TÂMARA PRADO DE MORAIS

CARACTERIZAÇÃO *in vitro* E *in planta* DE UMA PROTEÍNA QUIMÉRICA COM ATIVIDADE ANTIMICROBIANA À *Ralstonia solanacearum*

Tese apresentada à Universidade Federal de Uberlândia, como parte das exigências do Programa de Pós-graduação em Agronomia – Doutorado, área de concentração em Fitotecnia, para obtenção do título de "Doutor".

Orientador
Prof. Dr. José Magno Queiroz Luz

Coorientadores
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APROVADA em 18 de março de 2016.

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UBERLÂNDIA
MINAS GERAIS – BRASIL
2016

À comunidade científica,

Ofereço.

À minha família,

Dedico.

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"Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível."

Charles Chaplin

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RESUMO

MORAIS, TÂMARA PRADO. **Caracterização *in vitro* e *in planta* de uma proteína quimérica com atividade antimicrobiana à *Ralstonia solanacearum***. 2016. 148f. Tese (Doutorado em Agronomia / Fitotecnia) – Universidade Federal de Uberlândia, Uberlândia.[1]

A fitobactéria *Ralstonia solanacearum* [(SMITH, 1896) YABUUCHI et al. 1996], agente causal da murcha-bacteriana e da doença do Moko, é considerada um dos mais destrutivos patógenos de plantas em todo o mundo. No Brasil, sua ocorrência compromete o rendimento de culturas agronomicamente importantes, destacando a necessidade de estratégias eficazes para o manejo da doença, até então limitadas a ações preventivas. Peptídeos antimicrobianos (AMPs) participam da defesa inata de inúmeros organismos e são considerados potenciais agentes terapêuticos no combate a ampla variedade de patógenos, em virtude de suas propriedades antivirais, antifúngicas e antibacterianas. Visto isso, são candidatos promissores para o desenvolvimento de novas terapias no controle de *R. solanacearum*. Mediante o uso de ferramentas de bioinformática, vários AMPs foram selecionados baseando-se na estrutura e função da cecropina B, um conhecido peptídeo antimicrobiano α-helicoidal (AH-AMP), e testados *in vitro* contra a bactéria. Dentre os peptídeos identificados, um AH-AMP derivado da enzima fosfoenolpiruvato carboxilase, denominado PPC20, destacou-se como o mais eficiente para controlar o patógeno, simultaneamente configurando baixa toxicidade a células humanas. No intuito de verificar se a combinação de duas funções imunes inatas presentes na mesma molécula potencializa seu efeito antimicrobiano, esse domínio lítico foi fusionado a uma elastase putativa derivada de plantas (a proteína relacionada à patogênese, SlP14a), resultando no desenvolvimento de uma quimera. A caracterização e validação dessa nova proteína quimérica foi realizada por bioensaios conduzidos *in vitro* e *in planta*. Os genes SlP14a e SlP14a-PPC20 foram clonados e expressos em células bacterianas e em plantas de tabaco (expressão transiente). As proteínas extraídas e purificadas de ambos os sistemas de expressão apresentaram atividade antibacteriana *in vitro* através da inibição do crescimento de *R. solanacearum*. A fim de verificar a função biológica *in vivo* da quimera (SlP14a-PPC20), linhagens transgênicas de tomate (cultivar MoneyMaker) foram obtidas e inoculadas com *R. solanacearum*. Os índices de sobrevivência e a redução dos sintomas da murcha-bacteriana foram significativamente mais elevados em plantas transgênicas quando comparados com aqueles relativos às plantas não transformadas. Este estudo propõe uma estratégia alternativa para o controle da murcha-bacteriana mediante a expressão de uma nova proteína terapêutica antimicrobiana em plantas de tomate.

Palavras-chave: biotecnologia vegetal, proteína terapêutica antimicrobiana, concentração mínima inibitória, murcha-bacteriana.

[1] Comitê Orientador: José Magno Queiroz Luz – UFU (Orientador), Rafael Nascimento – UFU e Nilvanira Donizete Tebaldi – UFU

ABSTRACT

MORAIS, TÂMARA PRADO. ***In vitro* and *in planta* characterization of a chimeric antimicrobial protein against the phytopathogen *Ralstonia solanacearum***. 2016. (Doctor's Degree in Agronomy / Crop Science) – Federal University of Uberlandia, Uberlandia.[2]

The phytobacterium *Ralstonia solanacearum* [(SMITH, 1896) YABUUCHI et al. 1996], causative agent of bacterial wilt and Moko disease, is considered one of the world's most destructive plant pathogen. In Brazil this xylem-restricted bacterium reduces yields of agriculturally important crops and calls for effective disease management strategies, so far limited to preventive actions. Antimicrobial peptides have been considered powerful compounds for plant protection due to their antiviral, antifungal, and antibacterial activities. Hence, they are promising candidates to the development of novel rationally-designed therapies for the control of *R. solanacearum*. Mirroring the function and properties of cecropin B, a well-studied α-helical antimicrobial peptide (AH-AMP), several candidates were selected by bioinformatic tools and tested *in vitro* against the bacterium. The identified peptides included a linear AH-AMP within the existing structure of phosphoenolpyruvate carboxylase, named PPC20. This peptide stood out as the most efficient in killing the pathogen without jeopardizing human cells. In order to investigate whether the combination of two innate immune functions provides a robust class of antimicrobial therapeutics, this lytic domain was combined to a putative plant-derived elastase (the pathogenesis-related protein SlP14a), leading to the development of a chimeric protein. To characterize and validate this novel antimicrobial chimera as a biocontrol agent, bioassays were conducted *in vitro* and *in planta*. SlP14a and SlP14a-PPC20 were expressed in both bacterial and plant (transient expression) systems. Purified proteins showed *in vitro* antibacterial activity by inhibiting *R. solanacearum* growth. In order to explore the *in vivo* biological function of SlP14a-PPC20, transgenic lines of tomato cultivar MoneyMaker were obtained and characterized. To assess whether these lines acquired enhanced tolerance to the pathogen, they were challenged with *R. solanacearum* by stem inoculation. The survival rates and the reduction of disease symptoms were significantly higher in transgenic plants compared with the non-transgenic ones. This study proposes an alternative strategy for bacterial wilt control based on expression of a newly designed therapeutic antimicrobial protein in tomato plants.

Keywords: plant biotechnology, therapeutic antimicrobial protein, minimum inhibitory concentration, bacterial wilt.

[2] Supervising Committee: José Magno Queiroz Luz – UFU (Major Professor), Rafael Nascimento – UFU, and Nilvanira Donizete Tebaldi – UFU.

1 INTRODUÇÃO GERAL

A murcha-bacteriana, causada por *Ralstonia solanacearum* [(SMITH, 1896) YABUUCHI et al. 1996], é considerada a principal doença vascular de etiologia bacteriana encontrada no mundo. O patógeno é quarentenário em vários países europeus (OEPP/EPPO, 2004) e foi incluso na lista de Agentes de Bioterrorismo dos Estados Unidos no ano de 2002 (USDA, 2012); desde então, medidas têm sido adotadas para prevenir seu estabelecimento nesses países. No Brasil, *R. solanacearum* foi relatada em todos os Estados e é responsável por expressivos declínios de produtividade em culturas agronomicamente importantes e pela condenação de campos de cultivo, em especial aqueles dedicados à certificação de batata-semente (LOPES, 2005).

Pelo fato de o patógeno atuar nos vasos do xilema, ser habitante do solo, estar associado a um grande número de espécies botânicas e apresentar ampla variabilidade genética, o controle da doença é extremamente difícil. Dentre as estratégias recomendadas destacam-se a adoção de medidas preventivas e o uso de variedades resistentes. No entanto, o melhoramento para obtenção de plantas resistentes é complicado devido à ausência de boas fontes de resistência nas espécies vegetais e à diversidade genética da bactéria (LOPES, 2005; REMENANT et al., 2010). Explorar a capacidade inerente das plantas em se defenderem contra fatores bióticos, aliada à engenharia genética, torna-se, portanto, uma alternativa interessante para o manejo da murcha-bacteriana.

Sabe-se que a resposta imune inata é a primeira linha de defesa do hospedeiro contra a invasão por patógenos. Essa resposta ocorre logo após o reconhecimento do agente etiológico pelas células do hospedeiro, mediante sinalização intracelular, e culmina com a expressão de moléculas efetoras – tais como peptídeos líticos antimicrobianos, citocinas e espécies reativas de oxigênio – que estão direta ou indiretamente envolvidas na eliminação do patógeno (JANEWAY; MEDZHITOV, 2002). Ainda assim, alguns patógenos conseguem superar a defesa imune inata, estabelecendo o processo infeccioso e causando doenças (DE WIT, 2007; KRAUS; PESCHEL, 2008).

A hipótese que norteou esta pesquisa foi a de que a combinação de duas funções imunes inatas presentes na mesma molécula poderia potencializar seu efeito antimicrobiano (KUNKEL et al., 2007). Especificamente, cogitou-se que a combinação sinérgica entre a proteína que reconhece o patógeno e o peptídeo lítico em uma quimera

poderia impedir a infecção e, portanto, constituir-se em uma classe de proteínas terapêuticas.

Em pesquisas preliminares, o grupo do Prof. Dr. Abhaya Dandekar, na Universidade da Califórnia (UC-Davis), desenvolveu uma proteína quimérica antimicrobiana constituída por dois domínios bioativos – um proveniente da proteína elastase dos neutrófilos humanos (NE; domínio de reconhecimento) e outro da cecropina B de insetos (CecB; domínio lítico) – ligados por um peptídeo flexível. A proteína (NE-CecB) apresentou propriedade bactericida e foi eficiente em restringir a infecção causada pela fitobactéria *Xylella fastidiosa* em plantas transgênicas de videira (KUNKEL et al., 2007; DANDEKAR et al., 2009; 2012). Atualmente, algumas dessas linhagens estão sendo testadas em condições de campo em duas localidades no Estado da Califórnia (Estados Unidos).

A presença de proteínas de origem humana e de insetos nas plantas, porém, pode gerar dúvidas quanto ao seu potencial alergênico e desencadear aversão por alguns grupos da sociedade contrários a organismos geneticamente modificados. Uma estratégia para amenizar essa preocupação seria substituir os componentes NE e CecB por equivalentes naturalmente encontrados em plantas. Tal alteração, contudo, não pode comprometer a atividade antimicrobiana da nova proteína quimérica.

Como modelo, propôs-se estudar a interação entre a bactéria fitopatogênica *R. solanacearum* e plantas de tomate (*Solanum lycopersicum* L.). Por meio de análises de bioinformática, proteínas homólogas à NE e à CecB foram selecionadas, denominadas, respectivamente, SlP14a e PPC20. A identificação dessas proteínas em plantas foi feita de acordo com similaridades conformacionais, utilizando as metodologias CLASP (*CataLytic Active Site Prediction*) e SCALPEL. O objetivo deste trabalho foi caracterizar a atividade antimicrobiana da proteína quimérica SlP14a-PPC20 à *R. solanacearum,* propondo-a como uma nova alternativa ao controle da murcha-bacteriana do tomateiro.

CAPÍTULO 1

REFERENCIAL TEÓRICO

2 REFERENCIAL TEÓRICO

2.1. A fitobactéria *Ralstonia solanacearum*

O gênero *Ralstonia* pertence à subdivisão β das proteobactérias, ordem Burkholderiales, família *Ralstoniaceae* (LUDWIG et al., 1995; KERSTERS et al., 1996; GENIN; BOUCHER, 2002; EUZÉBY, 2014) e ao grupo homólogo II (rRNA) das *Pseudomonas*, que engloba as bactérias fitopatogênicas não fluorescentes (PALLERONI et al., 1973). *R. solanacearum* compreende isolados Gram-negativos, em forma de bastonete medindo 0,5-0,7 x 1,5-2,5µm, com um ou vários flagelos polares, não esporogênicos, não fluorescentes, estritamente aeróbicos e capazes de produzir pigmento difusível marrom quando cultivados *in vitro* (BRINGEL; TAKATSU; UESUGI, 2001; AGRIOS, 2005). Acumulam poli-β-hidroxibutirato (PHB) como material de reserva (EU, 1998), não formam levana a partir de sacarose e apresentam hidrólise negativa ou fraca de gelatina, assim como de amido. Praticamente todos os isolados reduzem nitrato, sendo que alguns são capazes de produzir gás (denitrificação). Testes de oxidase e catalase são positivos, ao passo que os de arginina e lipase são negativos. A maioria dos isolados produz tirosinase; as principais exceções são aqueles obtidos a partir de plantas da família *Musaceae*.

Cultivados em meios de cultura, isolados virulentos de *R. solanacearum* desenvolvem colônias de coloração branca, retas, irregulares e fluidas, enquanto formas avirulentas são pequenas, circulares, não fluidas e de cor branco-creme. Em meios contendo cloreto de trifeniltetrazólio [(KELMAN, 1954), BG (BOUCHER et al., 1985) e SMSA (ENGLEBRECHT, 1994)], as colônias são vermelhas com halo branco (EU, 2006).

O complexo específico *R. solanacearum* compreende ampla variedade de isolados que diferem em aspectos relacionados à agressividade, sobrevivência e latência (JAUNET; WANG, 1999; FEGAN; PRIOR, 2005). Na tentativa de caracterizar essa variabilidade intraespecífica, a bactéria é classificada em cinco raças patogênicas (de acordo com a gama de hospedeiros), em seis biovares (em virtude de propriedades bioquímicas) e em quatro filotipos e sequevares (1 ao 52), baseados em análises genotípicas (SIRI et al., 2011; ALBUQUERQUE et al., 2014).

O genoma de *R. solanacearum* é organizado em dois *replicons*: um cromossomo de 3,7 megabases (Mb) e um megaplasmídeo de 2,1 Mb (SALANOUBAT et al., 2002).

Ambos os *replicons* têm estrutura em mosaico evidenciando a aquisição de genes através de transferência horizontal, que está associada à evolução da bactéria e à agressividade dos isolados (FALL et al., 2007; COUPAT et al., 2008; GUIDOT et al., 2009; REMENANT et al., 2010, 2011, 2012). No megaplasmídeo são encontrados vários genes envolvidos no controle da patogenicidade. Os principais fatores de virulência são efetores secretados pelo sistema de secreção tipo III (BOUCHER et al., 1985; COLL; VALLS, 2013) e o exopolissacarídeo, também responsável pelo processo de colonização bacteriana nas plantas e pela oclusão dos vasos do xilema, que culmina com os sintomas de murcha (PEETERS et al., 2013). Detalhamento de outros fatores de virulência pode ser consultado em Genin e Denny (2012) e em Peeters et al. (2013).

A bactéria está amplamente distribuída em regiões temperadas, de clima tropical e subtropical (Figura 1) e afeta diversas culturas, incluindo tanto plantas monocotiledôneas como dicotiledôneas pertencentes a 50 famílias botânicas (ELPHINSTONE, 2005; CUEVA et al., 2013; NISHAT et al., 2015). Sua disseminação pode ocorrer pelo solo, água ou material de propagação contaminado, como tubérculos de batata e mudas de espécies ornamentais. As plantas são infectadas pelo sistema radicular, à exceção de alguns isolados de bananeira que, aparentemente, podem ser transmitidos por insetos, infectando partes florais.

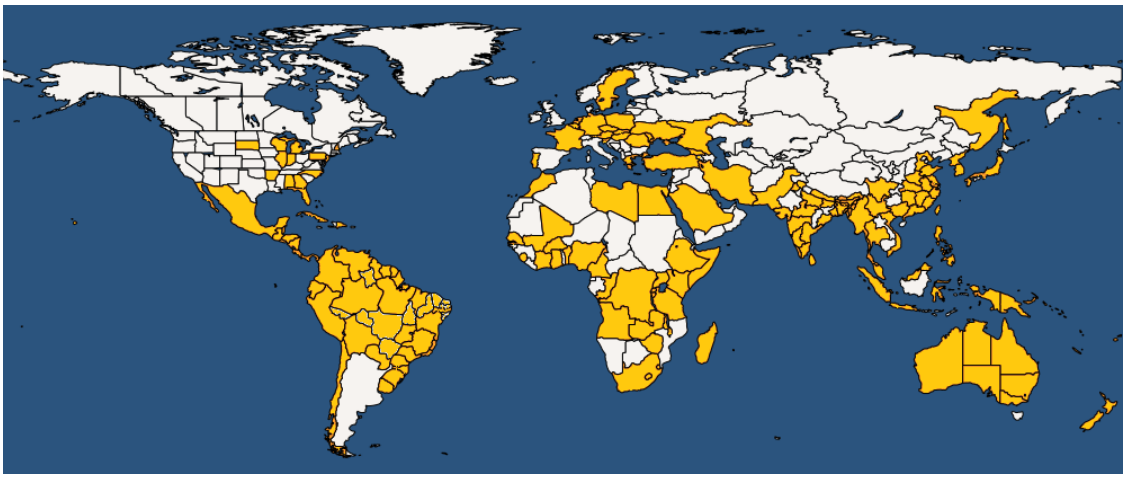

Figura 1. Distribuição mundial de *Ralstonia solanacearum*. Adaptado de OEPP/EPPO *Global Database* (2016).

