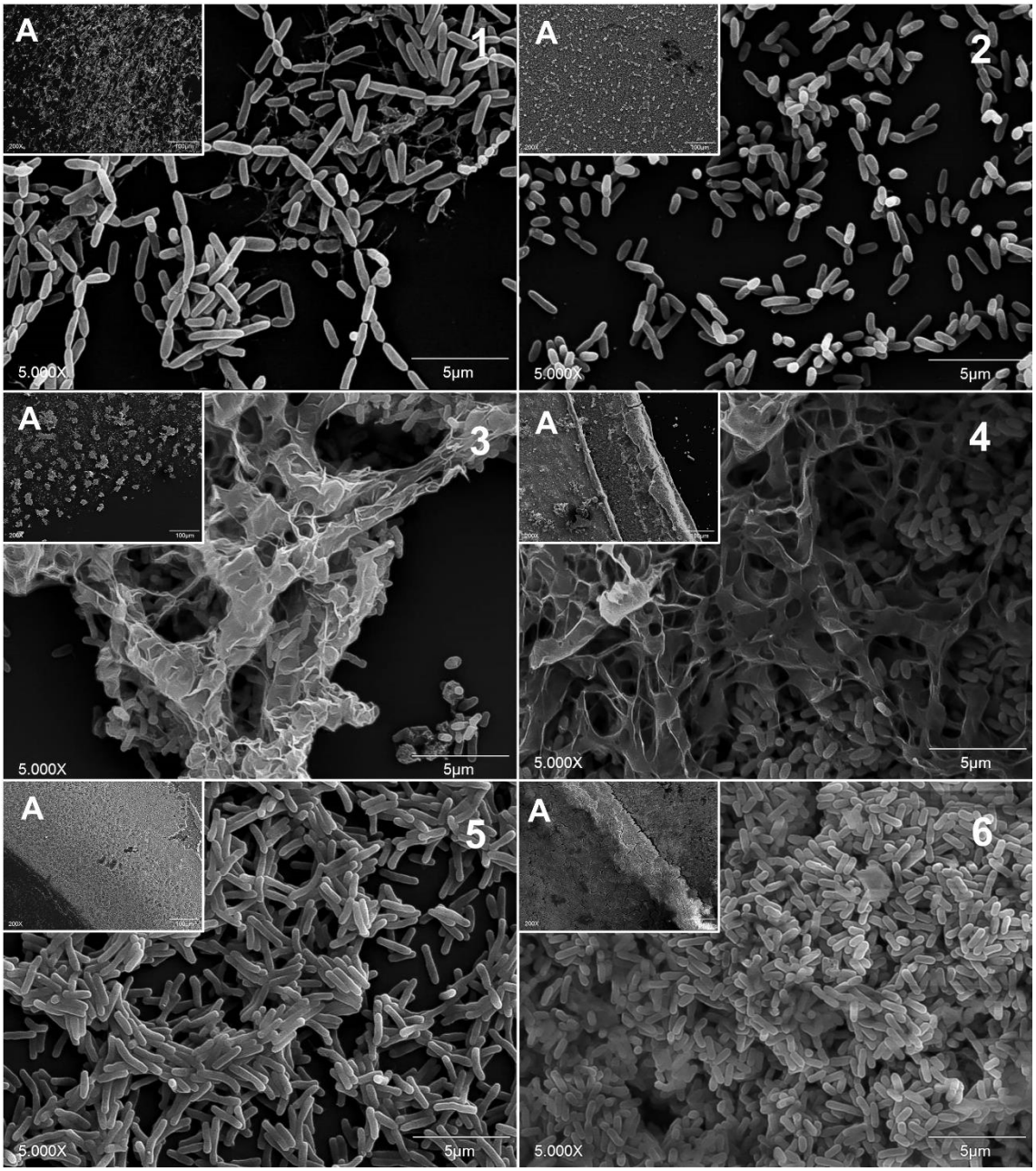


Fig. 2 SEM of biofilms in media NYD on Permanox™: *Ralstonia solanacearum* B5-5 (1), CGM10 (2), CGH8 (3), CGH26 (4) and *Acidovorax citrulli* Aac1.43 (5), Aac1.73 (6) at 5.000× (Inserts A at 200×).

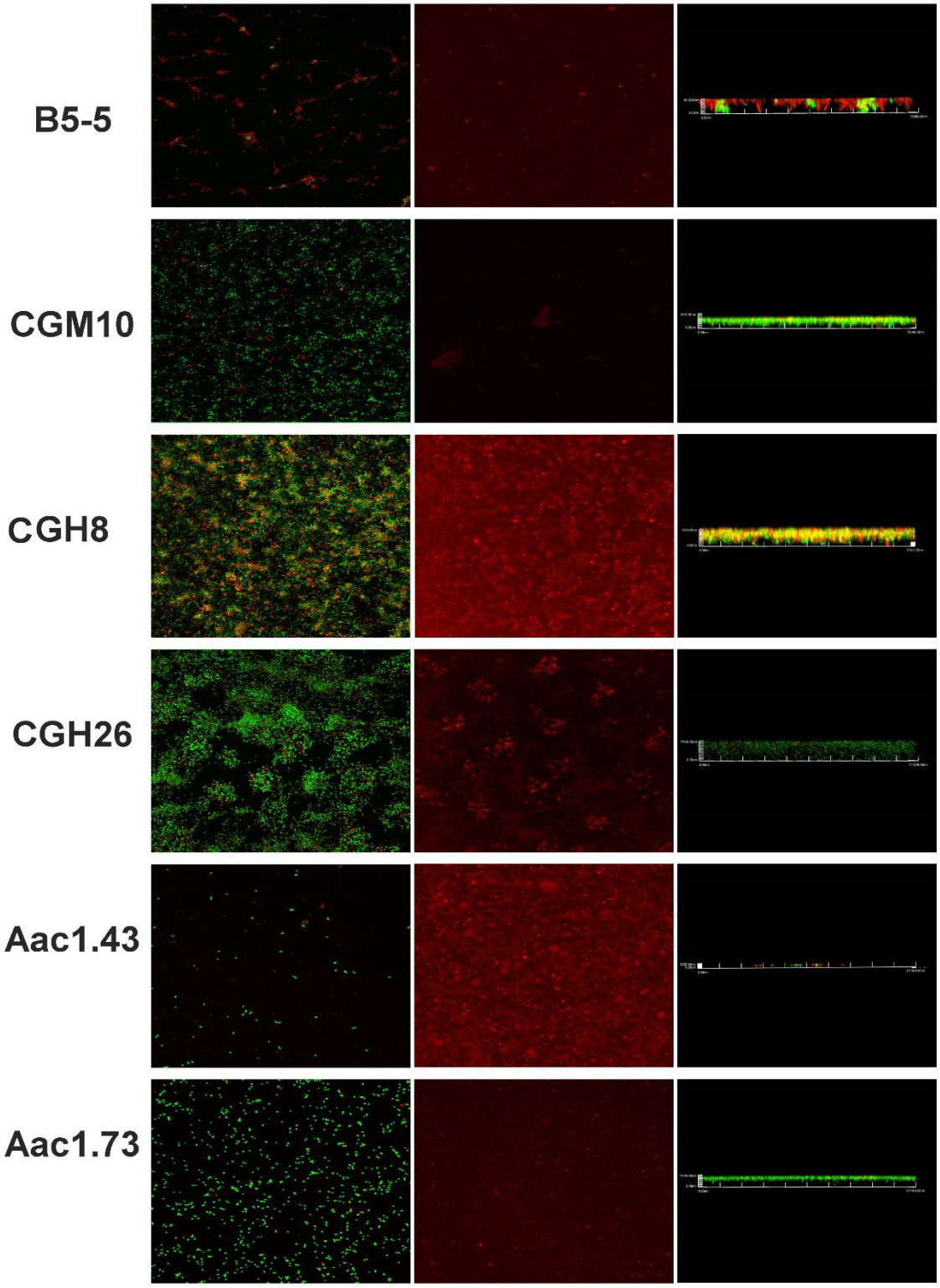


Fig. 3 Biofilm structure for *Ralstonia solanacearum* (B5-5, CGM10, CGH26 and CGH8) and *Acidovorax citrulli* (Aac1.43 and Aac1.73), after 24 h of growth in liquid culture medium NYD analyzed by Confocal Laser Scanning Microscopy (CLSM). Stained with A – Live/Dead BacLight (Invitrogen) and B – concanavalin A (Invitrogen). In C: biofilm thickness observed with Live/Dead stain.

4. Discussion

Currently, it is known that in most environments the microorganisms may change from a life-free state to a sessile lifestyle in order to form biofilms, exhibiting specific properties such as increased tolerance to adverse conditions including desiccation resistance and high concentrations of antimicrobial agents (19). Further, in several plant pathogens such as *Xylella fastidiosa* (20-22), *Xanthomonas campestris pv. campestris* (23, 24), *Pantoea stewartii subsp. stewartii* (25, 26) and *Clavibacter michiganensis* (27, 28), biofilm formation has been reported as an important factor associated with virulence, since it allows a greater resistance to plant defenses and competition with other species (1).