A doença causada pela bactéria *R. solanacearum* é conhecida por murcha-bacteriana (exceto quando acomete a bananeira, situação na qual recebe o nome de Moko). De maneira geral, os sintomas iniciais caracterizam-se por escurecimento vascular, mais visível na região próxima ao colo, epinastia e murcha de folhas, podendo

haver recuperação das plantas nas horas mais frescas do dia. Com a progressão da doença, esse quadro de murcha afeta a planta toda, resultando em sua morte (Figura 2B). O fluxo bacteriano, uma massa branca e viscosa da bactéria exsudada a partir dos vasos do xilema, pode ser visualizado pelo teste do copo através da imersão da haste de plantas infectadas em água. Esse teste é utilizado para diagnóstico rápido do patógeno no campo (Figura 2C).

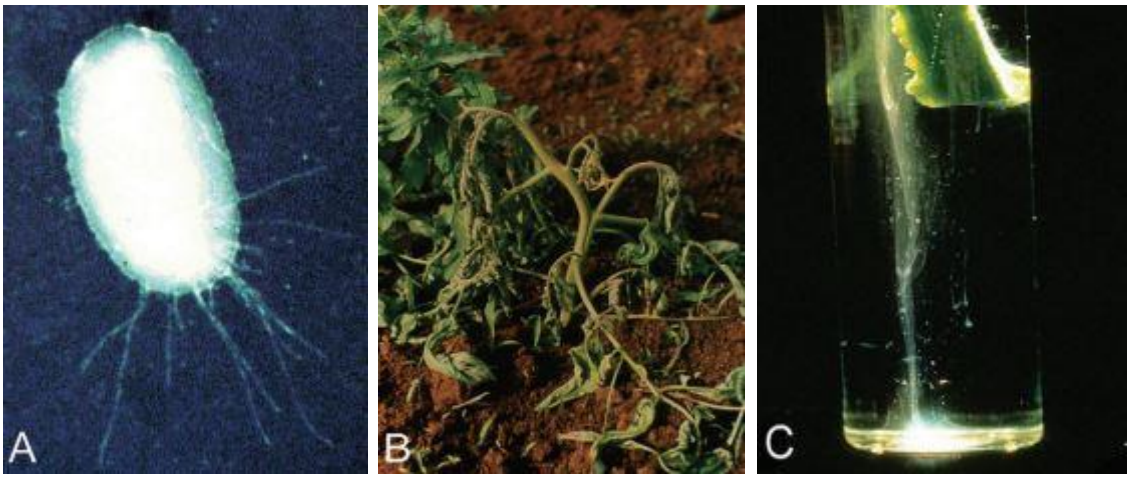

Figura 2. *Ralstonia solanacearum* (A, fotografia por C. Boucher e J. Vasse), sintomas da murcha-bacteriana em tomateiro (B) e teste do copo evidenciando o exsudado bacteriano (C). Mansfield et al. (2012).

Os prejuízos econômicos diretos decorrentes da murcha-bacteriana variam de acordo com a cultura hospedeira, condições edafoclimáticas e isolado bacteriano. Mundialmente, em áreas em que a doença ocorre, perdas de produtividade são estimadas em 33 a 90% na cultura da batata, 10 a 30% em lavouras de tabaco, 80 a 100% em bananeiras, até 20% na cultura do amendoim e até 91% em tomateiros (ELPHINSTONE, 2005 *apud* YULIAR; NION; TOYOTA, 2015), gerando prejuízos de bilhões de dólares por ano (ALBUQUERQUE et al., 2015).

Dentre as estratégias para o controle de *R. solanacearum*, a utilização de cultivares resistentes é considerada a mais importante (HAYWARD, 1991). No entanto, o melhoramento para obtenção de cultivares resistentes é complicado devido à ausência de boas fontes de resistência nas espécies vegetais e à diversidade genética do patógeno (LOPES, 2005; REMENANT et al., 2010). Soma-se a isso o fato de que a resistência genética não tem demonstrado estabilidade em relação ao tempo e ao local, principalmente devido a alterações climáticas (TUNG et al., 1990; LOPEZ; BIOSCA, 2004) e ao surgimento de novas linhagens bacterianas que superam a resistência

(JANSKY, 2009). Nesse contexto, o objetivo de melhoristas é desenvolver novas variedades com resistência duradoura e de largo espectro ao complexo específico *R. solanacearum*.

Aliada ao melhoramento clássico, a biotecnologia pode desempenhar papel importante na proteção vegetal. Doenças bacterianas podem ser controladas em plantas por engenharia genética mediante expressão de genes encontrados em fungos, insetos, animais e outras plantas (PATIL; GOPAL; SINGH, 2012). Dentre os potenciais genes que conferem resistência às plantas transgênicas, destacam-se aqueles que sintetizam peptídeos antibacterianos. Essa estratégia tem sido uma das formas estudadas para se controlar a murcha-bacteriana em plantas de tabaco (JAYNES et al., 1993), batata (JIA et al., 1998; LIANG; HE, 2002; BOSHOU, 2005) e tomateiro (JAN; HUANG; CHEN, 2010).

2.2. Peptídeos antimicrobianos

Peptídeos antimicrobianos (AMPs) são pequenas moléculas – em sua maioria menores que 10kDa, catiônicas e com predominância de aminoácidos hidrofóbicos – que apresentam atividade inibitória a vários patógenos. Os AMPs têm sido divididos em grupos baseados em seu tamanho, estrutura secundária e terciária, bem como na presença ou ausência de pontes de dissulfeto. Os principais grupos englobam: (a) peptídeos que formam estruturas em alfa-hélice; (b) peptídeos ricos em resíduos de cisteína; (c) peptídeos que formam padrão estrutural de folha-beta (com pontes de dissulfeto); (d) peptídeos ricos em aminoácidos regulares, tais como histidina, arginina, prolina e triptofano; e (e) peptídeos compostos por aminoácidos raros e modificados, por exemplo, lantionina, 3-metil-lantionina, dehidroalanina e dehidrobutirina (REDDY; YEDERY; ARANHA, 2004).

Os AMPs participam da defesa inata de inúmeros organismos, desde micróbios a plantas e animais (BROWN; HANCOCK, 2006). Nas últimas décadas, têm sido reconhecidos como potenciais agentes terapêuticos no controle a ampla variedade de patógenos, em virtude de suas propriedades antibacterianas, antivirais e antifúngicas (THEVISSEN et al., 1996; ZASLOFF, 2002; HANCOCK, 2003; MANGONI; SHAI, 2009, 2011; PASUPULETI; SCHMIDTCHEN; MALMSTEN, 2012). Uma vez que os AMPs diferem estruturalmente dos antibióticos convencionais produzidos por bactérias e fungos, oferecem novos moldes para o desenvolvimento de compostos farmacêuticos.

Em muitos casos, esses peptídeos são efetivos mesmo contra micro-organismos resistentes a antibióticos ou fungicidas (MUÑOZ et al., 2007).

Os AMPs atuam em membranas celulares, comprometendo sua integridade e, consequentemente, causando o extravasamento do conteúdo celular (ZASLOFF, 2002; HANCOCK, 2003). Com base nesse alvo, três principais modelos foram propostos para elucidar o mecanismo de ação dos peptídeos. O primeiro, modelo de aduelas (*barrel-stave model*), descreve a formação de canais transmembrana, ou de poros, por feixes de α-hélices anfipáticas, de tal modo que as suas superfícies hidrofóbicas interagem com o núcleo lipídico da membrana e suas superfícies hidrófilas posicionam-se internamente, produzindo um poro aquoso (Figura 3A) (MATSUZAKI et al., 1998). O segundo modelo é denominado tapete (*carpet model*). Os peptídeos são eletrostaticamente atraídos pela cabeça aniônica dos fosfolipídios e cobrem diversos pontos da superfície da membrana, como um tapete de moléculas. Em altas concentrações, os peptídeos desestruturam a bicamada, assemelhando-se à ação de um detergente, eventualmente conduzindo à formação de micelas (Figura 3B) (SHAI, 1999; LADOKHIN; WHITE, 2001). No terceiro modelo, de poros toroidais (*toroidal-pore model*) (Figura 3C), as hélices dos peptídeos antimicrobianos inserem-se na membrana e induzem ao dobramento das monocamadas lipídicas através dos poros, de modo que estes ficam revestidos tanto pelos peptídeos inseridos como pelas cabeças lipídicas dos fosfolipídios (MATSUZAKI et al., 1996). Esse modelo difere do primeiro apresentado, uma vez que, nos poros toroidais, os peptídeos sempre estão associados à cabeça lipídica da membrana, mesmo se forem inseridos perpendicularmente à bicamada.

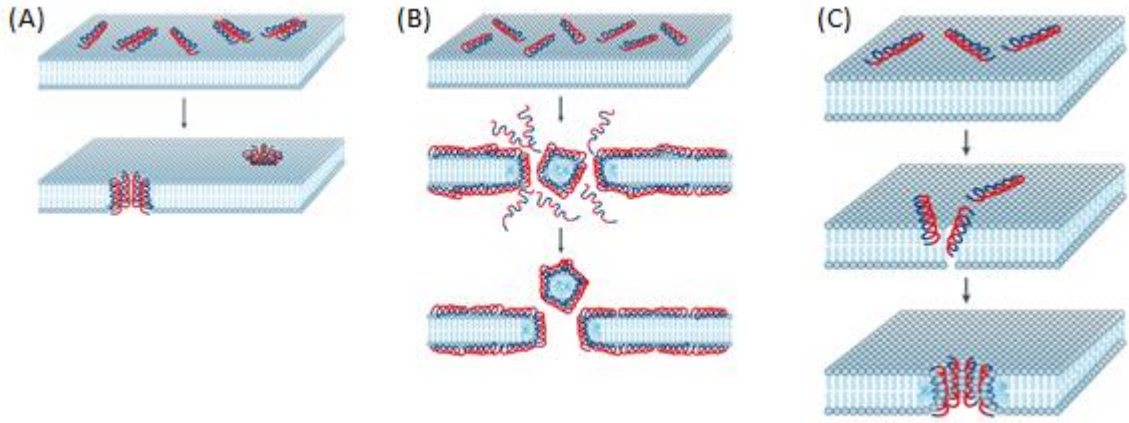

Figura 3. Modelos representativos dos mecanismos de ação dos peptídeos antimicrobianos (AMPs). Modelo de aduelas (*barrel-stave model*) (A), tapete (*carpet model*) (B) e poros toroidais (*toroidal-pore model*) (C). Adaptado de Brogden (2005).

Alternativamente, alguns AMPs podem atravessar a membrana plasmática sem destruí-la (PARK et al., 2000; ZELEZETSKY; TOSSI, 2006) e exercer sua atividade pela interação com alvos intracelulares – por exemplo, através da ligação e inibição de ácidos nucleicos (LEHRER et al., 1989; YONEZAWA et al., 1992; BOMAN; AGERBERTH; BOMAN, 1993; PARK; KIM; KIM, 1998; SUBBALAKSHMI; SITARAM, 1998; CUDIC; OTVOS, 2002; PATRZYKAT et al., 2002), inibição da síntese de proteínas (LEHRER et al., 1989; BOMAN; AGERBERTH; BOMAN, 1993; SUBBALAKSHMI; SITARAM, 1998; PATRZYKAT et al., 2002), inibição de atividade enzimática (ANDREU; RIVAS, 1998; OTVOS et al., 2000) e inibição da síntese de parede celular (BROTZ et al., 1998).

A capacidade de micro-organismos tornarem-se resistentes aos AMPs é pequena [para maiores detalhes, consultar Steinberg et al. (1997), cujo estudo demonstrou que a resistência à protegrina – obtida de porcos – é mais difícil que a seleção de mutantes resistentes à vancomicina], uma vez que, para tal, teriam de redesenhar suas membranas, modificando a composição e/ou organização dos lipídios. Entretanto, a resistência pode ser adquirida por meio da síntese de proteases (capazes de degradar os peptídeos) ou mediante ligação dos AMPs a determinados envoltórios ou compostos celulares que reduziriam o efeito antimicrobiano (ZEITLER et al., 2013). Assim, apesar da resistência aos AMPs por micro-organismos ser pequena, não é improvável que ocorra. Em um experimento de seleção, a multiplicação de *Escherichia coli* e de *Pseudomonas fluorescens* em meio de cultura suplementado com pexiganan (um análogo à magainina) configurou no surgimento de organismos resistentes ao peptídeo após sucessivas repicagens (PERRON; ZASLOFF; BELL, 2006). Visto isso, para prevenir problemas, como os encontrados devido à utilização irregular de antibióticos convencionais, AMPs devem ser usados correta e sensatamente.

2.3. Peptídeos antimicrobianos no controle de doenças de plantas

A produção agrícola pode ser drasticamente comprometida por fitopatógenos. Por essa razão, agrotóxicos são utilizados com frequência nas plantas para o manejo de doenças, objetivando reduzir perdas. No entanto, muitos produtos são tóxicos e/ou carcinogênicos e podem causar sérios problemas ambientais. Soma-se a isso o fato de que sua eficácia pode ser reduzida em virtude do surgimento de patógenos resistentes aos ingredientes ativos (KNIGHT et al., 1997; MAKOVITZKI et al., 2007; MARCOS et al.,

2008). A incessante demanda por alimentos, aliada aos preceitos de sustentabilidade, requer, portanto, produtos com elevada atividade antimicrobiana, não tóxicos e seguros ao meio ambiente para substituírem os agrotóxicos tradicionalmente utilizados na proteção de plantas.

A participação dos AMPs na defesa do hospedeiro contra patógenos é bem conhecida, e seu emprego na agricultura foi proposto desde sua descoberta. AMPs derivados de animais foram avaliados *in vitro* e *ex vivo* (em folhas ou frutos destacados) quanto ao seu potencial de proteção de plantas contra fitopatógenos. Dentre os AMPs estudados, destacam-se a magainina (de sapos), a cecropina (derivada de mariposa) e quimeras ou formas modificadas desses dois peptídeos (CAVALLARIN; ANDREU; SAN SEGUNDO, 1998; OSUSKY et al., 2000; ALAN; EARLE, 2002; YEVTUSHENKO et al., 2005; COCA et al., 2006).

Em ensaios *in vitro*, o peptídeo sintético MSI-99, derivado da magainina, é eficaz contra o oomiceto *Phytophthora infestans* e o fungo *Alternaria solani*, e contra bactérias fitopatogênicas (ALAN; EARLE, 2002). Pep3, uma quimera entre cecropina e melitina, tem atividade contra *P. infestans*, *Thielaviopsis basicola* e duas espécies de *Fusarium* (ANDREU et al., 1992; CAVALLARIN; ANDREU; SAN SEGUNDO, 1998). Um peptídeo análogo à cecropina B, denominado D4E1, apresenta efeito inibitório sobre *T. basicola*, *Verticillium dahliae*, *Fusarium moniliforme*, duas espécies de *Phytophthora* e sobre as bactérias *Pseudomonas syringae* pv. *tabaci* e *Xanthomonas axonopodis* pv. *malvacearum* (DeLUCCA; WALSH, 1999). Outro análogo à cecropina B, MB-39, é eficaz contra *Pectobacterium carotovorum* subsp. *betavasculorum*, *Clavibacter michiganensis*, três patovares de *P. syringae* e dois patovares de *X. campestris*, além de inibir o desenvolvimento do oomiceto *P. infestans* e do fungo *Rhizoctonia solani* (OWENS; HEUTTE, 1997).

A ação dos AMPs na proteção vegetal, mediante pulverização, é proposta na literatura por vários autores (KEYMANESH; SOLTANI; SARDARI, 2009; CHE et al., 2011; ZEITLER et al., 2013). No estudo de Che et al. (2011), plantas de tabaco, tomate e arroz foram pulverizadas preventivamente com uma proteína quimérica contendo os domínios ativos da melitina e da cecropina A (Hcm1). Após inoculação artificial, as plantas apresentaram resistência contra virose (*Tobacco mosaic virus* – TMV, em plantas de tabaco), infecção bacteriana (*R. solanacearum*, em tomateiro) e doença fúngica (*Magnaporthe grisea*, em plantas de arroz). Diante destes resultados, os autores propuseram o uso dessa quimera como ingrediente ativo de agrotóxicos. Cabe salientar,

no entanto, que o desenvolvimento de compostos para a agricultura, utilizados como ingredientes ativos de agrotóxicos, apresenta diversos entraves, principalmente devido à toxicidade intrínseca e à baixa estabilidade de alguns compostos, bem como ao desenvolvimento de formulações adequadas para a tecnologia de aplicação e à viabilidade econômica. Sendo assim, pesquisas devem ser conduzidas no intuito de prover compostos menos tóxicos e mais estáveis, além de produzi-los em larga escala a custos reduzidos.