Garcia (29) observed that *R. solanacearum* isolates were strongly virulent against pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*) and tobacco (*Nicotiana tabacum*), confirming the results obtained in our study for the same isolates, since most of the they were moderate or strong biofilm producers. In addition, our results confirm previous studies that reported the biofilm formation on abiotic surfaces by *R. solanacearum* (30). However, for CGM6 and SCN21 isolates, which were poor producers of biofilm in both media tested, there was not a direct correlation between virulence and biofilm formation. According to Silva (31), *A. citrulli* Aac5.3 isolate shows low virulence in melon fruits (*Cucumis melo*) while Aac1.43 has moderate virulence and the others isolated used in this study had high virulence and was not observed correlation between these data and the biofilm formation.

The biofilm formation is a complex process regulated by several environmental features. One of the most important factors influencing its formation is the surface properties that allow bacterial adhesion, the first step of

the process (32), and the cell surface hydrophobicity also affect bacterial adhesion (33). It was previously shown that microorganisms, including *Pseudomonas syringae* isolates, adhere in greater numbers to hydrophobic materials (34), which in our work seems to have a positive influence to the biofilm formation of *R. solanacearum* and a negative influence to *A. citrulli*. Further, the medium composition also has influence in the biofilm formation. Nutritive media can influence the persistence of planktonic lifestyle, while a poorest media favor the biofilm formation due to stress condition that the bacteria are submitted (35). This may also have influenced the absence of biofilm formation by most of the *A. citrulli* isolates that were tested in this study.

In *R. solanacearum* SEM analysis, the CGM10, CGH8 and CGH26 isolates show the monolayer pattern for the biofilm formation, in which a cell layer adheres to the surface. This type of structure is favored when cell surface interactions are stronger than the cell-cell interactions (36). This process was shown in Figure 2 (panels 1, 2 and 3), in which bacteria aggregation and multiplication promotes the biofilm formation. The same was observed for *A. citrulli* isolates that developed multilayered biofilm, which bacteria were able to adhere to a surface and also other bacterial cell. This process is known as cell recruitment by *quorum sensing* and involves the release of attraction factors by the cells adhered to a surface, favoring the deposition of others bacteria, which multiply, thus promoting the increase of biomass (36, 37).

CLSM analysis showed the same biofilm structure observed by SEM for CGM10, CGH8 and CGH26 isolates, with viable cells inside of the extracellular polymeric matrix (EPM). Cell adhesion and cohesion, nutrition and protection are some of the several advantages of life in biofilm (38), however not all biofilm cells

252 profit these features, as noted in competitive relations between polymer
253 producers and non-producers. An evolutionary advantage of the strong
254 producers is probably due to formation of polymers which allows access of the
255 cells to oxygen rich environments (38, 39).

256 Bacterial surface structures and components, such as flagella, pili, fimbriae
257 and lipopolysaccharide (LPS) display a crucial role in the physical processes,
258 during the primary stages of the biofilm formation (interaction to the surface), and
259 the virulence development as observed in *Xylella fastidiosa* (40) and
260 *Xanthomonas oryzae* pv. *oryzicola* (41). It was shown in previous studies the
261 presence of pili type IV in *R. solanacearum* (30) and *A. citulli* (42, 43) which
262 connected to adhesion of these species, and LPSs mutations linked to LPS
263 synthesis in phytopathogenic bacteria, such as *Pseudomonas syringae* (44),
264 *Xanthomonas axonopodis* (45) and *Xanthomonas citri* (46) caused reductions in
265 biofilm formation and in virulence.

266 Although, in CLSM, B5-5 has shown cell adhesion, there was no matrix
267 formation. Further, it was observed a low adhesion and biofilm matrix formation
268 by *A. citrulli* isolates, probably due to the hydrophilic characteristics of glass slides
269 surface, which negatively influenced the biofilm formation in many species, since
270 that hydrophobic interaction is essential for the primary adhesion (6).

271

272 **5. Conclusions**

273 According to the results we conclude that, despite being an important factor
274 in the virulence for many pathogen, the biofilm formation not appear to be strictly
275 related in the tested species. We also conclude that the biofilm appears
276 structurally distinct such as observed for isolates of *R. solanacearum*. Most

277 isolates of *A. citrulli* not seem to be well suited to adhesion and biofilm formation
278 on hydrophobic abiotic surfaces. The biofilm formation is an important feature for
279 bacteria due to their natural formation and influence in the surrounding
280 environment, giving to the microorganisms high tolerance to adversity. Further
281 studies will be necessary to understand the mechanism of different biofilm
282 formation profile obtained. *R. solanacearum* and *A. citrulli* depict a great potential
283 to become natural models to study plant biofilm infections due to the high number
284 of host species. The recognition of the biofilm concept in plant pathology is likely
285 to have profound impact in the field, as it had in medical microbiology, with vast
286 implications in the development of novel strategies to prevent and eliminate these
287 and other plant diseases.
288
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299

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Effects of Caatinga plant extracts in planktonic growth and biofilm formation in *Ralstonia solanacearum*

Carolina Barbosa Malafaia[1*], Ana Cláudia Silva Jardelino[1], Ana Paula Sant'Anna da Silva[1]; Mariana Oliveira Barbosa[2]; Alexandre Gomes da Silva[3], Elineide Barbosa de Souza[4], Alexandre José Macedo[5], Maria Tereza dos Santos Correia[1], Márcia Vanusa Silva[1]

[1] Departamento de Bioquímica, Universidade Federal de Pernambuco, Recife, Pernambuco, Brasil.
[2] Programa de Pós Graduação em Biologia Vegetal, Universidade Federal de Pernambuco. Recife, Pernambuco, Brasil.
[3] Instituto Nacional do Semiárido, Campina Grande, Paraíba, Brasil.
[4] Departamento de Biologia, Universidade Federal Rural de Pernambuco, Recife, Pernambuco, Brasil
[5] Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brasil.

* Autor correspondente: Carolina Barbosa Malafaia.
Email: carol08malafaia@hotmail.com
Tel.: +55 81 2126 8547; fax: +55 81 2126 8576.

Abstract

Ralstonia solanacearum, the causal agent of bacterial wilt, a disease of major negative impact on the agriculture world, presents serious difficulties in handling due to its resistance to common control forms. Based on this context, the aim of this work is investigate alternative substances for control of the biofilm and the growth of this pathogen from Caatinga plants. Twenty-two aqueous extracts of plants collected in the Vale do Catimbau were evaluated for antibiofilm and antibacterial activity through the crystal violet method and antibacterial activity in microplate, considering as positive values higher than 50 % of inhibition. From the obtained results, the organic extracts from *Myroxylon peruiferum* were prepared using an increasing polarity series and evaluated for the above-mentioned tests. The organic extracts were qualitatively assessed by thin layer chromatography. The results of the screening showed that *Jacaranda rugosa* presented antimicrobial activity higher than 90 %, while the antibiofilm activity of branches of *Harpochilus neesianus* and leaves of *M. peruiferum* was higher than 50%, both for all tested *R. solanacearum* isolates. Among the organic extract of the *M. peruiferum* there was detected antibiofilm activity in the cyclohexane extract, whereas the ethyl acetate extract showed antibiotic activity, both at the inhibitory minimum concentration of 3 mg/ml. This study describes the first antibiofilm and antibacterial screening for plants from Caatinga against *R. solanacearum*. The evaluation presented in this study provides data as basis for the development of new natural agents for control of phytopathogens.

Key-words: Phytopathogens, antibiofilm, *Myroxylon peruiferum*, *Harpochilus neesianus*, *Jacaranda rugosa*.

1. Introduction

Ralstonia solanacearum is one of the most destructive pathogens identified until the present moment due to its capacity to induce rapid and fatal symptoms in host plants (Yuliar *et al*. 2015). This bacterium is Gram-negative, has rapid growth, and agricultural and economic importance to colonizing the vascular system in several arable plant species (Meng, 2013). The infection caused by this pathogen begins in the roots and reaches the xylem vessels. Once there, it produces large amounts of exopolysaccharides that are viscous macromolecules secreted by the bacterial cells and responsible for the formation of biofilm on the walls of the xylem, inhibiting their functions through physical blockage and preventing the water circulating in the plant, thus causing wilt symptoms and consequent death of the plant (Mansfield *et al*. 2012; Bae *et al*. 2015). The difficulties related to the control of this pathogen are associated with their ability to endophytically grow, survive in the soil, especially in the deeper layers, being carried by the water and infect weeds (Yuliar *et al*. 2015).

The biofilm production has been described as an important virulence factor in a wide variety of phytopathogens, including *R. solanacearum* (Kang *et al*. 2002; Dow *et al*. 2003; Ramey *et al*. 2004; Joe *et al*. 2015), since this form of life becomes the microorganisms tolerant to adverse environmental conditions, such as lack of water and nutrients, drastic and sudden pH variations, and interspecific competition, in addition especially to resistance against defense strategies imposed by the host, such as production of antimicrobial (Dow *et al*. 2003; Muranaka *et al*. 2012). This resistance is due to the biofilm structure, which is formed by a exopolysaccharidic matrix (EPS) mainly composed of exopolysaccharides, extracellular proteins and extracellular DNA (Stoodley *et al*. 2002; Trentin *et al*. 2013).

Nowadays there is a strong commitment to the discovery of new natural antibacterial agents in order to replace synthetic molecules with harmful intends to health and the environment (Venuroso *et al*. 2011). Plants have been a suitable choice to be important sources of bioactive natural products from secondary metabolism. These compounds are synthesized in response to extreme environmental conditions, such as high temperatures, high incidence of UV light, high concentration of salts in the soil and limited water availability (Bita and

Gerats 2013); they are also used as chemical defenses against microorganisms (Trentin *et al*. 2013). In what they concern, plant-derived compounds have gained wide interest in seeking to identify alternatives for microbial control (Essawi and Srour 2000).

Previous research based on bioactive plant components have focused almost exclusively on the effects of these compounds against planktonic bacteria. With less emphasis on biofilms that are more resistant to antimicrobial agents, and therefore more difficult to control and eradicate (Sandasi *et al*. 2010). In Caatinga, located in the Northeast of Brazil, medicinal plants are an important social and cultural component. Since plants produce chemical defense against environmental microbes, they constitute an alternative to the necessity to find antibacterial with innovative action modes, including antibiofilm compounds (Silva *et al*. 2015).

A range of natural products received an increased attention due to its antibiofilm and antimicrobial activities against a wide variety of human pathogens (Kawsud *et al*. 2014; Nikolić *et al*. 2014), while few studies reported the effect of these compounds on phytopathogens. The aim of this study was to investigate the effects of 22 aqueous extracts of 16 plants collected in the Caatinga ecosystem on the bacterial growth and biofilm formation of *R. solanacearum*.

2. Materials and methods

2.1. Plant material

The plant material was collected at various points within the Catimbau National Park, Pernambuco, Brazil, in November 2014 (Table 1). The species were identified in the herbarium of the Instituto Agronômico de Pernambuco (IPA) and a voucher for each specimen was deposited. The plant tissues collected were separate and dried at 40 °C for 3 to 4 days. Subsequently, the material was pulverized in a blender.

2.2. Aqueous extracts

The extracts were prepared according to Trentin *et al.* (2011) with modifications, where the dried powdered plant was mixed for 15 min with sterile water [1:9; (w:v)] and kept in the dark at room temperature (22 °C) for 24 h. After

this period the mixture was filtered and the filtrate was liofilized. A 4 mg/mL and 40 mg/mL aqueous solution were filtered through a 0.2 µm pore membrane and stored at −20 °C.

Table. 1 List of plant tested against *Ralstonia solanacearum* strains collected from region of semi-arid Pernambuco, Brazil (Caatinga).

Species	Popular Name	Family	Plant Tissue
Anadenanthera colubrina (Vell.) Brenan var. *colubrina*	Angico	Fabaceae (Mimosoideae)	Stem bark
Bauhinia acuruana Moric.	Mororó, Pata de vaca	Fabaceae (*Caesalpinioideae)*	Branches and fruits
Commiphora leptophloeos (Mart.) J.B. Gillett	Imburana, amburana, imburana de cambão	Burseraceae	Stem bark
Cronton sp.	Capixingui, Velame	Euphorbiaceae	Leaves and stembark
Dalbergia nigra (Vell.) Allemão ex Benth.	Jacarandá da Bahia	Fabaceae (Papilionoideae)	Leaves and fruits
Eugenia brejoensis Mazine	Cutia	Myrtaceae	Leaves
Harpochilus neesianus Mart. ex Nees	Pau de gume	Acanthaceae	Branches, inflorescences and leaves
Hymenaea courbaril var. *courbaril* L.	Jatobá	Fabaceae (*Caesalpinioideae)*	Leaves
Jacaranda rugosa A.H.Gentry	Pindaíba-do-cerrado, Pindaibinha	Bignoniaceae	Leaves
Libidibia ferrea (Mart. exTul.) L.P. Queirozvar.*férrea*	Pau ferro, jucá	Fabaceae (Caesalpinioideae)	Leaves and fruits
Myroxylon peruiferum L. f.	Balsamo do perú	Fabaceae (Papilionoideae)	Leaves
Ouratea blanchetiana (Planch.) Engl.	Batiputá	Ochnaceae	Leaves
Parkinsonia aculeate L.	Cina-cina, Espinho-de-judeu	Fabaceae (Papilionoideae)	Leaves
Pityrocarpa moniliformis (Benth.) Luckow & R. W. Jobson	Canzenzo, angico de bezerro, quipembe	Fabaceae (Mimosoideae)	Leaves
Polygala boliviensis A.W. Benn.	Arrozinho	Polygalaceae	Aerial part
Turnera melochioides Cambess.	Piriqueta	Passifloraceae	Leaves

2.3. Organic extracts

The dried and crushed leaves (100g) of *Myroxylon peruiferum* were subjected to organic extraction using an increasing polarity series of solvents (cyclohexane, chloroform, ethyl acetate and methanol) in Soxhlet (45 to 80 °C), subjected to reflux until saturation. Then, the extracts were filtered and subsequently concentrated until complete removal of the solvent on a rotary evaporator at 45 °C under reduced pressure. Finally, the extracts were analyzed for phytochemical and antimicrobial activities.

2.4. Phytochemical Screening

Organic extracts from *M. peruiferum* leaves were evaluated for the presence of secondary metabolites. The analysis of alkaloids, triterpenoids, flavonoids, and tannins were performed by thin layer chromatography using a silica gel plate (Merck), according to Wagner and Bladt (2009), while the presence of saponins was evaluated according to Silva *et al*. (2010).

2.5. Bacterial isolate and culture conditions

Three *R. solanacearum* isolates that are biofilm formers (B5-5, CGH26 and CGH8) obtained by Culture Collection of the Phytobacteriology Laboratory of the Agronomic Department of the Universidade Federal Rural de Pernambuco (UFRPE), Brazil. They were isolated, identified, biochemically characterized, and grown in NYDA (5 g/L yeast extract, 3 g/L meat extract, 5 g/L peptone, 10 g/L dextrose and 18 g/L Agar) during 24h, at 28 °C. A bacterial suspension in 0.9% NaCl, corresponding to 1 McFarland scale (3×10^8 CFU/mL), was used in the assays.

2.6. Antibiofilm and antibacterial assays

A protocol adapted from Trentin *et al*. (2011), employing crystal violet in sterile 96-well microtiter plates, polystyrene flat-bottom (Costar 3599), spurchased from Corning Inc. (USA), was used. Forty microliters of the bacterial suspension, 20 µl of the aqueous extract (concentration of 0.4 mg/ml or 4.0 mg/ml in the wells) or organic extract (concentration of 0.5mg/ml to 4 mg/ml) and 140 µl of medium broth NYD were added to wells of the microtiter plates. After incubation at 28 °C for 24 h, the content of the wells was removed and the wells were washed three times with sterile saline. The remaining attached bacteria were heat-fixed at 60 °C for 1 h. The adherent biofilm layer formed was stained with 0.4 % crystal violet for 15 min at room temperature. The stain bound to the cells was solubilized with 99.5 % ethanol and after 30 min the absorbance was measured at 570 nm (Spectramax M2e Multimode Microplate Reader, Molecular Devices, USA). The biofilm formation in control was considered to represent 100 % of biofilm formation, and the extracts were replaced by 20 µl of water in 96-well

microtiter plates. Values higher than 100 % represent a stimulation of biofilm formation in comparison to the control.

The planktonic bacterial growth was evaluated by the difference between the OD_{600} absorbance measured at the end and at the beginning of the incubation time in polystyrene 96-well microtiter plates. As a control for bacterial growth, the extracts were replaced by 20 µl of water, with this being considered to represent 100 % of planktonic bacterial growth. Values higher than 100 % represent a stimulation of bacterial growth in comparison to the control.

2.7. Statistical analysis

All microbiological experiments were performed at least in triplicate. The results were expressed as percentage of mean ± standard deviation. Differences between groups were evaluated by Student's t-test and the values were considered statistically significant if compared to the significance level of $p < 0.05$.

3. Results

Table 2 shows the screening of 22 aqueous extracts from 16 different plants. Their antibacterial and antibiofilm activities were tested against three isolates that form biofilm of *R. solanacearum*. The results showed the inhibition of the growth of all *R. solanacearum* isolates (growth less than 50 % compared to control) by five extracts: *Croton* sp. leaves, *Dalbergia nigra* fruits, and *Jacaranda rugosa* leaves at a concentration of 4 mg/ml, the latter with better inhibition percentage (above 90 %) for the three tested strains, and *Eugenia brejoensis* leaves and *Libidibia ferrea* fruits at a concentration of 0.4 mg/ml.

Table. 2 Biological activity of aqueous extracts of Caatinga plants against biofilm formation and growth of tree strain of *Ralstonia solanacearum*.