A biotecnologia pode ser empregada para o desenvolvimento de plantas transgênicas que expressem genes que codificam para a síntese de compostos antimicrobianos, conferindo-as resistência vertical ou horizontal a fitopatógenos (Tabela 1). Em ensaios conduzidos em casa de vegetação, linhagens transgênicas de videira expressando uma proteína quimérica contendo cecropina B apresentaram ausência ou redução de sintomas da doença de Pierce, causada pela bactéria *X. fastidiosa*: menor bloqueio do xilema pela massa bacteriana e restrita necrose foliar (DANDEKAR et al., 2012). Apesar da eficácia observada nesse ensaio, novas variedades de videira resistentes à doença de Pierce não estão disponíveis no mercado, porque o comportamento das linhagens mais promissoras precisa ser testado em condições de campo e as plantas transgênicas submetidas a uma série de estudos regulamentados.

Plantas transgênicas expressando AMPs devem ser avaliadas criteriosamente antes de sua liberação comercial. Análises de biossegurança fazem-se necessárias para resguardar a saúde humana e o meio ambiente. Nesse escopo, possíveis impactos ambientais decorrentes do uso de plantas com AMPs têm sido foco de alguns estudos. Como exemplo, cita-se o trabalho de O'Callaghan et al. (2005), que compararam a microbiota associada a plantas de batata expressando magainina com aquela encontrada em associação a cultivares de batata não transgênicas. Esse tipo de experimento, juntamente com regulares avaliações de biossegurança, deve ser conduzido de forma organizada para cada cultura de modo a estabelecer protocolos confiáveis de avaliação de riscos, o que poderia acelerar a liberação dos transgênicos. Em alguns países tropicais, a ausência de normas de biossegurança entrava ensaios de campo com centenas de linhagens transgênicas de *Musa* sp. expressando AMPs (TRIPATHI, 2003 *apud* KEYMANESH; SOLTANI; SARDARI, 2009).

Em se tratando da murcha-bacteriana, o primeiro estudo abordando a expressão de peptídeos antimicrobianos em plantas foi conduzido na década de 1990 (JAYNES et al., 1993). Análogos à cecropina B (SB-37 e Shiva-1) foram clonados em plantas de tabaco que, após inoculação com uma linhagem virulenta de *R. solanacearum*, apresentaram

reduzida severidade da doença quando comparadas ao controle não transformado. A cecropina B também foi expressa em plantas de tomate para o controle dessa fitobactéria (JAN; HUANG; CHEN, 2010). Na cultura da batata, plantas transgênicas continham um AMP derivado de uma variedade de *Solanum tuberosum* L. resistente à murcha-bacteriana (LIANG; HE, 2002). O destaque do estudo de Liang e He (2002) refere-se à utilização de um peptídeo proveniente da mesma espécie que fora submetida à transformação genética.

Tabela 1. Peptídeos antimicrobianos expressos em plantas transgênicas (Adaptado de Montesinos, 2007, Breen et al., 2015 e Holásková et al., 2015).

AMP	Fonte	Planta transformada	Resistência	Referência
Hordotionina	Cevada	Tabaco	*Clavibacter michiganensis* e *Pseudomonas syringae* pv. *tabaci*	Carmona et al., 1993
		Macieira	*Venturia inaequalis*	Krens et al., 2011
		Batata-doce	*Ceratocystis fimbriata*	Muramoto et al., 2012
SB-37, Shiva-1	Análogos à cecropina	Tabaco	*Ralstonia solanacearum*	Jaynes et al., 1993
		Batata/Macieira	*P. syringae* pv. *tabaci*	Huang et al., 1997
			Pectobacterium carotovorum subsp. *atrosepticum*	Arce et al., 1999
		Anthurium	*Xanthomonas axonopodis* pv. *dieffenbachia*	Kuehnle et al., 2004
		Paulownia	Fitoplasmas	Du et al., 2005
Rs-AFP2	Defensina de rabanete	Tabaco/Tomate	*Alternaria longipes*	Terras et al., 1995
		Arroz	*Magnaporthe grisea* e *Rhizoctonia solani*	Jha; Chattoo, 2010
		Trigo	*Fusarium graminearum* e *R. cerealis*	Li et al., 2011a
Taquiplesina	Caranguejo (hemolinfa)	Batata	*P. carotovorum*	Allefs et al., 1996
		Girassol	*Sclerotinia sclerotiorum*	Lu, 2003
Sarcotoxina IA	Mosca das frutas (hemolinfa)	Tabaco	*P. syringae* pv. *tabaci* e *P. carotovorum* subsp. *carotovorum*	Ohshima et al., 1999
DRR206	Defensina de ervilha	Canola/Tabaco	*Leptosphaeria maculans*	Wang et al., 1999
Spi1	Defensina de pinheiro	Tabaco	*Heterobasidium annosum*	Elfstrand et al., 2001
MB-39	Análogo à cecropina	Macieira	*E. amylovora*	Liu et al., 2001

Attacin E Attacin A	Mariposa (hemolinfa)	Pereira	*Erwinia amylovora*	Reynoird et al., 1999
		Macieira		Norelli et al., 2000
		Laranjeira	*X. axonopodis* pv. *citri*	Boscariol et al., 2006
Defensina	*Brassica rapa*	Arroz	Inseto (cigarrinha-marrom)	Choi et al., 2009
Alf-AFP	Defensina de alfafa	Batata Tomate	*Verticillium dahliae* *F. oxysporum*	Gao et al., 2000 Abdallah et al., 2010
D4E1	Sintético	Tabaco Álamo	Diversos patógenos	Cary et al., 2000 Mentag et al., 2003
		Algodoeiro	Fungos	Rajasekaran et al., 2005
Magainina	Pele de sapo	Tabaco	Diversos fungos e bactérias	De Gray et al., 2001
		Milheto	*Sclerospora graminicola*	Ramadevi; Rao; Reddy, 2014
Cecropina A, B	Mariposa (hemolinfa)	Arroz	*X. oryzae*	Sharma et al., 2000
			M. grisea	Coca et al., 2006
			F. verticillioides e *Dickeya dadantii*	Bundó et al., 2014
		Tomate	*R. solanacearum* e *X. vesicatoria*	Jan; Huang; Chen, 2010
		Videira	*Xylella fastidiosa*	Dandekar et al., 2012
Myp30	Análogo à magainina	Tabaco	*Peronospora tabacina*	Qingshun et al., 2001
Mi-AMP1	Sementes de macadâmia	Canola	*L. maculans*	Kazan et al., 2002
AMP1	Clone de batata MS42.3	Batata	*R. solanacearum*	Liang; He, 2002
Ac-AMP1.2/ ESF12	Sementes de amaranto/ Sintético	Álamo	*Septoria musiva*	Liang et al., 2002
Heliomicina/ drosomicina	Defensina de insetos	Tabaco	*Botrytis cinerea*	Banzet et al., 2002
BSD1	Defensina de repolho	Tabaco	*Phytophthora parasitica*	Park et al., 2002
WT1	Defensina de wasabi	Arroz	*M. grisea*	Kanzaki et al., 2002
		Citrullus colocynthis	*A. solani* e *F. oxysporum*	Ntui et al., 2010
Pn-AMP	Heveína de *Ipomoea nil*	Tabaco	*P. parasitica*	Koo et al., 2002
Esculentina-1	Pele de sapo	Tabaco	*P. syringae* pv. *tabaci*, *P. aeruginosa* e *P. nicotianae*	Ponti et al., 2003
AFP	Defensina de fungos	Arroz	*M. grisea*	Coca et al., 2004

Mj-AMP1	Defensina de Jalapa	Tomate	*A. solani*	Schaefer et al., 2005
MSI-99	Análogo à magainina	Bananeira	*F. oxysporum* f. sp. *cubense* e *Mycosphaerella musicola*	Chakrabarti et al., 2003
		Tomate	*P. syringae* pv. *tomato*	Alan; Blowers; Earle, 2004
		Videira	*Rhizobium radiobacter*	Vidal et al., 2006
		Batata	*Aspergillus niger*	Ganapathi et al., 2007
MsrA1/ MsrA2/ MsrA3/ CEMA	Quimera cecropina-melitina	Tabaco	*F. solani*	Yevtushenko et al., 2005
		Tabaco/Batata/ Álamo	Fungos	Yevtushenko; Misra, 2007; 2012
		Batata	*P. infestans*, *P. erythroseptica* e *Fusarium*	Osusky et al., 2005
			P. carotovorum, *P. infestans* e *A. solani*	Vutto et al., 2010
			P. carotovorum	Yevtushenko; Misra, 2012
		Mostarda-castanha	*A. brassicae* e *S. sclerotiorum*	Rustagi et al., 2014
Dm-AMP1	Defensina de dália	Berinjela	*B. cinerea* e *V. alboatrum*	Turrini et al., 2004
Rev4	Análogo à indolicidina	Tabaco/ Arabidopsis	*P. tabacina*, *P. syringae* pv. *tabaci* e *P. carotovorum*	Xing et al., 2006
Pep1	Arabidopsis	Arabidopsis	*Pythium irregulare* e *P. dissotocum*	Huffaker; Pearce; Ryan, 2006.
PV5	Defensina de límulo	Tabaco	*Tobacco mosaic virus*, bactérias e fungos	Bhargava et al., 2007
ESF39A	Sintético	Ulmeiro (*Ulmus americana* L.)	*Ophiostoma novo-ulmi*	Newhouse et al., 2007
Cecropina P1	*Ascaris*	Batata	*P. infestans* e *S. sclerotiorum*	Zakharchenko et al., 2007
		Tabaco	*P. carotovorum* e *S. sclerotiorum*	Zakharchenko et al., 2009
		Falso-linho e Colza	*P. carotovorum* e *F. sporotrichioides*	Zakharchenko et al., 2013a, b
Magainina D	Análogo à magainina	Batata	*P. carotovorum*	Barrell; Conner, 2009
DEF2	Defensina de tomate	Tomate	*B. cinerea*	Stotz; Spence; Wang, 2009
MTK	*Drosophila melanogaster*	Cevada	*F. graminearum*	Rahnamaeian et al., 2009
			Blumeria graminis f. sp. *hordei*	Rahnamaeian; Vilcinskas, 2012
Defensina	Tabaco	Tabaco/Batata	Diversos fungos e bactérias	Portieles et al., 2010

Tanatina	*Podisus maculiventris* (inseto)	Arroz Milho	*M. grisae* *A. flavus*	Imamura et al., 2010 Schubert et al., 2015
PG1	Protegrina de porcos	Tabaco	*P. carotovorum*	Lee et al., 2011
Pen4-1	Camarão (*Litopenaeus setiferus*)	*Agrostis stolonifera*	*S. homoecarpa* e *R. solani*	Zhou et al., 2011
Tionina	Plantas	Batata	*B. cinerea*	Hoshikawa et al., 2012
hCAP18	Neutrófilos humanos	Repolho chinês	*P. carotovorum* subsp.*carotovorum*, *F. oxysporum* f. sp. *lycopersici*, *Colletotrichum higginsianum* e *R. solani*	Jung et al., 2012
BP100 e derivados	Sintético	Arroz	*D. chrysanthemi* e *F. verticillioides* Fitobactérias	Nadal et al., 2012 Company et al., 2014
SmAMP1.1a SmAMP2.2a	Heveína de morugem	Tabaco/ Arabidopsis	*Bipolaris sorokiniana* e *Thielaviopsis basicola*	Shukurov et al., 2012
TvD1	Defensina de *Tephrosia villosa*	Tabaco	*R. solani*	Vijayan et al., 2013
Dermaseptin e quimeras	Sapo	Batata Laranjeira	Fungos e bactérias *X. axonopodis*	Rivero et al., 2012 Furman et al., 2013
NaD1	Defensina de tabaco	Algodoeiro	*F. oxysporum* e *V. dahliae*	Lay et al., 2012 Gaspar et al., 2014

2.4. Proteínas relacionadas à patogênese (proteínas RP)

As plantas resistem ao ataque de patógenos utilizando defesas constitutivas e induzidas. Além de peptídeos antimicrobianos, o reconhecimento de elicitores do patógeno pela planta pode desencadar a síntese de proteínas relacionadas à patogênese (proteínas RP). São grupos de proteínas de defesa cuja síntese é induzida em resposta ao ataque de micro-organismos ou decorrente de estresses abióticos, reações de hipersensibilidade e resistência sistêmica adquirida (SAR) (RAMADEVI; RAO; REDDY, 2011). Possuem baixo peso molecular, são termoestáveis, altamente resistentes a proteases (VAN LOON; VAN STREIN, 1999) e apresentam atividade antimicrobiana (TONON et al., 2002; ANAND et al., 2004).

As proteínas RP foram descobertas por dois grupos independentes (VAN LOON; VAN KAMMEN, 1970; GIANINAZZI; MARTIN; VALLEE, 1970) que constataram o acúmulo de numerosas proteínas em extratos de folhas de tabaco com hipersensibilidade ao TMV (*Tobacco mosaic virus*). *A priori*, as proteínas RP foram agrupadas em cinco famílias (VAN LOON; VAN KAMMEN, 1970; VAN LOON, 1985), cada uma contendo duas subclasses: uma subclasse básica encontrada nos vacúolos e uma ácida localizada nos espaços extracelulares (KITAJIMA; SATO, 1999). Tais famílias incluíam quitinases, glucanases, osmotinas e proteínas homólogas à taumatina. Posteriormente, uma nova nomenclatura foi proposta, agrupando as proteínas de acordo com relações sorológicas, sequências de aminoácidos e similaridades enzimática ou biológica. Atualmente, 17 famílias (RP-1 a RP-17) foram reconhecidas e classificadas (AGARWALA et al., 2014; GAO et al., 2015).

O primeiro relato do desenvolvimento de plantas transgênicas expressando proteínas RP foi feito por Broglie et al. em 1991. *Nicotiana tabacum* e *Brassica napus*, contendo uma proteína da família RP-3, apresentaram resistência a *Rhizoctonia solani*. A introdução de proteínas RP em trigo também resultou em plantas resistentes a doenças fúngicas (BLIFFELD et al., 1999; SCHWEIZER; CHRISTOFFEL; DUDLER, 1999; BIERI; POTRYKUS; FUTTERER, 2000; OLDACH; BECHER; LORZ, 2001). Plantas transgênicas expressando proteínas RP foram desenvolvidas por distintos grupos de pesquisa. Algumas são apresentadas na Tabela 2.

Tabela 2. Proteínas relacionadas à patogênese expressas em plantas transgênicas (Adaptado de Ramadevi, Rao e Reddy, 2011, Balasubramanian et al., 2012 e Cletus et al., 2013).