Species	Plant tissue	B5-5				CGH26				CGH8			
		0.4mg/ml		4mg/ml		0.4mg/ml		4mg/ml		0.4mg/ml		4mg/ml	
		Biofilm formation	Bacterial growth	Biofilm formation	Bacterial growth	Biofilm formation	Bacterial growth	Biofilm formation	Bacterial growth	Biofilm formation	Bacterial growth	Biofilm formation	Bacterial growth
Anadenanthera colubrina	Stem bark	42.8 ± 16.7*	61.2 ± 12.0*	34.0 ± 11.9*	21.2 ± 5.8*	58.7 ± 11.9*	61.0 ± 8.5*	45.9 ± 15.5*	34.8 ± 22.8*	175.6 ± 20.7*	100.3 ± 13.3	33.3 ± 5.2*	52.0 ± 30.9*
Bauhinia acuruana	Branches	116.2 ± 29.6	88.5 ± 16.5	81.7 ± 22.3	56.1 ± 9.8*	221.3 ± 94.6*	78.1 ± 5.9*	133.1 ± 30.7*	63.4± 2.4*	115.7 ± 34.8	88.5 ± 3.7*	175.5 ± 23.2*	79.8 ± 20.2
Bauhinia acuruana	Fruits	170.9 ± 36.6*	90.9 ± 10.4	151.5 ± 12.7*	48.4 ± 4.1*	117.8 ± 13.1*	97.8 ± 5.6	52.5 ± 11.1*	56.3 ± 12.0*	107.3 ± 20.9	97.9 ± 10.5	132.4 ± 39.4*	53.7 ± 12.9*
Commiphora leptophloeos	Stem bark	47.9 ± 6.9*	72.3 ± 18.1*	35.8 ± 8.7*	48.7 ± 11.4*	48.3 ± 16.4*	71.6 ± 6.1*	43.3 ± 23.2*	52.0 ± 0.3*	177.1 ± 19.5*	55.3 ± 20.4*	162.5 ± 15.9*	48.5 ± 13.1*
Cronton sp.	Leaves	203.8 ± 57.9*	88,4 ± 11.3	19.6 ± 15.5*	44.5 ± 5.9*	86.6 ± 33.0	105.5 ± 15.0	8.2 ± 0.3*	49.4 ± 0.4*	69.6 ± 17.1*	67.0 ± 9.8*	23.1 ± 7.0*	49.2 ± 5.0*
Cronton sp.	Stembark	145.4 ± 50.3	93.1 ± 3.5*	152.6 ± 58.3	49.6 ± 3.1*	107.9 ± 5.0*	89,8 ± 2.5*	23.9 ± 12.2*	73.0 ± 16.1*	84.2 ± 14.6*	89.7 ± 5.3*	97.7 ± 1.0*	57.6 ± 6.5*
Dalbergia nigra	Leaves	58.2 ± 24.9*	96.3 ± 11.3	162.7 ± 27.8*	44.5 ± 5.9*	113.1 ± 7.6*	98.8 ± 4.6	32.3 ± 9.2*	57.2 ± 10.5*	115.7 ± 23.4	87.4 ± 10.5*	93.3 ± 19.3	61.6 ± 7.9*
Dalbergia nigra	Fruits	90.6 ± 17.2	67.1 ± 0.8*	58.8 ± 11.3*	35.2 ± 7.7*	93.7 ± 30.1*	76.4 ± 2.0*	16.3 ± 8.3*	45.4 ± 7.9*	111.0 ± 2.2*	77.0 ± 11.5*	81.7 ± 9.0*	35.0 ± 3.5*
Eugenia brejoensis	Leaves	21.8 ± 4.1*	35.9 ± 3.0*	21.9 ± 4.6*	49.3 ± 11.3*	10.8 ± 0.8*	39.1 ± 1.1*	12.7 ± 1.8*	60.8 ± 10.5*	48.2 ± 3.0*	42.5 ± 6.2*	14.9 ± 3.9*	54.6 ± 6.7*
Harpochilus neesianus	Branches	128.1 ± 39.6	99.6 ± 10.1	16.6 ± 3.0*	101.6 ± 11.6	125.2 ± 2.6*	89.2 ± 5.6*	9.8 ± 0.9*	135.5 ± 32.9*	91.2 ± 8.5*	116.6 ± 23.0	9.3 ± 0.2*	180.1 ± 33.4*
Harpochilus neesianus	Inflorescences	128.1 ± 14.4*	108.6 ± 11.5	130.7 ± 19.6*	47.4 ± 2.4*	101.8 ± 12.5	101,6 ± 3.7	19.6 ± 6.7*	63.2 ± 11.6*	86.9 ± 8.5*	139.7 ± 24.6*	79.6 ± 27.4	61.2 ± 9.8*
Harpochilus neesianus	Leaves	143.1 ± 3.5*	72.1 ± 0.5*	147.0 ± 7.1*	135.0 ± 4.6*	75.5 ± 7.6*	64.6 ± 5.8*	19.2 ± 6.5*	104.1 ± 23.5	97.8 ± 8.0	87.7 ± 8.6*	42.1 ± 6.1*	173.8 ± 29.5*
Hymenea courbaril var. *courbaril*	Leaves	82.0 ± 3.8*	83.6 ± 6.5*	39.1 ± 16.0*	39.4 ± 6.5*	73.3 ± 9.5*	101.3 ± 12.2	14.7 ± 2.0*	56.0 ± 15.1*	113.3 ± 7.9*	91.7 ± 6.5*	102.4 ± 8.0	42.6 ± 21.3*
Jacaranda rugosa	Leaves	56.4 ± 11.4*	69.6 ± 13.0*	16.8 ± 3.6*	4.9 ± 8.0*	110.0 ± 32.4	75.0 ± 6.6*	22.1 ± 8.7*	9.2 ± 9.9*	105.6 ± 28.3	62.3 ± 4.8*	17.5 ± 10.2*	1.3 ± 1.2*
Libidibia ferrea	Leaves	21.9 ± 4.6*	18.9 ± 4.1*	62.5 ± 10.9*	87.9 ± 13.7	27.7 ± 8.4*	17.6 ± 0.0*	74.1 ± 25.6*	74.9 ± 0.8*	65.9 ± 19.6*	23.8 ± 4.7*	65.3 ± 37.3	166.6 ± 36.3*
Libidibia ferrea	Fruits	22.1 ± 5.9*	24.2 ± 7.5*	48.4 ± 13.0*	27.9 ± 7.9*	22.5 ± 15,9*	23.1 ± 0.9*	46.7 ± 11.4*	29.0 ± 6.0*	122.1 ± 1.5*	32.5 ± 7.9*	31.7 ± 3.1*	80.4 ± 11.9*
Myroxylon peruiferum	Leaves	51.7 ± 7.0*	77.2 ± 2.9*	49.7 ± 0.4*	98.6 ± 4.3	14.9 ± 8.1*	102.8 ± 6.8	16.5 ± 6.9*	127.6 ± 10.0*	15.4 ± 1.1*	134.3 ± 11.5*	15.6 ± 1.5*	177.9 ± 7.8*
Ouratea blanchetiana	Leaves	114.4 ± 10.9	83.1 ± 11.4*	78.9 ± 12.7*	46.9 ± 1.7*	141.2 ± 81.0	80.8 ± 6.7*	75.5 ± 10.2*	54.7 ± 5.6*	166.9 ± 11.1*	110.2 ± 10.1	172.7 ± 17.3*	70.3 ± 19.6*
Parkisonia aculeata	Leaves	141.1 ± 23.1*	87.2 ± 24,9	52.8 ± 10.9*	63.1 ± 17.3*	200.8 ± 2.9*	137.1 ± 19.5*	80.9 ± 21.4	65.0 ± 5.4*	185.9 ± 20.4*	290.2 ± 38.7*	178.6 ± 21.8*	241.8 ± 56.2*
Pityrocarpa moniliformis	Leaves	40.2 ± 7.8*	61.6 ± 18,3*	33.4 ± 7.4*	39.6 ± 17.5*	58.0 ± 26.1*	66.0 ± 6.8*	46.8 ± 25.9*	42.9 ± 1.8*	164.7 ± 31.5*	83.1 ± 19.6	108.1 ± 25.5	61.7 ± 25.0*
Polygala boliviensis.	Aerial part	84.1 ± 18.7	153.0 ± 23.3*	156.4 ± 79.4	107.0 ± 12.7	64.2 ± 5.9*	158.5 ± 17.2*	16.1 ± 3.0*	111.1 ± 9.6*	95.3 ± 28.6	134.1 ± 11.1*	106.2 ± 33.3	90.1 ± 8.9
Turnera meloquioides	Leaves	148.9 ± 44.3*	74.5 ± 6.4*	35.6 ± 2.5*	39.3 ± 4.1*	175.4 ± 56.1*	119.0 ± 14.9*	71.0 ± 7.8*	112.6 ± 10.3*	122.9 ± 30.9	99.4 ± 13.1	39.9 ± 14.3*	190.8 ± 4.4*

Results represent mean ± standard deviation of three experiments. * Represents significant difference in relation to control ($p<0.05$).

Biofilm formation results demonstrated that five extracts presented potential antibiofilm activity (reduction biofilm formation over than 50 %) of at least one isolate: *Harpochilus neesianus* branches (4 mg/ml) showed activity against all three isolates B5-5 (83,4 %), CGH26 (90.2 %) and CGH8 (90.7 %), and leaves (4 mg/ml) had antiformation activity against CGH26 (80.8 %) and CGH8 (58.9 %); *Myroxylon peruiferum* leaves too showed activity against all three isolates for B5-5 (50.3 %) at 4 mg/ml concentration and CGH 26 (85.1%) and CGH8 (84.6%) at a concentration of 0.4 mg/ml; the aerial part of *Polygala boliviensis* presented antiformation activity for biofilm only against CGH26 (83.9 %), as well as *Turnera meloquioides* leaves only against CGH8 (60.1 %) (both at 4 mg/ml). The two latter extracts revealed no significant antibacterial activity.

Since biofilms are an important mechanism of resistance and permanence in a common environment for many microorganisms, including *R. solanacearum*, the *Myroxylon peruiferum* leaf extract was selected and analyzed by organic extraction for the identification of potential phytochemicals groups with antibiofilm and/or antimicrobial activities against CGH8 isolate strong biofilm former (Figure 1). The ethyl acetate extract showed a 53.5 % inhibition on bacterial growth; at this concentration the extract did not affected biofilm formation. Antibiofilm activity was observed in the cyclohexane extract, which inhibited biofilm formation on a statistical difference between the concentrations of 3 mg/ml (54.1 %) and 4 mg/ml (63.7 %), determining a minimum inhibitory concentration of 3 mg/ml, by a mechanism that does not involve bacterial killing, since there was a stimulation of cell growth (up to 115 %).

Table 3 presents the preliminary qualitative phytochemical screening of these extracts, which indicated the presence of flavonoids in all extracts, coumarin and triterpenes in all extracts except the methanolic, and only the latter presented saponins and tannins.

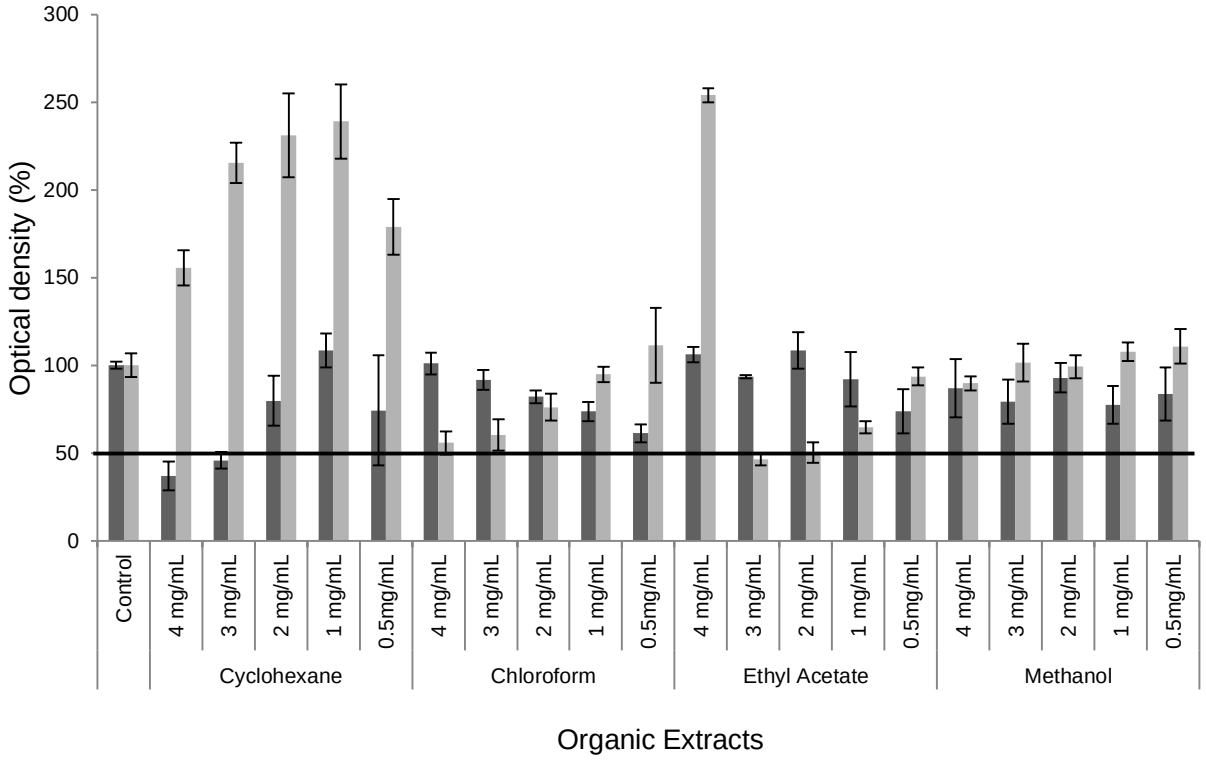

Fig 1. Pure culture biofilm formation assay in NYD in presence of organic extracts of *Myroxylon peruiferum* isolate CGH8; ■ Biofilm formation and ■ bacterial growth of *Ralstonia solanacearum*. The bars represent the mean values (n = 12).

Table. 3 Phytochemical analysis of organic extracts of leaves *Myroxylon peruiferum*.

Class of compounds	Extracts			
	CH	CF	EA	M
Alkaloids	-	-	-	-
Coumarins	+	+	+	-
Flavonoids	+	+	++	+++
Saponins	-	-	-	+
Tannins	-	-	-	+
Triterpenes	++	+++	++	-

(-) Absence, (+) weak, (++) average, (+++) Strong.
CH: Cyclohexane, CF: Chloroform, EA = Ethyl Acetate, M = Methanol

4. Discussion

The antibiotic resistance to plant pathogenic bacteria has increased in recent years, and the World Health Organization prohibited increasingly the use of many pesticides in agriculture due to the high toxicity to humans and the environment (Barnard, 1997; Minz *et al*. 2012). Many studies reported the antibacterial activity of plant extracts against human pathogens and their

pharmaceutical application (Gibbons, 2005), nevertheless the knowledge about such activity against plant pathogens is still scarce. Inhibition of biofilm formation, such that bacterial growth is adversely affected, comprises an alternative and attractive approach in controlling diseases, since it can hinder the rapid development selective pressure of bacterial resistance (Rasko and Sperandio, 2010; Silva *et al*. 2015).

Vegetables are abundant sources of many bioactive compounds with a great potential for the development of new antimicrobial agents (Muttalib *et al*. 2015). The plants analyzed in this study have popular and well-known applications for the treatment of infectious diseases and inflammation, indicating a potential antimicrobial feature that can be applied in fields such as the control of phytopathogens.

Myroxylon peruiferum is used medicinally as anti-inflammatory and expectorant, been used also in cases of asthma, asthmatic bronchitis, cystitis, pulmonary disease, headache, external wound healing, weakness, sore throat, cough and airway symptoms (Lorenzi and Matos, 2008). According to Gonçalves *et al*. (2005), hydro-alcoholics extracts of *M. peruiferum* showed higher antibacterial activity amplitude against *Streptococcus pyogenes*, *Staphylococcus aureus* and *Shigella sonnei*.

Commonly, organic fractions present greater biological activity than those observed in aqueous fraction in what concern the inhibition of biofilm formation of bacteria species (Muttalib *et al.* 2015), however our results presented other point of view, suggesting that the separation of compounds caused by increasing polarity series may have separated components that increase antibiofilm activity when in synergy.

Preliminary phytochemical screening of the active extract of *Myroxylon peruiferum* leaves revealed the presence of coumarins, flavonoids and triterpenoids, secondary metabolites produced by plants that, among other functions, are used in defence against pathogens (Cowan, 1999). Flavonoids are aromatic substances and coumarins are the largest group of substances extracted from plants with antimicrobial activity already reported (Cowan, 1999). These chemical classes of secondary metabolites have been described with a great antibiofilm activity, as evidenced by Riihinen *et al*. (2014), where flavonoids isolated from *Vaccinium vitis-idaea* were active against biofilm formation of

Streptococcus mutans and *Fusobacterium nucleatum*. Awolola *et al*. (2014) reported that purified flavonoids and triterpenes from *Ficus sansibarica* showed antibacterial and antibiofilm activities against *Staphylococcus aureus* and *Escherichia coli*.

5. Conclusões

The antimicrobial properties of most plants display their characteristics as valuable sources for research related to the development of new bioactive compounds safe for the environment, with less contamination for humans and animals, and effectively in the protection of arable regions. The search for a viable alternative in a diverse environment and still scarce studied as Caatinga leadeis to *M. peruiferum* and *Harpochilus neesianus*, a plants used for medicinal purposes that contains phytochemicals also effective in inhibiting biofilm formation by phytopathogens. The action of this antibiofilm extract against *R. solanacearum* is probably associated with the inhibition of cell adhesion process. This in vitro screening is important to guide the development of new products in addition to guide research studies of bioactive compounds.

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Conflict of interests

The authors declare that they have no competing interests.

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Selection of a protein solubilization method suitable for phytopathogenic bacteria: a proteomics approach

Carolina B Malafaia[1*†], Myrzânia L Guerra[2†], Túlio D Silva[1], Patrícia MG Paiva[3], Elineide B Souza[4], Maria TS Correia[3] and Márcia V Silva[3]

Abstract

Background: Finding the best extraction method of proteins from lysed cells is the key step for detection and identification in all proteomics applications. These are important to complement the knowledge about the mechanisms of interaction between plants and phytopathogens causing major economic losses. To develop an optimized extraction protocol, strains of *Acidovorax citrulli*, *Pectobacterium carotovorum* subsp. *carotovorum* and *Ralstonia solanacearum* were used as representative cells in the study of phytopathogenic bacteria. This study aims to compare four different protein extraction methods, including: Trizol, Phenol, Centrifugation and Lysis in order to determine which are more suitable for proteomic studies using as parameters the quantity and quality of extracted proteins observed in two-dimensional gels.

Results: The bacteria studied showed different results among the tested methods. The Lysis method was more efficient for *P. carotovorum* subsp. *carotovorum* and *R. solanacearum* phytobacteria, as well as simple and fast, while for *A. citrulli*, the Centrifugation method was the best. This evaluation is based on results obtained in polyacrylamide gels that presented a greater abundance of spots and clearer and more consistent strips as detected by two-dimensional gels.

Conclusions: These results attest to the adequacy of these proteins extraction methods for proteomic studies.