Gene	Planta transformada	Resistência	Referência
Quitinase	Tabaco e *Brassica napus*	*Rhizoctonia solani*	Broglie et al., 1991
RP1a	Tabaco	*Peronospora tabacina* e *Phytophthora parasitica* var. *nicotianae*	Alexander et al., 1993
Quitinase	Tabaco	*Cercospora nicotianae*	Zhu et al., 1994
Osmotina	Batata	*Phytophthora* sp.	Liu et al., 1994
Glucanase e quitinase	Tomate, tabaco e cenoura	*R. solani*	Jongedijk et al., 1995
Quitinase	Arroz	*Magnaporthe grisea*	Nishizawa et al., 1999
RP-5	Arroz	*R. solani*	Datta et al., 1999
Quitinase	Amendoim	*C. arachidicola*	Rohini e Rao, 2001
Taumatina	Laranjeira	*P. citrophthora*	Fagoaga et al., 2001
Taumatina	Cenoura	*Botrytis cinerea* e *Sclerotinia sclerotiorum*	Chen e Punja, 2002
Taumatina	Tabaco	*Alternaria alternata*	Velazhahan e Muthukrishnan, 2003
Glucanase	Linho	*Fusarium oxysporum* e *F. culmorum*	Wrobel-Kwiatkowska et al., 2004
Quitinase (*RCC2*)	*Lolium multiflorum*	*Puccinia coronata*	Takahashi et al., 2005
CABPR1	Tabaco	*P. nicotianae, Ralstonia solanacearum* e *Pseudomonas syringae* pv. *tabaci*	Sarowar et al., 2005
Glucanase	Tomate	*A. solani*	Schaefer et al., 2005
Quitinase	Soja	*Rizhopterius solani*	Salehi et al., 2005
Quitinase	Algodoeiro	*Verticillium dahliae*	Tohidfar, Mohammadi e Ghareyazie, 2005
Quitinase	Morango	*B. cinerea*	Vellice et al., 2006
Glucanase e *alfAFP*	Tomate	*R. solanacearum*	Chen, Liu e Zou, 2006
Quitinase	Cenoura	*A. radicicola* e *B. cinerea*	Jayaraj e Punja, 2007
Glucanase	Trigo	*F. graminearum*	Mackintosh et al., 2007
Quitinase (*Mcchit1*)	*Nicotiana benthamiana* Algodoeiro	*P. nicotianae* *Verticillium* sp.	Xiao et al., 2007
Glucanase	Mostarda	*A. brassicae*	Mondal et al., 2007
Glucanase	Bananeira	*F. oxysporum*	Maziah, Saraih e Sreeramanan, 2007
Glucanase	Festuca (gramínea)	*M. grisea* e *R. solani*	Dong et al., 2007
RP-4	Tabaco	*P. nicotianae*	Fiocchetti et al., 2008

Quitinase e glucanase	Arroz	*R. solani*	Sridevi et al., 2008
Quitinase (*alAFP*)	Tomate	*B. cinerea*	Chen et al., 2009
Glucanase e quitinase	Cenoura	*B. cinerea* e *S. sclerotiorum*	Wally, Jayaraj e Punja, 2009
Glucanase	Amendoim	*C. arachidicola* e *Aspergillus flavus*	Sundaresha et al., 2010
Quitinase	Tomate	*F. oxysporum*	Girhepuje e Shinde, 2011
Quitinase	Tomate	*P. infestans*	Khaliluev et al., 2011
Quitinase	Milho	*Exserohilum turcicum*	Zhu, Zhao e Zhao, 2011
RP-1	Tabaco	*P. syringae* pv. *tabaci*	Li et al., 2011b
Glucanase e quitinase	Ervilha	*Trichoderma harzianum*, *C. acutatum*, *B. cinerea* e *Ascochyta pisi*	Amian et al., 2011
RP-5 e RP-12	Amendoim	*Phaeoisariopsis personata*	Vasavirama e Kirti, 2012
Quitinase	Amendoim	*C. arachidicola*	Iqbal et al., 2012
Quitinase	Capim-pé-de-galinha	*Pyricularia grisea*	Ignacimuthu e Ceasar, 2012
Quitinase	Algodoeiro	*V. dahliae*	Tohidfar et al., 2012
Quitinase	Lichia	*Phomopsis* sp.	Das e Rahman, 2012
Quitinase	Videira	*Plasmopara viticola*	Nookaraju e Agarwal, 2012
Quitinase	Trigo	*Fusarium* sp.	Liu et al., 2012
Quitinase	*Brassica juncea*	*A. brassicae*	Chhikara et al., 2012
Taumatina	Bananeira	*F. oxysporum*	Mahdavi, Sariah e Maziah, 2012
Quitinase	Amendoim	*A. flavus*, *Cercosporidium personatum* e *P. arachidis*	Prasad et al., 2013
Taumatina	Batata	*Macrophomina phaseolina* e *P. infestans*	Acharya et al., 2013
Quitinase	Arroz	*R. solani*	Shah, Singh e Veluthambi, 2013
Quitinase	Melão	*R. solani* e *F. oxysporum*	Bezirganoglu et al., 2013
Quitinase	Bananeira	*Mycosphaerella fijiensis*	Kovacs et al., 2013
Quitinase	Trigo	*P. striiformis* f. sp. *tritici*	Huang et al., 2013
Glucanase	Tabaco	*Phomopsis* sp., *Alternaria* sp. e *Fusarium* sp.	Liu et al., 2013
Glucanase	Berinjela	*V. dahliae* e *F. oxysporum*	Singh et al., 2014
RP-1	Trigo	*P. triticina*	Gao et al., 2015
RP-10a	Tabaco	*M. phaseolina*	Agarwal et al., 2016

Quitinases e glucanases têm sido extensivamente estudadas e o principal alvo da transgenia visa ao controle de doenças fúngicas (Tabela 2). Dentre as várias famílias de proteínas RP, há de se dar destaque também à RP-1, devido ao seu potencial efeito sobre fitobactérias (SAROWAR et al., 2005; LI et al., 2011b).

A família RP-1 representa o grupo mais abundante (até 2% do total de proteínas foliares) e é altamente conservada no reino vegetal (EDREVA, 2005). Genes que codificam algumas proteínas RP-1 foram inicialmente descobertos em plantas de tabaco (*Nicotiana tabacum* L.) (ANTONIW et al., 1980) e, posteriormente, em várias mono- e dicotiledôneas (MITSUHARA et al., 2008; LI et al., 2011b).

Proteínas RP-1 são induzidas por estresses bióticos e abióticos, como infecção por patógenos, fito-hormônios (ácido salicílico, etileno ou ácido abscísico), salinidade, seca e metais pesados (THIERRY et al., 1995; MITSUHARA et al., 2008; LE et al., 2009; SABATER et al., 2010; HOU et al., 2012; YANG; ZHANG; ZHENG, 2013), sendo comumente utilizadas como marcadores de SAR. Possuem efeito inibitório sobre *Phytophthora infestans* e *Uromyces fabae* em plantas de tomate e de feijão-fava, respectivamente (NIDERMAN et al., 1995; RAUSCHER et al., 1999). Sua expressão constitutiva confere resistência a *Peronospora tabacina* e *P. parasitica* var. *nicotianae* em plantas transgênicas de tabaco (ALEXANDER et al., 1993), ao passo que o silenciamento gênico da RP-1 em cevada aumenta a susceptibilidade das plantas à infecção por *Blumeria graminis* f. sp. *hordei* (SCHULTHEISS et al., 2003).

Apesar da reconhecida atividade antifúngica e do potencial efeito antibacteriano, não existem evidências sobre a função das proteínas RP-1 (ALEXANDER et al., 1993; NIDERMAN et al., 1995; SUDISHA et al., 2012), que demanda, portanto, mais estudos acerca de seu papel na proteção de plantas. Ainda, seria interessante verificar se a expressão de uma proteína RP-1 juntamente com um peptídeo antimicrobiano confere às plantas resistência a doenças de difícil controle, como a murcha-bacteriana causada pela fitobactéria *Ralstonia solanacearum*.

Postulou-se neste trabalho que a combinação desses dois domínios bioativos em uma única molécula configuraria em uma nova classe de proteínas terapêuticas. Essa hipótese baseou-se na atividade antimicrobiana da quimera NE-CecB, composta por uma elastase humana e um peptídeo lítico, validada no controle da fitobactéria *Xylella fastidiosa* (DANDEKAR et al., 2012). Assim, propôs-se substituir os domínios da proteína quimérica NE-CecB por homólogos naturalmente encontrados no genoma vegetal, compreendendo uma proteína RP-1 fusionada a um AMP derivado de plantas. O

objetivo deste trabalho foi caracterizar a atividade antimicrobiana dessa nova proteína quimérica à *R. solanacearum*. O conhecimento gerado poderá ser utilizado para desenvolver princípios ativos para defesa de plantas ou cultivares resistentes à murcha-bacteriana.

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CAPÍTULO 2

OCCURRENCE AND DIVERSITY OF *Ralstonia solanacearum* POPULATIONS IN BRAZIL[3]

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3 OCCURRENCE AND DIVERSITY OF *Ralstonia solanacearum* POPULATIONS IN BRAZIL

3.1. Abstract

Ralstonia solanacearum is a Gram-negative soil-borne bacterium capable of infection of hundreds of vegetable species over more than 50 botanical families, causing bacterial wilt – except for bananas, for which the disease is called Moko. It deserves special attention, among all plant pathogenic bacteria, because of its high phenotypic and genotypic plasticity, a characteristic that makes disease control extremely difficult. Frequent and necessary surveys have been carried out in an attempt to genotype the prevailing strains of *R. solanacearum* in each region where the disease has been reported. However, knowledge about occurrence and diversity of *R. solanacearum* in Brazil is fragmented and in some cases based on inconclusive studies with few strains, little representative of a given region. The need to obtain a greater picture guided this review. The occurrence of this bacterium in Brazilian states and the possible causes for its dissemination are presented, with emphasis on studies of genetic variability of populations of *R. solanacearum* in the country. The compiled results report a wide distribution of the bacterium in Brazil and great variability of its populations throughout locations. Partly due to the difficulty of detecting small titers of bacteria in samples, information about the origin of inoculum is scarce for certain regions. Further information is necessary to detect the presence of the pathogen in asymptomatic plants, in potato tubers with latent infections, in soil, and water, which are the major causes of bacterial dissemination into areas without any disease history.

Keywords: bacterial wilt, phylotypes, genetic diversity, phenotypic characterization.

3.2. Introduction

Bacterial wilt, caused by *Ralstonia solanacearum* [(SMITH, 1896) YABUUCHI et al., 1996], was apparently first observed in tobacco plants in Japan at the end of the 17th century (KELMAN, 1953). Since then, several reports have suggested the introduction of the bacterium into new areas, the existence of different centers of origin for this pathogen, or the occupation of some soil, climate, and new host niches around the

world due to the evolution of the bacterium. In Brazil the disease was first reported by Von Parseval in 1922, in tobacco crops in the State of Rio Grande do Sul (TAKATSU; LOPES, 1997). Detailed information about the disease history and pathogen dissemination around the world can be found in Lopes (2005).

The pathogen causing bacterial wilt was first described as *Bacillus solanacearum* by Smith (1896). Subsequently, it was classified as *Bacterium solanacearum* (CHESTER, 1898), *Pseudomonas solanacearum* [(SMITH, 1896) SMITH, 1914], *Phytomonas solanaceara* [(SMITH, 1896) BERGEY et al., 1923], and *Burkholderia solanacearum* [(SMITH, 1896) YABUUCHI et al., 1992]. Three years after the last classification, the species was moved to the present genus *Ralstonia* [(SMITH, 1896) comb. nov. YABUUCHI et al., 1995] and its validation published in 1996 (YABUUCHI et al., 1996).

Ralstonia solanacearum is a vascular pathogen widely distributed in tropical, subtropical, and temperate climate regions, where it affects several crops, including monocots and dicots (BUDDENHAGEN; KELMAN, 1964; HAYWARD, 1994). In Brazil the most affected species are *solanaceae* such as potato, tomato, bell pepper, eggplant, tobacco, and gilo, alongside banana, heliconia, eucalypt, and castor beans, among others (MALAVOLTA JÚNIOR et al., 2008). Such wide geographical distribution and host range can be attributed to the species genetic heterogeneity, including divergent strains with over 30% dissimilarity (REMENANT et al., 2010), and explains its definition as a species complex (FEGAN; PRIOR, 2005).

Ralstonia solanacearum can be disseminated by soil adhered to machinery and implements, by water, and by propagation materials such as potato tubers, rhizomes, and seedlings. Except for some strains from banana that can be transmitted by insects visiting flowers, plants are usually infected from the root system. By penetrating through wounds, which can be minimal such as those caused by the emergence of secondary roots, the bacteria quickly colonize the xylem vessels. Colonized vessels become inoperative for water transport from roots to shoots resulting in brown discoloration of vascular tissues, stunting, wilt, and death of the infected plant.

Control of bacterial wilt is difficult, since the pathogen can survive for many years in infested soil and weeds. Plant breeding for resistant cultivars, considered as the best control strategy for the bacteriosis, is troublesome due to the lack of good resistance sources among the vegetable species and the genetic diversity of the pathogen (LOPES, 2005; REMENANT et al., 2010).

In Brazil bacterial wilt has been reported in all states and is responsible for expressive decline in yields of agriculturally important crops and the condemnation of growing fields, especially those dedicated to the certification of potato seeds. Infested areas become useless for growing susceptible species such as potato, tomato, bell pepper, and banana.

Due to the economic losses caused by *R. solanacearum*, it is of essence to increase the body of knowledge about its regional occurrence and variability. Presently, great emphasis has been given to population genetic studies of this bacterium, which are fundamental for the understanding of the specific resistance of cultivars in certain locations and for the development of control strategies.

Knowledge about occurrence and diversity of *R. solanacearum* in Brazilian regions has been fragmented and in some cases based solely on few strains, often unrepresentative of the local variability. Thus, this review presents the bacterium distribution in the country, relating it to economically important vegetable species and discussing possible causes of its dissemination. It also includes studies about the genetic variability of *R. solanacearum* and the discrimination of Brazilian strains according to the current classification scheme for this bacterium.

3.3. *Ralstonia solanacearum* races and biovars in Brazil

Presently, *Ralstonia solanacearum* variability is represented by five pathogenic races (as a function of the host range) and six biovars (based on their ability to metabolize sugars and alcohols) (BUDDENHAGEN et al., 1962; BUDDENHAGEN, 1986; HAYWARD, 1991; HAYWARD, 1994; HAYWARD; FEGAN, 2004). In Brazil surveys carried out in several geographical regions have indicated the existence of races 1, 2, and 3, associated with several agriculturally important crops and some ornamental plants. Biovar 1 has been reported in all regions of the country, while biovar 2 predominates in mild climates (South, Southeast and Middle-West), and biovar 3 in the North and Northeast. Biovars 4 and 5 have not been reported in the country (Table 1).

Table 1. Occurrence of *Ralstonia solanacearum* biovars and races in Brazil.

Host	Biovar	Race	State/Region	Reference
Potato	1 2 2T	1 3	RS, PR, SC, Middle-West	Lopes et al., 1993; French et al., 1993; Maciel et al., 2001, 2004; Silveira et al., 2002, 2005; Santana et al., 2012
Tomato	1 2T 3	1	AM, RS, TO, RR, DF	Coelho Netto et al., 2003, 2004; Silveira et al., 2006; Costa et al., 2007; Lima Neto et al., 2009; Lima et al., 2010
Eggplant	1	1	RS, AM	Coelho Netto et al., 2004; Silveira et al., 2006
Eucalypt	1 2T	3	ES, SC, MA, MG, BA, PA, GO, AM	Sudo et al., 1983; Dianese and Takatsu, 1985; Dristig et al., 1988; Robbs et al., 1988; Alfenas et al., 2006; Auer; Santos; Rodrigues Neto, 2008; Mafia et al., 2012; Marques et al., 2012; Fonseca et al., 2013
Geranium	2	3	SP	Almeida et al., 2003
Bell pepper	1 3	1	AM, BA, ES, MA, PB, PE, PR, RJ, RO, RR, SP	Martins et al., 1988; Mariano et al., 1988, 1989; Coelho Netto et al., 2004; Lopes et al., 2005; Malavolta Júnior et al., 2008; Garcia et al., 2013
Capsicum chinense Jacq.	3	1	AM	Coelho Netto et al., 2004
Capsicum frutensens L.	3	1	AM	Coelho Netto et al., 2004
Gilo	2T 3	1	TO, AM	Coelho Netto et al., 2004; Lima Neto et al., 2009
Tobacco	1 3	1	RS, BA, PR, SC, PB, PE	Duarte et al., 2003; Silveira et al., 2006; Viana et al., 2012
Solanaceae	2T	3	GO, DF, MG, BA, PR	Santana et al., 2012
Heliconia	1	2	AP, PA, AM, PE, SE, RO, RR, DF	Assis et al., 2005; Zocolli et al., 2009; Rodrigues et al., 2011; Conaban, 2012
Banana	1	2	AP, BA, PA, AM, PE, SE, RO, RR	Tokeshi and Duarte, 1976; Freire et al., 2003; Vieira Júnior et al., 2010; Talamini et al., 2010; Rodrigues et al., 2011; Conaban, 2012
Castor bean	ND	ND	Northeast, PB	Mariano et al., 1998; Soares et al., 2010
Chicory	1		PA	Costa et al., 2007
Bean		1	RJ	Akiba et al., 1980
Cucumber	1 3		AM	Parente et al., 1988
Passion fruit	ND	ND	PA	Lopes et al., 1999

Squash	1	1	SP	Sinigaglia et al., 2001
Olive tree	1	1	MG	Tebaldi et al., 2014

ND: not determined.

Race 1, including biovars 1 and 3, is frequently found in the Northern region, which reinforces indications that *R. solanacearum* has its center of origin in the Amazon (HAYWARD, 1991). However, this race has affected many tomato, potato, bell pepper, gilo, and tobacco crops in all Brazilian regions (MARTINS et al., 1988; COELHO NETTO et al., 2003; DUARTE et al., 2003; LOPES et al., 2005; MALAVOLTA JÚNIOR et al., 2008; LIMA NETO et al., 2009; LIMA et al., 2010).

Dissemination of race 1 throughout the country may have resulted from the introduction of contaminated seedlings from other regions of the country or from abroad. After the cultivation of diseased plants, the soil also becomes an inoculum source infecting subsequent crops, especially those of *solanaceae*. However, Felix et al. (2012) stated that in soils where race 1 is not native, its survival in the lack of hosts is limited to up to 11 weeks. The authors evaluated 10 different soil types, but only a single bacterial strain (A1-9^{Rif}), which makes it difficult to generalize given the variability within race 1 and the efficacy of *Ralstonia* populations in extracting nutrients from the soil for their survival in the absence of hosts.