Keyword: *Acidovorax citrulli*, *Pectobacterium carotovorum subsp. carotovorum*, *Ralstonia solanacearum*, Proteome analysis, Two-dimensional gel electrophoresis

Background

The practice of agriculture brings as consequence the occurrence of plant diseases in levels that require their control. The most recommended method for this control is the use of genetic resistance [1]. However, not all plants are resistant to pathogens, and not every resistant variety is adapted to different regions of cultivation [2].

The bacteria *Acidovorax citrulli* (*Ac*), *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) and *Ralstonia solanacearum* (*Rs*), respectively cause bacterial fruit blotch, which is the main bacterial disease of melon culture, being responsible for heavy losses in production and depreciation of fruits [3]; soft rot in several hosts, among which lettuce, potatoes and tomatoes [4]; and bacterial wilt, which is the main worldwide vascular disease and attacks more than 50 botanical families, mostly the Solanaceae family, causing great economic losses [5,6].

The proteome is defined as the set of proteins expressed in a cell, tissue or any biological sample at a given time or under specific conditions [7]. The identification and characterization of these microorganisms using proteomic technologies can integrate the knowledge base necessary for the understanding of the mechanisms that phytobacteria use to cause diseases in their host [8]. In comparison with genomic studies, investigations at the proteome level provide detailed information, such as the abundance of proteins and post-transcriptional modifications [9,10].

* Correspondence: carol08malafaia@hotmail.com
†Equal contributors
†Programa de Pós-Graduação em Ciências Biológicas - Centro de Ciências Biológicas - Universidade Federal de Pernambuco, Rua Prof. Nelson Chaves s/n, Cidade Universitária, CEP 50670-901 Recife, PE, Brasil
Full list of author information is available at the end of the article

The extraction of proteins is challenging, inconsistent and has been a problem for scientists [11]. Many techniques, including physical methods and those based on detergents, are available for protein extraction and are used for various purposes [12]. In proteomic studies, the development of an extraction method that can produce high yields and result in the complete dissolution, breakdown, denaturing and reduction of the greatest possible number of proteins present in the sample is an absolutely essential step for good results in two-dimensional gel electrophoresis (2D-PAGE) and mass spectrometry [13]. However, there are few studies that have compared the efficiency of these methods [14,15].

In this study, we compared four extraction methods: Trizol, Phenol, Centrifugation and Lysis, to determine their effectiveness in the separation of proteins by 2D-PAGE of three important phytobacteria: *Acidovorax citrulli*, *Pectobacterium carotovorum* subsp. *carotovorum* and *Ralstonia solanacearum*.

Results

In this study, four different extraction methods were compared to determine which of them increase the protein solubilization of phytobacteria for subsequent analysis by 2D-PAGE. Considering that non-protein impurities can severely affect the quality of 2D-PAGE separation, this study was critical to evaluate, standardize and select efficient methods for protein analysis of *Acidovorax citrulli*, *Pectobacterium carotovorum* subsp. *carotovorum* and *Ralstonia solanacearum*.

The four extraction methods tested were effective in obtaining and concentrating proteins and the results are presented in Table 1. Although all methods presented appropriate yields for bacteria *Ac* and *Pcc*, the largest amount of proteins was obtained by the Centrifugation method. However, for *Rs* the best result was observed with the Lysis method, where there was a significant difference compared to the other methods tested.

The SDS-PAGE analysis showed that the extractions presented good quality proteins, with well-defined bands without signs of degradation (Figure 1). For bacteria *Rs* and *Pcc* it was noted that all methods seem well suited. However, for *Ac* the Lysis method presented a loss of proteins with a molecular weight above 38KDa in addition to little definition of the bands, which from this analysis suggest that this method is less efficient compared to the other methods tested for this specie. In this gel, it is possible to observe that there is a difference in the patterns and intensity of the bands observed amongst the methods in each of the phytobacteria. Thus, for the study in question, the best extraction method was considered the one that is most comprehensive, namely, the method that presents the greatest possible number of proteins with the best definition in 2D-PAGE gels.

The results of the two-dimensional gels (Figure 2 and Table 2) showed clarity and resolution, but were different for each of the bacteria. To define which method is best suited for the organism under study, one should consider the relative quality of the sample for analysis in 2D-PAGE and the number of protein spots obtained. *Ac* presented the best results with the Centrifugation method, showing 224 spot with a pH distribution between 4 to 7 and a molecular weight of 10 to 80 KDa; the bacteria *Pcc* and *Rs* presented respectively 212 spots, pH of 4 to 7 and molecular weight between 10 and 70 KDa and 369 spots, pH of 4 to 9 and a molecular weight of 20 to 70 KDa. These results showed a good range and are recommended for use in proteomic studies.

Discussion

Proteomic studies of high resolution depend mainly on a sample of good quality, so the method applied in the extraction of proteins is a key step to that end [11]. There is a great diversity of types of samples, therefore, an efficient protein isolation process for each one of them must be assumed empirically and tested in order to determine its real efficiency for the sample used, in order to obtain reproducible results in addition to the greatest possible representativeness of proteins in 2D gels [10]. During sample preparation in bacteria cells, problems may arise in cellular rupture due to the presence of thick cell walls and polysaccharide capsule in certain bacterial groups. Some bacteria can be lysed just by lysis buffer constituents, while others must be mechanically broken; in some cases it is necessary to use enzymes for the digestion of the cell wall [16].

Although many methods have been developed and reported, there is no single method for efficient isolation

Table 1 The mean ± SD of protein concentrations (μg/μl) of all strains obtained by four different methods from mass of bacteria growth of 1 × 10^7 CFU/ml

Strains	Methods			
	Trizol	Phenol	Centrifugation	Lysis
Acidovorax citrulli	8.83 ± 0.15	7.58 ± 0.17	11.34 ± 0.15	9.83 ± 0.18
Pectobacterium carotovorum subsp. *carotovorum*	8.90 ± 0.10	7.82 ± 0.08	10.54 ± 0.05	8.19 ± 0.18
Ralstonia solanacearum	8.74 ± 0.23	7.03 ± 0.13	8.61 ± 0.05	9.08 ± 0.00

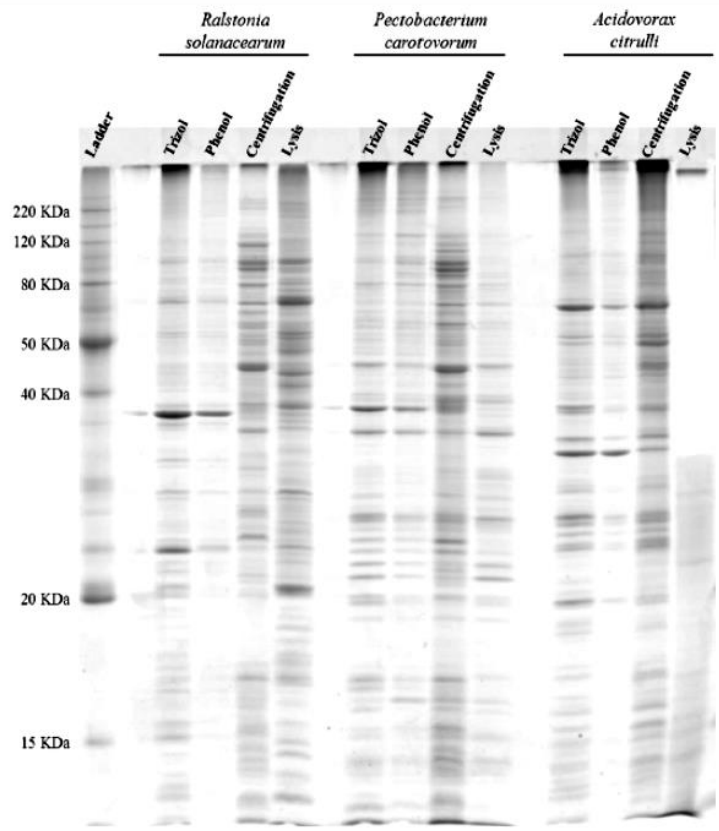

Figure 1 SDS-PAGE of the different extraction methods in phytopathogenic bacteria. SDS-PAGE stained with Coomassie brilliant blue illustrating the intracellular proteins of the phytobacteria *Ralstonia solanacearum*, *Pectobacterium carotovorum* subsp. *carotovorum*, and *Acidovorax citrulli* extracted by four different methods: Trizol, Phenol, Centrifugation and Lysis, Marker BenchMark™ Protein Ladder (Invitrogen) – KDa.

of all proteins of interest (cytosolic soluble or of a highly insoluble membrane) of an organism. Therefore, protein extraction methods continue to be a challenge for scientists in the accurate analysis of proteins [11]. In this regard, the chosen method should be simple and quick, with low cost and toxicity. These are important aspects in the selection of the method to be used, without selectively losing proteins while removing contaminants to the maximum extent possible [10].

The Lysis method has been applied in others organisms, such as sugarcane [17], soybean [11] and also in *Xanthomonas campestris* pv. *viticola* [18] with similar efficiency to that observed in this work. The lysis buffer composition allows quick access to the proteins, promoting denaturation, keeping them in the primary structure and thus protecting them against degradation agents. The preparing of protein samples consists in three fundamental steps, present in all methods: cell disruption, inactivation or removal of interfering and proteins solubilization [19].