R1Bv1 (Race 1, Biovar 1) was reported in *solanaceae* in the Midwestern region of Brazil by Takatsu et al. in 1984. Subsequently, its occurrence was reported in potato in South, although in lower frequency than R3Bv2 (LOPES et al., 1993; MACIEL et al., 2001; SILVEIRA et al., 2002; 2005). Moreover, under special conditions of moisture and temperature, it has caused significant losses in commercial eucalypt nurseries in the States of Espírito Santo, Santa Catarina, Maranhão, Minas Gerais, Bahia, Pará, and Goiás (ALFENAS et al., 2006; AUER; SANTOS; RODRIGUES NETO, 2008). Recent observations of bacterial wilt in eucalypts indicate the existence of potential primary inoculum sources in the formation and management of clonal mini-gardens. The hypotheses include the transmission of the pathogen through seedlings with latent or quiescent infections, through rooting substrate, through irrigation water, or even through weeds naturally present in the nurseries (MAFIA et al., 2012).

R3Bv2 is known as the “potato-race” and, differently from race 1, presents a restricted number of host species. It is most commonly found, with no exclusivity, in crops in the South and Southeast, where most of the potato is grown in the country

(FRENCH et al., 1993; LOPES et al., 1993; MACIEL et al., 2001; SILVEIRA et al., 2002). Besides affecting potato crops, this race was found associated with geranium (*Pelargonium zonale*) in the State of São Paulo (ALMEIDA et al., 2003). The occurrence of the bacterium in this ornamental plant is worrisome since Brazil exports geranium seedlings to several countries (ROSSATO, 2012). Although the source of contamination has not been determined, it may be associated with the substrate or to irrigation water, and the distribution of infected material may have been from a nursery or flower grower. Quarantine measures should be adopted and soil and irrigation systems should be inspected to both avoid exchange of contaminated plants and prevent the dissemination of the pathogen to other areas, including potato production-oriented areas.

The occurrence of a given *R. solanacearum* race or biovar in a region may not be exclusive. Trials performed in the State of Rio Grande do Sul (RS), for instance, between 1997 and 1999, characterized 94% and 6% of *R. solanacearum* isolates as R3Bv2 and R1Bv1, respectively, in different potato cultivars and planting seasons (MACIEL et al., 2001; SILVEIRA et al., 2002). High frequency of R3Bv2 was expected and confirms the hypothesis of prevalence of this biovar in that state, probably due to milder temperatures (14 to 22^{o}C) during the major growing season. In turn, the occurrence of R1Bv1 in Spring crops indicates that late plantings are less favorable to that biovar. However, R1Bv1 has been proved to be predominant in tomato, eggplant, and tobacco grown in that state (SILVEIRA et al., 2006). These results demonstrate that climate and soil conditions in RS account for the occurrence of both biovars, with the predominance of each of them being determined by the host plant and planting season.

In the Southern region of Brazil, the introduction of R1Bv1 strains through contaminated seed potato and the subsequent increase of its population in the soil is a potential explanation for its incidence. Thus, even though R1Bv1 apparently has less ability to persist as a latent infection in tubers than R3Bv2, it can be transmitted by contaminated propagation material and become prevalent in regions or planting seasons with higher temperatures.

Biovar 2 Tropical (2T), also known as N2, metabolically more versatile than biovar 2 Andine (2A), has been occasionally found in Brazil. This biovar was isolated from areas planted for the first time with *solanaceae* in the Amazon region, after the forest was felled, suggesting the presence of a yet non-identified native host for *R. solanacearum* in the forest (COELHO NETTO et al., 2004). Biovar 2T occurs in low altitude tropical climate regions and seems to have the Amazon region as its center or

origin, presenting lower soil survival than biovars 1 or 3 (COELHO NETTO et al., 2004). In Brazil, besides the Amazon, this biovar has been found in Distrito Federal and the States of Goiás, Minas Gerais, Bahia, and Paraná, infecting *solanaceae* (SANTANA et al., 2012). Marques et al. (2012) also described and characterized R3Bv2T in *Eucalyptus urophylla* x *E. grandis* forests in Alexânia county (State of Goiás). Determining how this biovar was disseminated to other Brazilian States is an audacious call, but some possibilities can be considered. The first one would be the introduction of contaminated propagation material from Amazon areas with history of bacterial wilt. The second one would be the natural occurrence of this pathogen in soils of those states in which the disease occurred in the presence of host plants and favorable climate conditions. Finally, it could be that some strains, previously described as biovar 2, are, in fact, biovar 2T. This hypothesis is based on the lack of trealose test in some published reports, since the use of this carbohydrate for biovar identification is not part of the usual protocol.

The studies mentioned above confirm the high adaptability, versatility, and host range of *R. solanacearum*, warning about possible foci of bacterial wilt in locations where disease had not occurred previously and in species, until then, not considered as hosts. Among "non-traditional" host plants reported in Brazil are common bean (AKIBA et al., 1980), eucalypt (DRISTIG et al., 1988), cucumber (PARENTE et al., 1988), passion fruit (LOPES et al., 1999), squash (SINIGAGLIA et al., 2001), soybean, peas (BRINGEL; TAKATSU; UESUGI, 2001), and olive trees (TEBALDI et al., 2014), besides several weeds (MALAVOLTA JÚNIOR et al., 2008). Coffee has also been included in this list under artificial inoculation conditions (LOPES et al., 2009).

Race 2 of *R. solanacearum* causes the disease known as the Banana's Moko, first reported in Brazil by Tokeshi and Duarte in the Federal Territory of Amapá (now State of Pará) in 1976. Since then, this race has been disseminated to some states of the Northern and Northeastern regions (FREIRE et al., 2003; COELHO NETTO et al., 2004; ANDRADE et al., 2009). Race 2 is considered as a present quarantine pest (A2), occurring in the States of Amapá, Amazonas, Pará, Rondônia, Roraima, and Sergipe, and is restricted only to banana (*Musa* spp.) and *Heliconia* spp. (CONABAN, 2012; MAPA, 2013). Survival of the bacteria in the lack of the host in dryland cropping areas, in contrast to floodplains of the Amazon, is two months long in the dry season and four months long in the rainy season (CONABAN, 2012), which shows that soil moisture is fundamental for its survival. Planting material has an important role in the dissemination of Moko disease, both for short and long range spread.

Surveys carried out in the the State of Rondônia between 2007 and 2010 showed the occurrence of Moko in several counties (VIEIRA JÚNIOR et al., 2010). However, according to the authors, disease dissemination within the state reduced in comparison with the surveys of 2004 and 2007 (first semester). Similarly, Talamini et al. (2010) observed that the disease is decreasing in Sergipe, indicating a satisfactory quarantine control. In contrast, the bacterium (R2Bv1) has been reported in heliconia and ornamental *Musa* sp. in Distrito Federal (ZOCCOLI et al., 2009). The introduction of contaminated seedlings, mostly from the Northern region of Brazil, is presumably the inoculum source.

A study carried out by Rodrigues et al. (2011) revealed that strains isolated from *Musa* or *Heliconia* (R2Bv1) are able to cause wilt symptoms in *Strelitzia*. This result indicates the pathogenic potential of the bacterium to this plant species or, at least, that *Strelitzia* seedlings can be used as test plants for the presumptive diagnosis of Moko disease in banana.

Castor bean plants (*Ricinus communis*) were found with wilt symptoms and dieback in an experimental area of the Universidade Federal da Paraíba (PB) in 2009. The causal agent was identified as *R. solanacearum* (SOARES et al., 2010). This was the first report of this disease in the micro-region of Areia/PB. Previously, the bacterium had been reported in castor bean by Mariano et al. in 1998. Although the studies do not report the biovar to which the strains belong, they presumably are Bv3, commonly found in the Northern and Northeastern regions of the country.

As to the occurrence of bacterial wilt in ornamental plants in Brazil, the first report is that by Gonçalves (1937) in *Dahlia* sp. Since then, the disease has been reported in 24 ornamental host plants, including economically important species such as begonia, geranium, chrysanthemum, and heliconia (ALMEIDA et al., 2003; MALAVOLTA JÚNIOR et al., 2008; ZOCCOLI et al., 2009). Importation of different flower varieties with latent infection may have been the cause for bacterium dissemination in ornamental plants.

3.4. Genetic diversity of *Ralstonia solanacearum* in Brazil

Despite its common use, the previously mentioned *R. solanacearum* classification into races and biovars has the inconvenience of inconsistency since it is based on phenotypical characteristics. The advances in molecular biology and genome sequencing of strain GMI1000 (SALANOUBAT et al., 2002) now allows for genotypically

characterize the bacterium and study its variability. In this context, a new hierarchical classification scheme has been proposed, with four taxonomic levels: species, phylotype, sequevar and clone (FEGAN; PRIOR, 2005).

Polymerase chain reaction (PCR) stands out among the techniques used for molecular characterization of *R. solanacearum* populations. Several PCR protocols and specific primers have been designed for detection or identification of the species and for phylotyping (Table 2) (SEAL et al., 1993; ELPHINSTONE et al., 1996; OPINA et al., 1997; FEGAN et al., 1998; BOUDAZIN et al., 1999; PASTRIK; MAISS, 2000; POUSSIER; LUISETTI, 2000; WELLER et al., 2000). Classification into phylotypes is performed by PCR Multiplex with the Nmult series primers (based on ITS region), and the classification into sequevar is performed by partially sequencing gene *egl* (encoding the enzyme endoglucanase).

Four phylotypes and 51 sequevares of *R. solanacearum* habe been described (XU et al., 2009; FONSECA et al., 2013). Analysis of genetic diversity can be conducted based on repetitive sequences (rep-PCR), comprised by the elements BOX, ERIC, and REP, by randomly amplified DNA (RAPD), by amplification of restriction fragments (AFLP), repeated simple sequences (SSR) and by polymorphisms based on restriction fragment size (RFLP) (JAUNET; WANG, 1999; POUSSIER et al., 1999; COENYE; VANDAMME, 2003; YU et al., 2003; KUMAR et al., 2004; SILVEIRA et al., 2005; COSTA et al., 2007; IVEY et al., 2007).

Table 2. Primers used for molecular analysis of *Ralstonia solanacearum*.

Objective		**Oligonucleotides**	**Amplicon (bp)**	**Reference**
Identification		OLI1 - 5’GGGGGTAGCTTGCTACCTGCC3’ Y2 - 5’CCCACTGCTGCCTCCCGTAGGAGT3’	287	Seal et al., 1993
		759 - 5’GTCGCCGTCAACTCACTTTCC3’ 760 - 5’GTCGCCGTCAGCAATGCGGAATCG3’	280	Opina et al., 1997
Phylotype	I	Nmult:21:1F - 5’CGTTGATGAGGCGCGCAATTT3’	144	Fegan and Prior, 2005
	II	Nmult:21:2F - 5’AAGTTATGGACGGTGGAAGTC3’	372	
	III	Nmult23:AF - 5’ATTACS*AGAGCAATCGAAAGATT3’	91	
	IV	Nmult:22:Inf - 5’ATTGCCAAGACGAGAGAAGTA3’	213	

	Nmult22:RR - 5’TCGCTTGACCCTATAACGAGTA3’	-	
Sequevar	Endo-F - 5’ATGCATGCCGCTGGTCGCCGC3’ Endo-R - 5’GCGTTGCCCGGCACGAACACC3’	720	Ji et al., 2007

*Degenerated base: C+G.

Silveira et al. (2005) investigated the genetic variability of *R. solanacearum* strains obtained from different potato producing areas in the State of Rio Grande do Sul, using RAPD and repetitive sequences, differentiating biovars1 and 2 by ERIC and BOX-PCR. In this case, only BOX-PCR could confirm the variability within strains of R1Bv1. The authors concluded that RAPD (using the primer oligonucleotide OPO-10 (5’TCA GAG CGC C3’) clearly demonstrated the separation of *R. solanacearum* biovars, proving that the profiles were characteristic of the regions where the strains were obtained and that local variability was small. However, the ability of RAPD to detect polymorphisms depends on the selection of primer oligonucleotides that will reveal greater variability among and within strains of the biovar being studied. While the population of *R. solanacearum* in the State of Rio Grande do Sul has been described as quite homogeneous (SILVEIRA et al., 2005), bacterial strains from the Amazon region have been reported with a high degree of polymorphism by BOX-PCR, with no correlation among genome profiles and source host, biovar, ecosystem or collection location (COSTA et al., 2007).

Several studies have identified phylotypes of Brazilian strains of *R. solanacearum* (FEGAN; PRIOR, 2005; VILLA et al., 2005; PEREZ et al., 2008; GUIDOT et al., 2009; CELLIER; PRIOR, 2010; LEBEAU et al., 2011). In contrast, classification into sequevars has been explored only recently. Strains of R3Bv2, obtained from several potato producing regions in Brazil, have been classified as biovars 2A and 2T, phylotype II and, mostly, sequevar 1 (SANTANA et al., 2012). Such genetic uniformity is favorable to the development of resistant cultivars and of pathogen detection methods.

One hundred twenty strains from tobacco (*Nicotiana tabacum* L.), collected in 13 counties of the State of Paraná, 24 of Santa Catarina, 13 of Rio Grande do Sul, one of Paraíba, and two of Pernambuco, have been characterized into biovar, phylotype and by genetic diversity using the repetitive sequences BOX, ERIC, and REP (rep-PCR) (VIANA et al., 2012). All studied strains belonged to R1Bv1 and phylotype II, corroborating the information presented in this review about the prevalence of this race/biovar in the Southern region of Brazil, except when associated with potato crops.

Although the authors found homogeneity in biovar and phylotype, the results of rep-PCR divided the strains into six groups, with maximum similarity of 61%.

A study performed by Santiago et al. (2012), with 120 *R. solanacearum* strains (from 19 Brazilian states and 12 host species), classified them as biovar 1 (42.5%), 2 (45%), and 3 (12.5%). Biovar determination was done by biochemical tests. Moreover, the strains were grouped into phylotype II (95.8%) and phylotype I (4.2%, all from the North of the country). Sequencing the gene *egl* identified sequevars 1, 4A, 5, 6, 18, and 36.

Classification into sequevar is not always possible. For instance, out of 33 *R. solanacearum* strains collected from several hosts (19 strains from race 2, 14 from race 1, and 15 strains associated with banana plants), 82% have been classified as phylotype II (including all strains from banana). However, it has not been possible to characterize most strains into sequevars, and, possibly, the banana strains belong to a yet undetermined sequevar (PINHEIRO et al., 2011). This observation was also reported by Albuquerque et al. (2014) who described a new sequevar associated with Moko, named IIA-53. Neither have strains from eucalypt plants been grouped into known sequevars (FONSECA et al., 2013).

The prevalence of phylotype II in characterization studies of Brazilian strains of *R. solanacearum* confirms its correspondence to the American continent, as proposed by Fegan and Prior (2005). However, due to the exchange of plant material across continents, infection of host plants by strains from other regions of the world may occur, which may explain reports of phylotype I in the country (COELHO NETTO et al., 2003, 2004; SANTIAGO et al., 2012; GARCIA et al., 2013).

Mistakenly, Pinheiro et al. (2011) published the characterization of four *R. solanacearum* strains (two from tomato plants from Guaraí-TO and Nova Friburgo-RJ, one from eggplants from Gurupi-TO, and one from bell pepper from Camocin S. Felix-PE) as positive for phylotype III. However, the analysis of the amplicon size reveals correspondence to phylotype I, of 144 bp (and not 91 bp as mentioned by the authors). Thus, it was not the first report of phylotype III in the country, but again a confirmation of the occurrence of Asian strains in Brazil.

Some studies about the genetic variability of this bacterial population in Brazil report that the existence of diversity among strains oftentimes is correlated with its geographical origin (SILVEIRA et al., 2005; FONSECA et al., 2013), similarly to what was established for the classification into phylotypes. Therefore, the lower local bacterial

variability allows for disease control through the use of resistant cultivars recommended for each region of the country, although care should be taken against the dissemination of strains via propagation material.

The constant attempts to group *R. solanacearum* strains as they are identified open the avenue for the suggestion of new classification schemes. One of them is based on the identification of virulence patterns in specific groups of hosts (LEBEAU et al., 2011). According to this classification, pathogenic profiles (pathoprofiles) would group the behavior of strains within a group of host plants of several species, while the pathotypes would group the strains according to their virulence within a single host species. Another classification suggests the division of *R. solanacearum* into new species (REMENANT et al., 2011; ALLEN et al., 2014; SAFNI et al., 2014). In the first putative proposal (REMENANT et al., 2011), only phylotype II strains would be classified as *R. solanacearum*, while phylotypes I and III would be included in the new species *R. sequeirae* and phylotype IV in *R. haywardii*. This proposal, however, was based on the genome analysis of only eight strains of the species complex *R. solanacearum* and did not include phenotypical differentiations associated with the new species. Taxonomic reviews proposed by Allen et al. (2014) (74 strains) and by Safni et al. (2014) (68 strains) are more similar to each other (Table 3). Both of them suggest the division into three species according to significant biological (phenotypical and pathogenic) differences and to genomic divergences. Thus, *R. solanacearum* would include strains corresponding to phylotype II, *R. syzygii* to phylotype IV, and a new species would include strains from phylotypes I and III: *R. sequeirae* sp. nov. (ALLEN et al., 2014) and *R. pseudosolanacearum* sp. nov. (SAFNI et al., 2014). The authors also divide the species *R. syzygii* into three distinct groups (Table 3). Such propositions have not been adopted by scientific community yet, and there are no studies in Brazil reporting these classification schemes.

Table 3. Taxonomic reviews proposed for the species complex *Ralstonia solanacearum*.

Current classification	**Proposed taxon**	
(FEGAN; PRIOR, 2005)	Allen et al. (2014)	Safni et al. (2014)
Ralstonia solanacearum (Phylotype II)	*Ralstonia solanacearum*	*Ralstonia solanacearum*
Ralstonia solanacearum (Phylotype I)	*Ralstonia sequeirae* sp. nov.	*Ralstonia pseudosolanacearum* sp. nov.
Ralstonia solanacearum (Phylotype III)	*Ralstonia sequeirae* sp. nov.	*Ralstonia pseudosolanacearum* sp. nov.

Ralstonia solanacearum (Phylotype IV)	*Ralstonia syzygii* subsp. *haywardii* subsp. nov.	*Ralstonia syzygii* subsp. *indonesiensis* subsp. nov.
Ralstonia syzygii (Phylotype IV)	*Ralstonia syzygii* subsp. *syzygii*	*Ralstonia syzygii* subsp. *syzygii* ~~comb. nov.~~
BDB (Blood Disease Bacterium) (Phylotype IV)	*Ralstonia syzygii* subsp. *celebensis* subsp. nov.	*Ralstonia syzygii* subsp. *celebesensis* subsp. nov.

3.5. Conclusion

Studies about the occurrence and diversity of *Ralstonia solanacearum* provide a more consistent idea about the composition of prevailing populations in several agricultural areas in Brazil. Moreover, proper identification of bacteria is fundamental for a better understanding of pathogen ecology and etiology, as well as an aid for the establishment of control measures, including the use of resistant cultivars.

Successful disease management depends on the knowledge of which biovar, phylotype and sequevar of the species complex *Ralstonia* is present in the cropland. Such a dependence is due to differences among strains, especially in aspects related to aggressiveness, survival, and latency.

The literature reports a wide distribution of the bacterium in Brazil, with prevalence of R3Bv2 in potato in the South, general distribution of R1Bv1, R1Bv3 in the warmer regions of North, Northeast and Midwest, and the occurrence of biovar 2T out of the Amazon Basin. The prevalence of a given biovar, besides soil and climate characteristics, is due to the cultivated vegetable species in that region. Also, greater bacterial population variability has been observed across locations, suggesting certain homogeneity within the regions where disease occurs. As the studies expand with new and representative strains of *R. solanacearum*, greater insight will be gained into the pathogenic and molecular variability of the bacterium, providing a greater body of knowledge on epidemiological and ecological aspects needed for the proposition of control measures.

This review points to a paucity of records on the origin of vegetable material (mostly when dealing with species propagated by seedlings), the probable use of non-certified seed potato, and the need for tests to detect the bacterium in soil, water, and plants with latent infections. Such scarcity of information, alongside possible incorrect pathogen identification, limits epidemiological studies of bacterial wilt in Brazil.

3.6. References

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CAPÍTULO 3

THE PDB DATABASE IS A RICH SOURCE OF α-HELICAL ANTIMICROBIAL SEQUENCES PEPTIDES TO COMBAT DISEASE CAUSING PLANT PATHOGENS[4]

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4 THE PDB DATABASE IS A RICH SOURCE OF α-HELICAL ANTIMICROBIAL SEQUENCES PEPTIDES TO COMBAT DISEASE CAUSING PLANT PATHOGENS

4.1. Abstract

The therapeutic potential of α-helical antimicrobial peptides (AH-AMPs) to combat pathogens is fast gaining prominence. Based on recently published open access software for characterizing α-helical peptides (PAGAL), this paper describes a search methodology (SCALPEL) that leverages the massive structural data pre-existing in the PDB database to obtain AH-AMPs belonging to the host proteome. It provides *in vitro* validation of SCALPEL for plant pathogens (*Xylella fastidiosa*, *Xanthomonas arboricola*, and *Liberibacter crescens*) by identifying AH-AMPs that mirror the function and properties of cecropin B, a well-studied AH-AMP. The identified peptides include a linear AH-AMP within the existing structure of phosphoenolpyruvate carboxylase (PPC20), and an AH-AMP mimicking the properties of the two α-helices of cecropin B from chitinase (CHITI25). The minimum inhibitory concentration of these peptides are comparable to that of cecropin B, while anionic peptides used as control failed to show any inhibitory effect on these pathogens. The use of native structures from the same organism could possibly ensure that administration of such peptides will be better tolerated and not elicit an adverse immune response. The paper suggests a similar approach to target Ebola epitopes, enumerated using PAGAL, one in which suitable peptides are selected from the human proteome, especially in the wake of recent reports of cationic amphiphiles inhibiting virus entry and infection.

Keywords: SCALPEL, cecropin B, minimum inhibitory concentration, phytobacteria.

4.2. Introduction

The abundance of alpha-helical (AH) structures within proteins bears testimony to their relevance in determining functionality (AZZARITO et al., 2013). AHs are key components in protein-protein interaction interfaces (LEE et al., 2011), DNA binding motifs (LANDSCHULZ; JOHNSON; McKNIGHT, 1988), proteins that permeate

biological membranes (DATHE; WIEPRECHT, 1999), and antimicrobial peptides (AMPs) (WANG, 2008; 2014). Unsurprisingly, these AHs are the targets for antibody binding (LEE et al., 2008; CHAKRABORTY et al., 2014a) and therapeutic agents (HANCOCK; CHAPPLE, 1999). These therapies in turn use AH peptides against viral pathogens (JUDICE et al., 1997; CHAMPAGNE; SHISHIDO; ROOT, 2009; HONG et al., 2014), fungal (GOYAL et al., 2013), and bacterial pathogens (ZEITLER et al., 2013). It has been proposed that AMPs are superior to gene-mediated immunity since they directly target diverse microbial pathogens (GOYAL; MATTOO, 2014).

Some AHs have unique characteristics, which are strongly correlated with their significance in the function of a protein (CHAKRABORTY et al., 2014a). For example, hydrophobic residues aligned on one surface – characterized by a hydrophobic moment (EISENBERG; WEISS; TERWILLIGER, 1982) – is critical for virus entry into host cells (BADANI; GARRY; WIMLEY, 2014), and for the permeabilizing abilities of AH-AMPs (CHEN et al., 2007). Often, AHs have cationic residues on the opposite side of the hydrophobic surface, which helps them target bacterial membranes (BROGDEN, 2005; HUANG; HUANG; CHEN, 2010). We have previously implemented known methods (JONES; ANANTHARAMAIAH; SEGREST, 1992) of evaluating these properties, and provided this as open source software (PAGAL) (CHAKRABORTY; RAO; DANDEKAR, 2014). PAGAL was used to characterize the proteome of the Ebola virus (CHAKRABORTY et al., 2014a), and to correlate the binding of the Ebola protein VP24 (ZHANG et al., 2012) to human karyopherin (XU et al., 2014) with the immune suppression and pathogenicity mechanisms of Ebola and Marburg viruses (CHAKRABORTY et al., 2014b).

Plant pathogens like *Xylella fastidiosa* (Xf) (HOPKINS; PURCELL, 2002), *Xanthomonas arboricola* (Xa) (RYAN et al., 2011), and *Liberibacter crescens* (Lc) (LEONARD et al., 2012) are a source of serious concern for economic reasons (ALSTON et al., 2014). Specifically, we have been involved in developing novel strategies to counter the Pierce's disease causing Xf, having previously designed a chimeric protein with antimicrobial properties that provides grapevines with enhanced resistance against Xf (DANDEKAR et al., 2012). Cecropin B (CecB) is the lytic component of this chimeric protein (MOORE et al., 1996; SHARMA et al., 2000). However, the non-nativeness of CecB raises concerns regarding its viability in practical applications (SHELTON; ZHAO; ROUSH, 2002). The CecB sequence does not have any significant matches in the grapevine or citrus genomes. Also, the cationic amphipathic nature of CecB is not

encoded in the linear sequence, and can only be analyzed through its structure. However, a structural homology search of the PDB database through a tool like DALILITE (HOLM et al., 2008) results in many redundancies, since it does not include the amino-acid properties in the search algorithm. Thus, the development of new algorithms should incorporate the charge and amphipathic properties while searching for AMPs. Computational methods have been used for designing *de novo* AMPs (FRECER; HO; DING, 2004; FJELL et al., 2011) to complement comprehensive hand curated databases of AMPs (WANG, 2013). However, it remains a challenge to predict the folding of peptides (PIANA; KLEPEIS; SHAW, 2014), since their random coil conformations achieve helical structures only by interacting with anionic membrane models (MISHRA et al., 2013). Extracting AHs from known protein structures provides a degree of confidence in the likelihood of the target sequence displaying a helical structure in its independent form.

In an effort to replace CecB with an equivalent peptide from the grapevine/citrus genome, we present a design methodology to select AH-AMPs from any given genome – **S**earch **c**haracteristic **alp**ha h**el**ical peptides in the PDB database and locate it in the genome (SCALPEL). CecB consist of two AHs, joined by a small loop. The N-terminal AH is cationic and hydrophobic, while the C-terminal AH consists of primarily hydrophobic residues. Characterizing all available AHs from plant proteins in the PDB database allowed us to identify a peptide with a large hydrophobic moment and a high proportion of positively charged residues, present in both grapevine and citrus (our organisms of interest), mirroring the linear cationic CecB N-terminal AH. One such match was a twenty residue long AH from phosphoenolpyruvate carboxylase in sunflower (PAULUS; SCHLIEPER; GROTH, 2013). The sequence of this peptide was used to find homologous peptides in the grapevine and citrus genome (PPC20). Subsequently, we used the SCALPEL algorithm to detect two contiguous AHs connected with a loop, mirroring the properties of CecB in a chitinase (CHITI25) from *Nicotiana tabacum* (PDBid:3ALG) (OHNUMA et al., 2011). Subsequently, we demonstrate through bioassay experiments that PPC20 from the grapevine and citrus genome, and CHITI25 from the tobacco genome, inhibit Xf, Xa, and Lc growth. The minimum inhibitory concentration of these peptides are comparable to that of CecB, while anionic peptides used as controls failed to show any inhibitory effect with these pathogens. Further, we observed variation in the susceptibility of the pathogens to these peptides.

4.3. Materials and methods

4.3.1. *In silico*

The PDB database was queried for the keyword ‘plants’, and proteins with the exact same sequences were removed. This resulted in a set of ~2000 proteins (data not shown). These proteins were analyzed using DSSP (JOOSTEN et al., 2011) to identify the AHs, and AHs with the same sequence were removed. This resulted in ~6000 AHs. PAGAL was applied to this set of AHs. These data were refined to obtain peptides with different characteristics. We also computed the set of all pairs of AHs that are connected with a short (less than five residues) loop. This set is used to extract a pair of AHs, such that one of them is cationic with a large hydrophobic moment, while the other comprises mostly of hydrophobic residues. The PAGAL algorithm has been detailed previously (JONES; ANANTHARAMAIAH; SEGREST, 1992). Briefly, the Edmundson wheel is computed by considering a wheel with center (0,0), radius 5, first residue coordinate (0,5) and advancing each subsequent residue by 100 degrees on the circle, as 3.6 turns of the helix makes one full circle. We compute the hydrophobic moment by connecting the center to the coordinate of the residue and give it a magnitude obtained from the hydrophobic scale (in our case, this scale is obtained from Jones, Anantharamaiah and Segrest, 1992). These vectors are then added to obtain the final hydrophobic moment. The color coding for the Edmundson wheel is as follows: all hydrophobic residues are colored red, while hydrophilic residues are colored in blue (dark blue for positively charged residues, medium blue for negatively charged residues and light blue for amides). All protein structures were rendered by PyMol (http://www.pymol.org/). The sequence alignment was accomplished using ClustalW (LARKIN et al., 2007). The alignment images were generated using Seaview (GOUY; GUINDON; GASCUEL, 2010). Protein structures were superimposed using MUSTANG (KONAGURTHU et al., 2006).

4.3.2. *In vitro*

Synthesized chemical peptides were obtained from GenScript USA, Inc. The protein molecular weight was calculated per peptide and then diluted to 2000µM or 3000µM stock solutions with phosphate buffered saline. Stock solutions were stored at -20°C and thawed on ice before use.

Using the stock solutions, we made dilute solutions of 300µM, 250µM, 200µM, 150µM, 100µM, 75µM, 50µM, 30µM, 25µM, and 10µM to a final volume of 100µL of

phosphate buffered saline. Dilute peptide solutions were stored at -20°C and thawed on ice before use.

Xylella fastidosa 3A2 (PD3) (IONESCU et al., 2014), *Xanthomonas arboricola* 417 (TYS) (LINDOW; OLSON; BUCHNER, 2014), and *Liberibacter crescens* BT-1 (BM7) (FAGEN et al., 2014) media were prepared and autoclaved at 121°C for 15-30 minutes, then cooled and poured into 100 × 15mm sterile petri dishes. Kanamycin (50µg/mL) was added to PD3 medium to avoid contamination, since *Xylella* was allowed to grow for 5 days in liquid medium and 7-10 days after plated. This strain (Xf 3A2) is a mutant containing a kanamycin resistant gene.

Bacteria were inoculated and allowed to grow in liquid medium at 28°C: Xf (5 days), Xa (3 days), and Lc (3 days) to reach the exponential phase. The inoculum was diluted to a working concentration of 1×10^7 cells/mL. Then 10µL of the inoculum was plated with 90µL of liquid media and spread on the pre-made agar plates to create a confluent lawn of bacteria. The bacteria were given an hour to set at room temperature. Subsequently, 10µL of each peptide concentration was spotted onto a plate of agar preseeded with a layer of bacterium. After spotting, the plates were incubated at 28°C for 2 to 10 days till zones of clearance were clearly visible and the plates were scored for the minimum inhibitory concentration (MIC) as that beyond which no visible clearance was observed. Data were identical across triplicates.

4.4. Results

4.4.1. Existing AH-AMPs: the positive controls

Cecropin B (CecB) was used as a positive control, as it is known to target membrane surfaces and creates pores in the bacterial outer membrane (MOORE et al., 1996; SHARMA et al., 2000). CecB consists of a cationic amphipathic N-terminal with a large hydrophobic moment (Figure 1a) and a C-terminal consisting mostly of hydrophobic residues, which consequently has a low hydrophobic moment, (Figure 1b) joined by a short loop. Another positive control was a linear AH-AMP consisting of the residues 2-22 of the N-terminal in CecB (CBNT21) (Figure 1a). The sequences of these are shown in Table 1.