The solubilization of proteins is considered the most problematic step in preparing protein samples for proteomic studies. The better solution is the buffer with a combination of urea and thiourea, associated with appropriate detergents, as tested by Chan and collaborators [20] for *Prorocentrum triestinum*. They observed an increase in the number of spots in electrophoresis gels when using urea and an even greater increase when using the combination of urea and thiourea. This is due to the fact that urea is a chaotropic agent, efficient in the rupture of hydrogen bonds, denaturing proteins by breaking the non-covalent and ionic links between aminoacid residues [19], leading to the split and denaturation of proteins. In turn, thiourea is very suitable for breaking hydrophobic interactions, increasing the solubilization of membrane proteins [21,22]. CHAPS and DTT are two important components in the proteins solubilization because they prevent hydrophobic interactions and promote the re-oxidation of disulfide bonds, respectively, avoiding the loss of proteins by aggregation or precipitation.

There are several advantages to the use of the Lysis method in protein extraction: it is a method that is simple, fast (about 1 h), most interfering materials (non-protein components) are effectively removed, the proteins are protected against degradation by proteases,

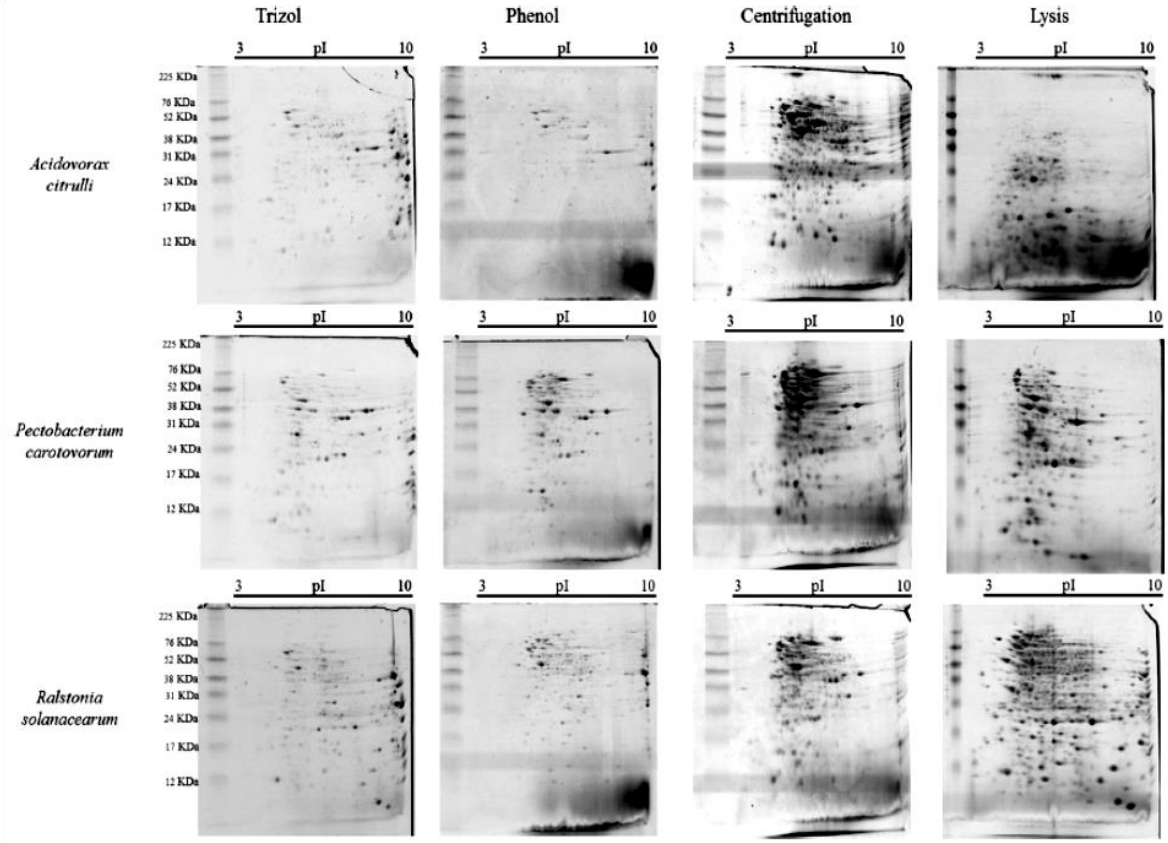

Figure 2 Abundance of proteins in different extraction methods. 2D-PAGE stained with Coomassie brilliant blue illustrating intracellular proteins, focused on 7 cm strips, pI 3–10, of the phytobacteria *Ralstonia solanacearum*, *Pectobacterium carotovorum* subsp. *carotovorum*, and *Acidovorax citrulli* extracted by four different methods: Trizol, Phenol, Centrifugation and Lysis, Marker Rainbow (GE Life Sciences).

thus not requiring the addition of protease inhibitors, in addition to having low toxicity. Furthermore, the composition of the extraction buffer ensures that proteins are under the same conditions as 2D-PAGE.

The Centrifugation method was very efficient for *Acidovorax citrulli*, result that suggests its utilization for specific studies with this specie. The presence of SDS in the extraction buffer used in this method allows access to the proteins by breaking the membrane and, associated with heating at 100°C, inactivating proteases. The use of DNAse I and RNAse A enzymes, with subsequent precipitation with acetone, guarantees the elimination of contaminating in the final sample [23].

Some studies have focused on comparing protocols for protein extraction from a wide variety of organisms. For example, a study of lactic acid bacteria, which presented the comparison of three extraction methods for sonication, Centrifugation and FastPrep, found the best results with the latter [24]. In aphids, the TCA/acetone-based method was the more efficient in comparisons for 2DE than detergent and phenol based methods [13]. Unlike the results obtained in this work, in dinoflagellates the Trizol method presented better results when compared to the Lysis method [25]. Proteomic studies conducted with the bacterial phytopathogen *Xanthomonas axonopodis* pv. *citri* showed that the Phenol method was employed with success [26]. In some cases, it is necessary to develop a new method due to peculiarities of the sample in question as noted by Barbarino and Lourenço in 2005 [27] due to high concentrations of salts present in the sample.

Table 2 Number of spots of all strains obtained by the four different methods

Strains	Methods			
	Trizol	Phenol	Centrifugation	Lysis
Acidovorax citrulli	$164^{a} \pm 5$	$43^{b} \pm 4$	$224^{c} \pm 8$	$126^{d} \pm 4$
Pectobacterium carotovorum subsp. *carotovorum*	$152^{a} \pm 3$	$151^{a} \pm 8$	$172^{b} \pm 5$	$212^{c} \pm 3$
Ralstonia solanacearum	$148^{a} \pm 3$	$196^{b} \pm 4$	$183^{c} \pm 5$	$369^{d} \pm 4$

Superscript letters (a-d) indicate statistically significant relationships between methods ($p < 0.05$).

Conclusions

For new proteomic studies with organisms that have not been registered in the literature, a pre-test of different methods for the preparation of the sample is strongly recommended in order to determine which is best suited for this type of analysis. In the case of the phytobacteria used in this study, the recommended methods are Centrifugation for *Acidovorax citrulli* and Lysis for *Pectobacterium carotovorum* subsp. *carotovorum* and *Ralstonia solanacearum*.

Methods

Growing conditions

Bacterial isolates were *Acidovorax citrulli* (*Aac* 1.12), *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc* 31), and *Ralstonia solanacearum* (*Rs* CGH 26), obtained by Culture Collection of the Phytobacteriology Laboratory of the Agronomic Department of Universidade Federal Rural de Pernambuco, Brazil. Were grown in 20 ml NYD medium (dextrose 10 g/l; meat extract 3 g/l; yeast extract 5 g/l; peptone 5 g/l) during 24 h at 28°C under constant agitation of 150 rpm for the formation of the pre-inoculate. Following this, 180 ml of the same media was added and the culture maintained under the same growth conditions for 24 h. Consequently, the bacterial growth was collected by centrifugation at 10.000 × g for 5 min, to obtain the cell mass for the extraction of total protein. Three biological replicas were made (independent cultures) and the samples were collected at an optical density ($OD_{600} = 0.5 \pm 0.05$) corresponding to the exponential phase 1 x 10^7 CFU/ml of each of the strains.

Extraction of proteins

Four different protein extraction methods were tested including modified Trizol, Phenol, Centrifugation and Lysis. After extraction, the supernatants containing the proteins of each of the methods were stored at − 20°C until later analysis.

Trizol method

Protein extraction followed the instructions set out by the manufacturer of Trizol (Invitrogen ®) with some modifications. Briefly, 500 μl Trizol reagent were added to the cell pellet and lyse cells in sample by pipetting up and down several times. Subsequently, 200 μl of chloroform were added to the cell lysate before shaking vigorously for 15 s. The mixture was allowed to stand for 5 min at 25°C before being centrifuged at 12000 × g for 15 min at 4°C. The aqueous phase was removed. 300 μl of ethanol were added in order to resuspend the reddish bottom layer and the mixture centrifuged at 8000 × g for 5 min at 4°C. Supernatant was transferred to a new tube and 1.5 ml of isopropanol were added. The mixture was allowed to stand for at least 20 min for protein precipitation at 25°C. It was then centrifuged at 12000 × g for 10 min at 4°C. The pellet obtained was briefly washed with 95% ethanol before allowed to air dry. Finally, the proteins were ressolubilized in 500 μl of sample preparation solution (7 M Urea; 2 M thiourea; 4% CHAPS).