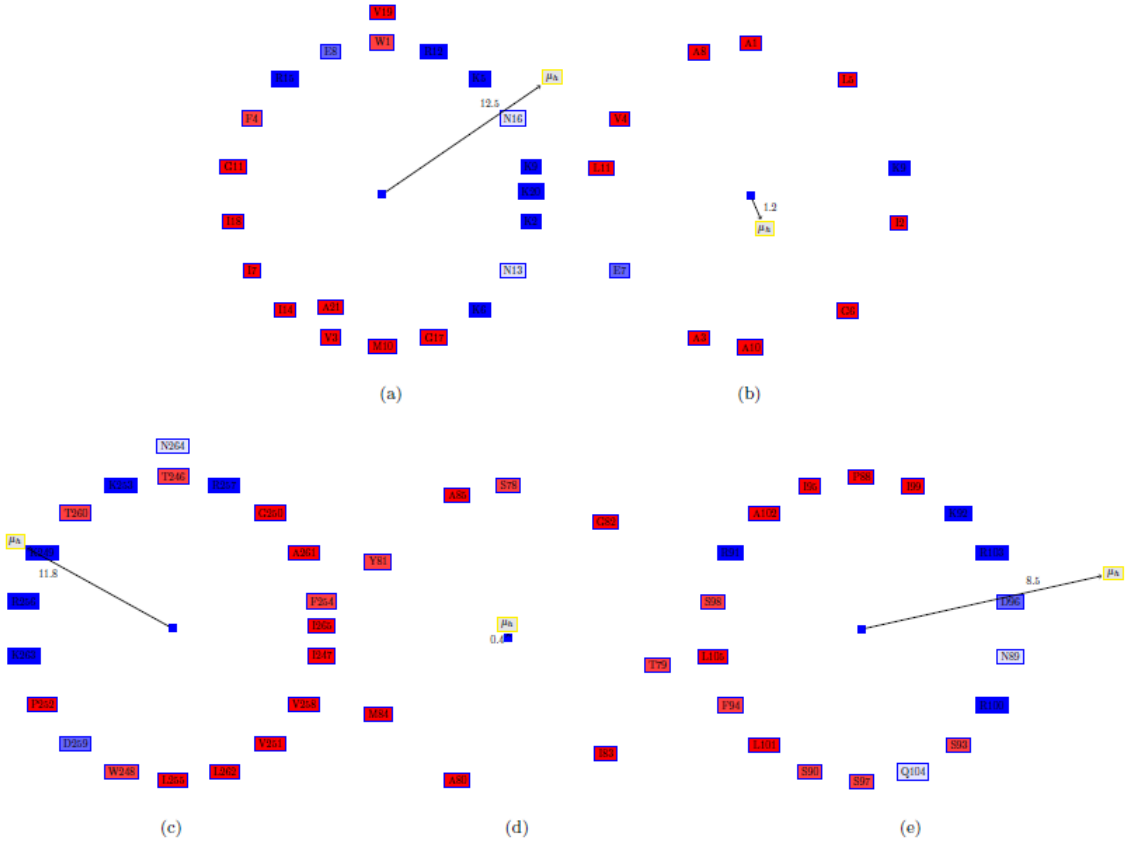

Figure 1. Edmundson wheel for AHs. The color coding for the Edmundson wheel is as follows: all hydrophobic residues are colored red, while hydrophilic residues are colored in blue (dark blue for positively charged residues, medium blue for negatively charged residues and light blue for amides). The hydrophobic moment arrow is not to scale. **(a)** N-terminal of Cecropin B (CecB) shows its amphipathic nature, with one side being cationic and the other side hydrophobic. The first lysine is omitted, since residues 2-22 of the N-terminal in CecB were used to construct the CBNT21 peptide. The first lysine reduces the hydrophobic moment from 12.5 to 11.1. **(b)** C-terminal of CecB consists mostly of hydrophobic residues, and thus has a low hydrophobic moment. **(c)** Edmundson wheel for a 20 amino acid AH from phosphoenolpyruvate carboxylase from sunflower (PDBid:3ZGBA.α11), PPC20. Two AHs within chitinase from *Nicotiana tabacum* (PDBid:3ALGA.α4 and 3ALGA.α5) connected by a short random coil such that one of the AHs is cationic and hydrophobic, while the other AH is comprised mostly of hydrophobic, uncharged residues. **(d)** Edmundson wheel for 3ALGA.α4, which corresponds to the C-terminal of CecB and consists mostly of hydrophobic residues (low hydrophobic moment). **(e)** Edmundson wheel for 3ALGA.α5, which corresponds to the cationic, N-terminal of CecB with a large hydrophobic moment.

Table 1. Sequences of peptides used in this study. CO: control peptides SC: SCALPEL generated peptides.

CO	CecB	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL full length CecB from *Hyalophora cecropia* (silk moth)
CO	CBNT21	WKVFKKIEKMGRNIRNGIVKA N-terminal CecB (minus the first lysine)

SC	PPC20	TIWKGVPKFLRRVDTALKNI linear cationic AH-AMP from phosphoenolpyruvate carboxylase (PDBid:3ZGBA)
SC	CHITI25	TAYGIMARQPNSRKSFIDSSIRLAR CecB-like AH-AMP from chitinase *Nicotiana tabacum* (PDBid:3ALGA)
SC	ISS15	TLDELELFTDAVERW linear anionic peptide from isoprene synthase from gray poplar (PDBid:3N0FA)

4.4.2. SCALPEL: Identifying native AH-AMPs from the host proteome

Linear AH-AMPs. In order to choose a peptide mimicking CBNT21 (cationic, amphipathic, with large hydrophobic moment), we directed our search to 'locate a small peptide with a large hydrophobic moment and a high proportion of positively charged residues' on the raw data computed using PAGAL. A small peptide is essential for quick and cost effective iterations. Table 2 shows the best matching AHs. Next, we used the sequence of these AHs to search the grapevine and citrus genomes, choosing only those that are present in both genomes. This allowed us to locate an AH from phosphoenolpyruvate carboxylase from sunflower, a key enzyme in the C4-photosynthetic carbon cycle which enhances solar conversion efficiency (PDBid:3ZGBA.α11) (PAULUS; SCHLIEPER; GROTH, 2013). Figure 2a shows the specific AH located within the protein structure, marked in green and blue. Although DSSP marks the whole peptide stretch as one AH, we chose the AH in blue due to the presence of a small π helix preceding that. We named this peptide PPC20 (Figure 2, Table 1). This peptide is fully conserved (100% identity in the 20 residues) in both grapevine (Accession id:XP_002285441) and citrus (Accession id:AGS12489.1). Figures 2b and 2c show the Pymol rendered AH surfaces of PPC20. The Asp259 stands out as a negative residue in an otherwise positive surface (Figure 2c). Since previous studies have noted dramatic transitions with a single mutation on the polar face, it would be interesting to find the effect of mutating Asp259 to a cationic residue (JIANG et al., 2008).

Table 2. Identifying AHs with cationic properties from plant proteins with known structures. All AHs in plant proteins are analyzed using PAGAL, and the data is pruned for AHs with a high proportion of positive residues, and finally sorted based on their hydrophobic moment. The first match is present in both grapevine and citrus (PDBid:3ZGBA.α11, which is a phosphoenolpyruvate carboxylase from sunflower). A small π AH is ignored in the beginning of this peptide comprising four residues. This peptide has been named PPC20. Len: length of α; HM: hydrophobic moment; RPNR:

relative proportion of positive residues among charged residues; NCH: number of charged residues.

PDB.α	Len	HM	RPNR	NCH
3ZGBA.α11 (PPC20)	24	12.6	0.8	8
4HWIA.α10	17	12.3	0.9	9
4BXHB.α11	23	12.3	0.8	8
2J376.α1	18	10.5	0.9	8
3J61R.α4	21	10.4	0.9	10
3J60G.α3	44	10.2	0.8	22
1W07A.α4	21	9.9	0.8	10
2WWBM.α1	17	9.5	0.9	8
1B8GA.α17	27	7.3	0.9	11
3J61L.α1	19	7.2	1	9

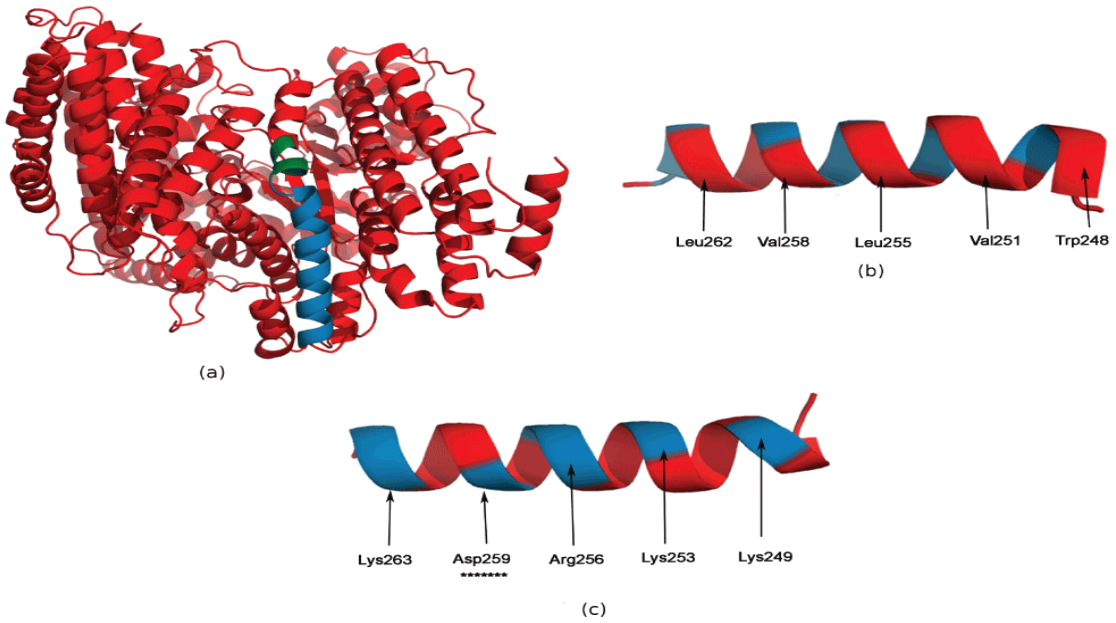

Figure 2. Peptide PPC20 from phosphoenolpyruvate carboxylase in sunflower (PDBid:3ZGBA.α11). **(a)** 3ZGBA.α11 is marked in green and blue. The π AH and the small AH preceding it (marked in green) were ignored. PPC20 is marked in blue. **(b)** hydrophobic surface of PPC20. **(c)** charged surface of PPC20. Asp259 stands out as a negative residue in an otherwise positive surface.

Non-linear AH-AMPs consisting of two AHs. Next, we located two AHs within chitinase from *Nicotiana tabacum* (PDBid:3ALGA.α4 and 3ALGA.α5) (OHNUMA et al., 2011) connected by a short random coil such that one of the AHs is cationic and hydrophobic, while the other AH is comprised mostly of hydrophobic, uncharged residues (CHITI25, Figure 3a, Table 1). This peptide mimics the complete CecB protein (Figure 3b). While the properties of the AHs in CHITI25 are reversed from that of CecB, we speculate that the order in which these AHs occur is not important for functionality

due to the inherent symmetry in the structure of a two AH peptide if it is abstracted in terms of the position of the side chains.

The multiple sequence alignment of CHITI25 from grapevine, citrus, and tobacco is shown in Figure 3c. CHITI25 from tobacco is the most cationic (five), followed by citrus (four) and grapevine (three). Thus, it is possible that the antimicrobial properties of CHITI25 from grapevine would be lower than those of CHITI25 from tobacco. These peptides can be subjected to mutations to enhance their natural antimicrobial properties in such a scenario (WANG et al., 2014).

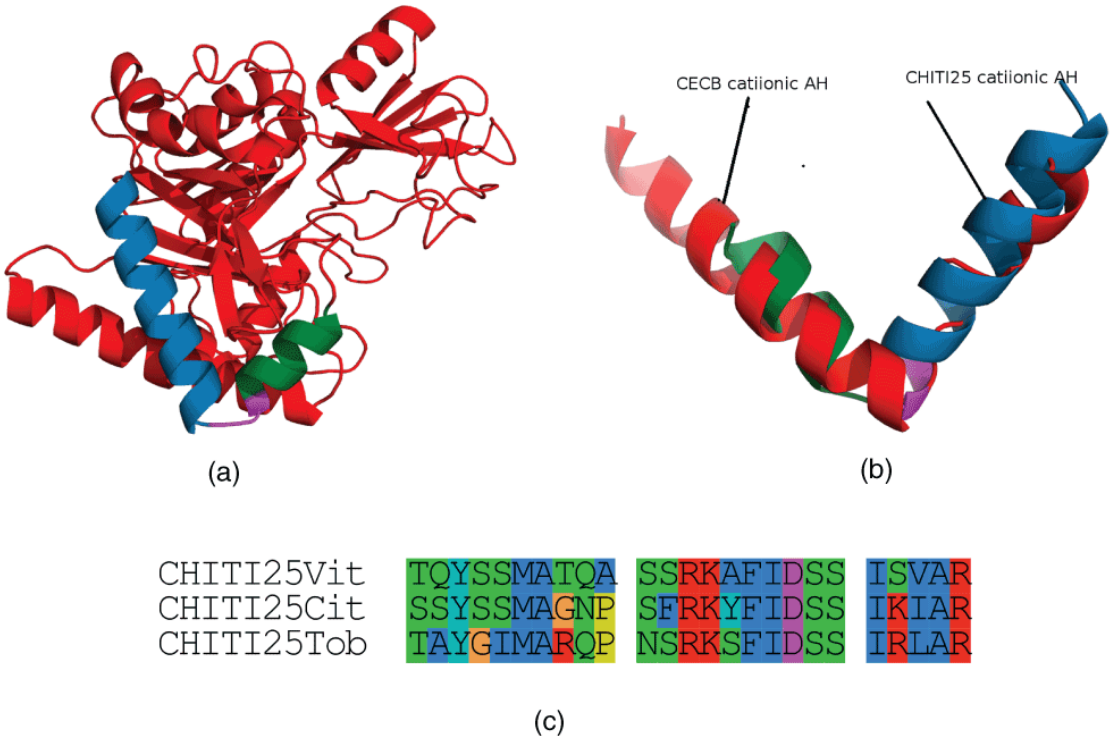

Figure 3. Peptide CHITI25 from chitinase in tobacco (PDBid:3ALGA). **(a)** PDBid:3ALGA.α4 in green, loop in magenta, and 3ALGA.α5 in blue. **(b)** superimposing CecB (PDBid:2IGRA) in red, with CHITI25 in green using MUSTANG (KONAGURTHU et al., 2006). Note, that the order of the AHs is reversed. **(c)** multiple sequence alignment of CHITI25 from grapevine (CHITI25Vit), citrus (CHITI25Cit) and tobacco (CHITI25Tob). CHITI25Tob is more cationic than CHITI25Vit or CHITI25Cit.

Negative control - an anionic AH-AMP. We also located an anionic AH-AMP using a similar strategy – a 13 residue peptide within the structure of isoprene synthase from gray poplar (*Populus* × *canescens*) (PDBid:3N0FA.α18) (KÖKSAL et al., 2010). We also used phosphate buffered saline as a negative control. We extended this helix on both terminals by including one adjacent residue from both terminals to obtain ISS15 (Table 1).

4.4.3. *In vitro* results

We have validated our peptides using plating assays (Table 3, Figure 4). CecB, the well-established AH-AMP, is the most potent among all the peptides tested, having minimum inhibitory concentrations of 25μM (for Xa) to 100μM (for Xf and Lc). This shows the variations in susceptibilities of different organisms. Understanding this differential susceptibility would require a deeper understanding of the underlying mechanism by which these AH-AMPs work (SHAI, 1999), as well as the difference in the membrane composition of these Gram-negative pathogens (KOEBNIK; LOCHER; VAN GELDER, 2000). Mostly, CBNT21 has a slightly lower potency, indicating a role for the C-terminal AH in CecB, which comprises of mostly hydrophobic residues, for Xf and Lc. These results corroborate a plausible mechanism suggested by others in which the anionic membranes of bacteria are targeted by the cationic N-terminal, and followed by the insertion of the C-terminal AH into the hydrophobic membrane creating a pore. PPC20 and CHITI25 have comparable potencies with CecB and CBNT21, although Lc appears to be resistant to CHITI25. Finally, the anionic peptide used as a negative control shows no effect on these pathogens.

Table 3. Minimum inhibitory concentration of peptides tested (μM). CecB is the most efficient among all the peptides for all three pathogens, while the anionic ISS15 does not show any effect even at higher concentrations. However, while CHITI25 is almost as effective as CecB for Xf, it fails to inhibit Lc growth. Also, Xa is much more susceptible to these peptides compared to the other two pathogens. Finally, the anionic ISS15 has no effect on these pathogens. Data are identical across triplicates. NoAct: no activity detected in the maximum concentration used (300μM).

	Bacteria	**CecB**	**CBNT21**	**PPC20**	**CHITI25**	**ISS15**
γ Proteobacteria	*Xylella fastidiosa* 3A2	100	200	150	100	NoAct
	Xanthomonas arboricola 417	25	25	50	150	NoAct
α Proteobacteria	*Liberibacter crescens* BT-1	100	200	200	NoAct	NoAct

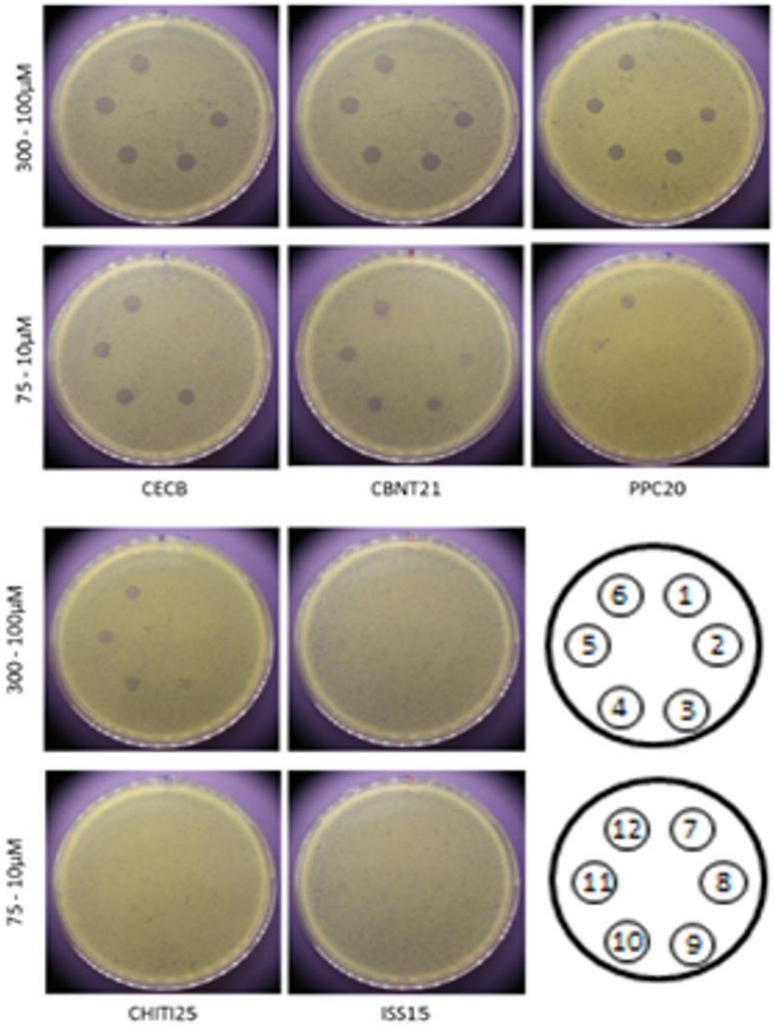

Figure 4. *In vitro* validation of SCALPEL methodology. Plating assay to determine minimum inhibitory concentration (MIC) of SCALPEL identified peptides for *Xanthomonas arboricola*. Counter-clockwise: (6) 300µM, (5) 250µM, (4) 200µM, (3) 150µM, (2) 100µM, (1) PBS, (12) 75µM, (11) 50µM, (10) 30µM, (9) 25µM, (8) 10µM, (7) PBS. CecB: MIC 25; CBNT21: MIC 25; PPC20: MIC 50; CHITI25: MIC 150; ISS15: no activity detected in the maximum concentration used (300µM).