Phenol method

Total protein extraction was done as described by Metha and Rosato in 2001 [28]. The cell pellets were washed in phosphate buffer (7 mM K_2HPO_4; 3 mM KH_2PO_4; 0.15 mM NaCl; pH 7.2) and 750 μl of extraction buffer were added (0.7 M sucrose; 0.5 M Tris–HCl; 30 mM HCl; 50 mM EDTA; 0.1 M KCl and 40 mM DTT; pH 8.5), followed by incubation for 15 min (25°C). The same volume of phenol was added, and after 15 min of agitation in a vortex, the suspension was centrifuged at 14.000 x g for 3 min at 4°C and the phenolic phase was recovered. This procedure was repeated two more times. Proteins were precipitated with the addition of 5 volumes of 0.1 M ammonium acetate in methanol. The precipitate was washed with 1 ml of 80% acetone and solubilized as previously described.

Centrifugation method

The pellets were resuspended in 500 μl of extraction buffer (0.3% SDS; 200 mM DTT; 48 mM Tris; 28 mM HCl; pH 8.8). The microcentrifuge tubes containing the cell suspension were agitated gently for 10 min at 4°C, followed by removal of the cells by centrifugation at 14.000 x g for 10 min at 4°C. The extraction was incubated at 100°C for 5 min and then cooled on ice. Subsequently, 24 μl of assay buffer (0.5 M Tris; 476 mM HCl; 50 mM $MgCl_2$ pH 8.5; 1 mg/ml DNAse I; 0.25 mg/ml RNAse A) were added and the extraction incubated again for 15 min on ice. The reaction was stopped by the addition of four volumes of acetone cooled on ice and precipitation of proteins was left to occur for 20 min on ice. The cellular debris were removed by centrifugation at 14.000 × g for 10 min at 4°C [29]. The proteins were resolubilized in in 500 μl of sample preparation solution (7 M Urea; 2 M thiourea; 4% CHAPS)

Lysis method

The centrifuged pellets of bacteria were resuspended in 500 μl of lysis buffer (7 M urea; 2 M thiourea; 4% CHAPS) and homogenized in a vortex for 5 min at 25°C. The homogenized sample was centrifuged at 10.000 x g for 30 min at 4°C. The supernatant was transferred to a new 1.5 ml tube [17].

Quantification of proteins

Total cellular protein concentrations were determined using a commercial protein colorimetric assay kit, 2D Quant Kit, according to the manufacturer's protocol

(GE Life Sciences®) with bovine serum albumin (BSA) as a standard of measurement and absorbance at 480 nm. The kit is reported to not interfere with any chemicals used during extraction protocols and is therefore compatible with isoelectric focusing (IEF). The samples and the standard were read in triplicate.

SDS-PAGE

100 μg of protein were applied in a 15% acrylamide separating gel was used with stacking at 4% for SDS-PAGE on SE 600 Ruby Standard Dual Cooled Vertical Unit. The acrylamide gel was run at 40 mA for 15 min and then at 100 mA for 2 h (Electrophoresis Power Supply – EPS 601 - GE Life Sciences) [30] in SDS buffer (124 mM Tris; 960 mM glycine; 17.5 mM SDS). 10 μl of protein molecular weight standard BenchMark™ Protein Ladder (Invitrogen) were used. At the end of electrophoresis, the gels were visualized by staining with Coomassie brilliant blue (5% acetic acid; 20% methanol; 0.2% Comassie Brilliant Blue R-250) and then decolorized with 0.5% acetic acid and 20% methanol.

2D-PAGE

Two-dimensional electrophoresis (2-DE) was carried out according to the method of Görg and collaborators [31]. In the first dimension isoelectric focusing, 100 μg proteins were added to a rehydration buffer (7 M urea; 2 M thiourea; 2% CHAPS; 2 mM DTT; 1% IPG buffer pH 3–10 and 0.2% bromophenol blue) for a final volume of 250 μl. The sample was loaded onto 13 cm pH 3-10NL immobiline DryStrips (GE Life Sciences) with overnight rehydration, followed by isoelectric focusing for a total of 15.500 V/hrs. Strips were equilibrated in SDS equilibration buffer (6 M urea, 30% glycerol, 2% SDS) for 15 min with 10 mg/ml DTT, then 15 min in fresh buffer with 25 mg/ml 15 min. The second dimension was performed in homogeneous vertical acrylamide gel 15%. The equilibrated strips were applied onto the gel and sealed with agarose 0.5% and bromophenol blue 0.01%. The proteins electrophoretic separation was performed at 15°C in two stages: the first at 15 mA per fixed gel for 20 min and the second at 45 mA per gel for approximately 2 hours. Rainbow was used the molecular weight standard (GE Life Sciences). After the second dimension electrophoresis, the proteins were stained as in the SDS-PAGE.

Rainbow was used as the molecular weight standard (GE Life Sciences). After the second dimension electrophoresis, the proteins were stained as in the SDS-PAGE.

Analysis of two-dimensional gels

After stained, the gels were scanned using an ImageScanner (Amershan Biosciences) scanner in transparency mode with a resolution of 300 dpi and images were saved in .mel format. These were analyzed using the ImageMaster Platinum v. 7.0 (Amershan Biosciences) computer program. The detection of each spot of protein was validated by manual inspection. The program provided the number of spots of each of the gels.

Statistical analysis

Data were analyzed by oneway analysis of variance (ANOVA) followed by Tukey's posthoc test and shown as mean and standard deviation. In all statistical analyses, $p < 0.05$ as taken as the level of significance.

Abbreviations

Ac: *Acidovorax citrulli*; *Pcc*: *Pectobacterium carotovorum* subsp. *carotovorum*; *Rs*: *Ralstonia solanacearum*; 2D-PAGE: Two-dimensional polyacrylamide gel electrophoresis; SDS-PAGE: Sodium dodecyl sulfate - polyacrylamide gel electrophoresis; 2DE: Two dimensional electrophoresis; TCA: Trichloroacetic acid; NYD: Nutrient yeast dextrose; OD_{600}: Optical density 600 nm; CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS: Sodium dodecyl sulfate; DTT: Dithiothreitol; BSA: Bovine serum albumin; IEF: Isoelectric focusing.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CBM, MLG and TDS carried out the experiments and analyzed the data. All authors contributed to writing of the manuscript. Experimental strategy was carried out by PMGP, EBS, MTSC and MVS who also supervised the project. All authors read and approved the final manuscript.

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Author details

[1]Programa de Pós-Graduação em Ciências Biológicas - Centro de Ciências Biológicas - Universidade Federal de Pernambuco, Rua Prof. Nelson Chaves s/n, Cidade Universitária, CEP 50670-901 Recife, PE, Brasil. [2]Programa de Pós-Graduação em Fitopatologia - Universidade Federal Rural de Pernambuco, Av. Dom Manoel de Medeiros, s/n - Dois Irmãos, CEP: 52171-900 Recife, PE, Brasil. [3]Departamento de Bioquímica, Universidade Federal de Pernambuco, Rua Prof. Moraes Rego s/n, Cidade Universitária, 50670-420 Recife, PE, Brasil. [4]Departamento de Biologia, Universidade Federal Rural de Pernambuco, Av. Dom Manoel de Medeiros, s/n - Dois Irmãos, CEP: 52171-900 Recife, PE, Brasil.

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Conclusões

Concluímos que a formação de biofilme não parece estar relacionados com a virulência em *R. solanacearum* e *A. citrulli*, e mesmo entre isolados da mesma espécie é possível observar biofilmes morfologicamente variadoa. A adesão microbiana de *A. citrulli* parece ser prejudicada em superfícies abióticas hidrofóbicas.

O screening *in vitro* revelou que cinco extratos de plantas da Caatinga possuem potencial antimicrobiano e outros cinco apresentam potencial antibiofilme contra *R. solanacearum*, patógeno economicamente importantes na agricultura, para guiar prospecção de compostos bioativos e o desenvolvimento de um produto a ser aplicado no controle da murcha bacteriana. *Jacaranda rugosa*, *Harpochilus neesianus* e *Myroxylon peruiferum*, plantas utilizadas para fins medicinais, contêm fitoquímicos eficazes na inibição do crescimento celular e da formação de biofilme de *R. solanacearum*.

Estudos de proteômica preconizam alta qualidade na preparação da amostra e o processo de extração de proteínas é crucial na qualidade dos resultados obtidos. Neste estudo recomenda-se a utilização dos métodos de Lise para extração de proteínas de *P. carotovorum* subsp. *carotovorum* e *R. solanacearum* e de centrifugação para *A. citrulli* em estudos futuros de investigação de proteínas direta e indiretamente envolvidas na formação de biofilme por essas fitobactérias.

Anexo I

Artigos publicados no período do doutorado

1. UCHÔA, A. D. A., OLIVEIRA, W. F., PEREIRA, A. P. C., SILVA, A. G., CORDEIRO, B. M. P. C., MALAFAIA, C. B., ALMEIDA, C. M. A., SILVA, N. H., ALBUQUERQUE, J. F. C., SILVA, M. V., CORREIA, M. T. S. Antioxidant Activity and Phytochemical Profile of *Spondias tuberosa* Arruda Leaves Extracts. **American Journal of Plant Sciences**. v.06, p.3038 - 3044, 2015.

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Anexo II

Aceite de artigos para publicação

1. SILVA T. D., ALMEIDA. C. M. A., MALAFAIA, C. B., OLIVEIRA, L. M. S., SILVA, M.V., CORREIA, M. T. S. Analysis of protein profile of the root of tomato infected with *Fusarium oxysporum* f. sp. *lycopersici*. **Genetics and Molecular Research.**