4.5. Discussion

The repertoire of defense proteins available to an organism is being constantly reshaped through genomic changes that entail resistance to pathogens. Genetic approaches aim at achieving the same goal of enhancing immunity through rational design of peptides (HANCOCK; SAHL, 2006; ZEITLER et al., 2013), which are then incorporated into the genome (SHARMA et al., 2000; GRAY et al., 2005; DANDEKAR et al., 2012). Also, it is important to ensure that these non-endogenous genomic fragments have minimal effect on humans for their commercial viability (SHELTON; ZHAO; ROUSH, 2002). Identifying peptides from the same genome helps allay these concerns

to a significant extent. The key innovation of the current work is the ability to identify peptides with specific properties (cationic AHs with a hydrophobic surface, linear or otherwise) from the genome of any organism of interest. Such peptides also present less likelihood of eliciting an adverse immune response from the host.

4.5.1. Alternate methods

Alternate computational methods for finding such new AMPs based on known AMPs could be of two kinds, although neither method is as effective in obtaining our results. Firstly, a sequence search using BLAST can be done to find a corresponding peptide in the genome, say for cecropin B. However, a BLAST of the cecropin sequence does not give any significant matches in the grapevine or citrus genomes, and is a dead end. In principle, what we need is a peptide with cecropin B-like properties – and that information is not encoded in the linear sequence, but in the Edmundson wheel of the AH. The second method for such a search is to find structural homology in the PDB database through a tool like DALILITE (HOLM et al., 2008). However, AHs are almost indistinguishable structurally, and the results will give rise to many redundancies. Thus, there are no existing methods tailored to incorporate the quantifiable properties of AHs in the search. We, for the first time, have proposed such a method in SCALPEL.

Computer-assisted design strategies have also been applied in designing *de novo* AMPs (FRECER; HO; DING, 2004; FJELL et al., 2011). Other hand curated comprehensive databases for ‘storing, classifying, searching, predicting, and designing potent peptides against pathogenic bacteria, viruses, fungi, parasites, and cancer cells’ (WANG, 2013) do not enjoy the automation and vastness of available data elucidated in the SCALPEL methodology.

4.5.2. Limitations and future directions

There are several caveats to our study. We are yet to ascertain the hemolytic nature of the identified peptides and will be performing these experiments in the near future. In fact, the selective cytotoxicity against human cancer cells might be used as a substitute therapy in place of conventional chemotherapy (MADER; HOSKIN; 2006; DOUGLAS; HOSKIN; HILCHIE, 2014). The development of a selective peptide with anti-cancer cell properties has been a challenge (GASPAR; VEIGA; CASTANHO, 2013). Although we have not measured the lipid permeabilizing abilities of our peptides, a recent study has found that potency in permeabilizing bacteria-like lipid vesicles does not correlate with

significant improvements in antimicrobial activity, rendering such measurements redundant (HE; KRAUSON; WIMLEY, 2014). The electrostatic context of a peptide is known to have a significant bearing on its likelihood to display an AH structure. The ability to predict the folding of peptides requires significant computational power and modelling expertise (PIANA; KLEPEIS; SHAW, 2014). Peptides often remain in random coil conformations, and achieve helical structures only by interacting with anionic membrane models (MISHRA et al., 2013). It is also possible to measure peptide helicity through circular dichroism spectroscopy (HUANG et al., 2012). However, our results have been all positive based on selected choices of peptides arising from our search results, and suggest a high likelihood of getting antimicrobial activity from these peptides. Additionally, we may have to resort to other innovative techniques that have been previously adopted to overcome thermodynamic instability or proteolytic susceptibility (CHAPMAN; DIMARTINO; ARORA, 2004; HARRISON et al., 2010; BIRD et al., 2010; 2014).

4.6. Conclusion

In sum, we established the presence of a large number of AH-AMPs 'hidden' in the universal proteome. We designed a methodology to extract such peptides from the PDB database – the 'Big Data' center in proteomics. We demonstrated our results on well-known plant pathogens – Xf, Xa, and Lc. The feasibility of using such peptides in cancer therapies is also strong (DOUGLAS; HOSKIN; HILCHIE, 2014; TYAGI et al., 2015). The ability to choose a peptide from the host itself is an invaluable asset, since nativeness of the peptide allays fears of eliciting a negative immune response upon administration. The problem of antibiotic resistance is also increasing focus on peptide based therapies (HANCOCK; CHAPPLE, 1999; OYSTON et al., 2009), since it is "an enigma that bacteria have not developed highly effective cationic AMP-resistance mechanisms" (PESCHEL; SAHL, 2006). Lastly, in face of the current Ebola outbreak (PIOT, 2014a,b), we strongly suggest the possibility of developing peptides derived from the human genome to target viral epitopes, such as those enumerated for the Ebola virus recently (CHAKRABORTY et al., 2014). A recent study has reported the inhibition of the Ebola virus entry and infection by several cationic amphiphiles (SHOEMAKER et al., 2013), suggesting the SCALPEL generated cationic peptides with the aid of cell penetrating peptides (MONTROSE et al., 2013) could achieve similar results.

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CAPÍTULO 4

THE PLANT-DERIVED PEPTIDE PPC20 IS MORE POTENT THAN CECROPIN B AGAINST THE BACTERIAL PHYTOPATHOGEN *Ralstonia solanacearum* WITH LESS TOXICITY TO HUMAN CELLS

5 THE PLANT-DERIVED PEPTIDE PPC20 IS MORE POTENT THAN CECROPIN B AGAINST THE BACTERIAL PHYTOPATHOGEN *Ralstonia solanacearum* WITH LESS TOXICITY TO HUMAN CELLS

5.1. Abstract

The phytobacterium *Ralstonia solanacearum*, causative agent of bacterial wilt in several agronomically important crops, has limited disease management strategies in place. The negligible effect of well-established antimicrobial peptides (AMPs), like cecropin B (CecB), on this pathogen calls for the development of novel rationally-designed therapies. Also, the traditionally successful strategy of generating transgenic resistant lines faces severe criticism for using non-native peptides, like the moth-derived CecB. Previously, the antimicrobial properties of several alpha-helical (AH) cationic peptides (PPC20, CHITI25, etc) encoded by plant genomes have been validated against three plant pathogens (*Xylella fastidiosa*, *Xanthomonas arboricola*, and *Liberibacter crescens*). In the current work, the effect of these peptides, as well as other AMPs derived from human proteins, are determined on *R. solanacearum*. Remarkably, PPC20 (a linear AH-peptide within the existing structure of phosphoenolpyruvate carboxylase) has a three-fold improved MIC on *R. solanacearum* compared to CecB (25μM vs 75μM) and lower toxicity (20% vs 48%) on human intestinal epithelial cells. The length of the linear-AMPs seemed to impact the efficacy, exemplified by the ineffectiveness of the AMP CATH12, corresponding to residues 18 to 29 of cathelicidin (LL-37), on *R. solanacearum*. Thus, PPC20 can be a promising candidate as a novel defense mechanism expressed by transgenic lines designed to be resistant to bacterial wilt.

Keywords: phosphoenolpyruvate carboxylase; α-helical antimicrobial peptides; kill-curves; MTT cell viability assay; bacterial wilt.

5.2. Introduction

Antimicrobial peptides (AMPs) are important components of natural defenses of most living organisms against invading pathogens. These peptides are broadly classified into five major groups namely (a) peptides that form α-helical structures, (b) peptides rich in cysteine residues, (c) peptides that form β-sheet, (d) peptides rich in regular amino

acids namely histidine, arginine, tryptophan, and proline, and (e) peptides composed of rare and modified amino acids, like lanthionine, 3-methyllanthionine, dehydroalanine, and dehydrobutyrine (REDDY; YEDERY; ARANHA, 2004). Most of these peptides are believed to act by disrupting the plasma membrane leading to cell lysis.

Cecropin B (CecB) is an alpha-helical (AH) antibacterial peptide originally identified in moths (*Hyalophora cecropia*) and later in pig intestine. It exhibits a broad spectrum of antimicrobial activities against both Gram-positive and Gram-negative bacteria but is unable to lyse normal eukaryotic cells (SATO; FEIX, 2006). Its mechanism of action relies on the amphipathic, cationic α-helix at the N-terminal that targets the bacterial membrane and disturbs bilayer integrity either by disruption or by pore formation (WU et al., 2009; LIU et al., 2010). CecB is active *in vitro* against a wide range of plant pathogenic Gram-negative bacteria, including *Rhizobium radiobacter*, *Xylella fastidiosa*, *Xanthomonas vesicatoria*, *Pseudomonas syringae* (three pathovars), *Pectobacterium carotovorum* subsp. *carotovorum*, and *Dickeya chrysanthemi* (ALAN; EARLE, 2002; LI; GRAY, 2003; JAN; HUANG; CHEN, 2010; DANDEKAR et al., 2012).

Although the relative efficacy of lytic peptides in inhibiting *in vitro* growth of various pathogenic bacteria has been determined (MOORE et al., 1996; ALAN; EARLE, 2002), there is a lack of information on their activity against xylem-limited bacteria such as *Ralstonia solanacearum*. *R. solanacearum* is probably the most destructive plant pathogenic bacterium worldwide, causing bacterial wilt disease in several agronomically important crops. It infects plants through wounds, which can be minimal such as those caused by the emergence of secondary roots, by nematodes or insects (AGRIOS, 2005). The bacteria subsequently colonize the root cortex, invade the xylem vessels and reach the stem and aerial parts of the plant through the vascular system (SAILE et al., 1997; VASSE et al., 2000). *R. solanacearum* can rapidly multiply in the xylem up to very high cell densities, leading to wilting symptoms and plant death. Disease management remains limited and is hampered by the ability of the pathogen to survive for years in wet soil, water ponds, on plant debris, or in asymptomatic weed hosts, which act as primary inoculum source. Breeding for resistance, although effective in a few cases, is hampered by the broad diversity of the pathogenic strains (REMENANT et al., 2010).

As an alternative to the control of bacterial wilt, cloning and recombinant expression of AMPs in heterologous plant host systems can lead to the production of disease resistant transgenic lines. Although the antibacterial effect of many AMPs has

been proven *in vitro*, their utility in plant protection is limited due to relatively high inhibitory concentrations, sensitivity to salts, cytotoxic effects, and difficulty to ensure a useful antibacterial activity *in vivo* (HANCOCK, 1999; LIU et al., 2007). In addition to that, their efficacy in killing the pathogen seems to vary based on the length of the peptide, though poor agreement between studies has been observed (DESLOUCHES et al., 2005; NIIDOME et al., 2005; LIU et al., 2007; PUSHPANATHAN et al., 2013; SUN et al., 2014).

Due to the potential application of α-helical AMPs in controlling plant pathogenic bacteria, this study proposes a search on promising peptides against *R. solanacearum*, displaying low inhibitory concentrations and low toxicity to human cells. The search was set up to identify peptides that were either derived from plants or from human proteins, using a recently validated methodology (SCALPEL) (CHAKRABORTY et al., 2015). The activities of the candidates were compared *in vitro* to those of CecB.

5.3. Materials and methods

5.3.1. Peptide synthesis

AH-AMPs are often amphipathic (quantified by a hydrophobic moment), aligning hydrophobic residues on one surface and charged residues on the others. The hydrophobic moment of AHs (JONES; ANANTHARAMAIAH; SEGREST, 1992) has been computed using open access software (PAGAL) (CHAKRABORTY; RAO; DANDEKAR, 2014), using the hydrophobic scale from Engelman, Steitz and Goldman (1986). The method for choosing AH-AMPs has been detailed in Chakraborty et al. (2015). In summary, 'plant' and 'human' tagged proteins in the PDB database were analyzed using DSSP (JOOSTEN et al., 2011) to identify putative AHs. These data were refined to obtain peptides with different characteristics. In order to choose a peptide mimicking linear AH-AMPs, the search was directed to find a small peptide which had a large hydrophobic moment and a high percentage of positively charged residues. To obtain a peptide that mimics the complete CecB protein (which has two AHs), the search was modified to look for two α-helices connected by a short random coil such that one of the AHs is cationic and hydrophobic, while the other AH is comprised mostly of hydrophobic, uncharged residues (Table 1).

Table 1. Sequences of peptides used in this study. Underlined peptides are derived from human proteins.

Peptide	Amino acid sequence	Source[1]
Cecropin B	KWKVFKKIEKMGRNIRNGIVKA GPAIAVLGEAKAL	Derived from *Hyalophora cecropia* (P01508)
CBNT21	WKVFKKIEKMGRNIRNGIVKA	N-terminal helix of CecB (P01508)
PPC20	TIWKGVPKFLRRVDTALKNI	Phosphoenolpyruvate carboxylase (Q20GR6[2])
PPC20 Mut	TIWKGVPKFLRRVNTALKNI	Change of aspartic acid by asparagine (Q20GR6[2])
ACX23	PRKELFKNTLRKAAYAWKRIIEL	The flavoenzyme acyl-CoA oxidase (A0A075EYT4)
GST26	PQMIARSQDNARQKLRVLYQRAD AHL	Glutathione-S-transferase from *Xylella fastidiosa* (V8L135)
CHITI25 Cit	SSYSSMAGNPSFRKYFIDSSIKIAR	Derived from *Citrus* chitinase (A0A067DG30)
CHITI25 Vit	TQYSSMATQASSRKAFIDSSISVAR	Derived from *Vitis vinifera* chitinase (D7SSN5)
GDS17	SPARVVRAVGELAKAIG	Geranylgeranyl diphosphate synthase (Q43133)
ISS15	TLDELELFTDAVERW	Isoprene synthase (Q9AR86)
CCR25	IQRNVQKLKDTVKKLGESGEIKAI G	Cytokine receptor (Q9GZX6)
STK20	IKAVRSYSQQLFLALKLLKR	Serine/threonine-protein kinase PRP4 homolog (Q13523)
BCR16	QRMSRNFVRYVQGLKK	Blood clotting regulator (P04275)
CATH12	KRIVQRIKDFLR	Cathelicidin (P49913)

[1] The UniProtID from source protein is shown in parenthesis;
[2] Since the protein sequence from *Helianthus anuus* is not available at UniProt, identification number is from *Pyrostegia venusta* (100% identity).

Synthesized chemical peptides were obtained from GenScript USA, Inc. The protein molecular weight was calculated per peptide and then diluted to 1000µM stock solutions with phosphate buffered saline (PBS). Stock solutions were stored at -20°C and thawed on ice before use.

Dilutions of 300, 250, 200, 150, 100, 75, 50, 30, 25, and 10µM were made to a final volume of 100µL of 0.2µm filtered PBS. Dilute peptide solutions were stored at -20°C and thawed on ice before use.

5.3.2. Bacterial strain and growth conditions

Antimicrobial activity of the peptides was tested against the phytobacterium *Ralstonia solanacearum* strain GMI1000 (phylotype I, biovar 3, kindly provided by C. A. Lopes, Embrapa Hortaliças, Brazil). Twenty percent glycerol stocks were prepared

and stored at -80°C. When needed, the bacterium was streaked in Luria-Bertani (LB) medium (5g yeast extract, 10g tryptone, 10g NaCl, 15g agar per liter) incubated for 36-48h at 28°C and then transferred to LB broth to adjust cell cultures for assays as described below.

5.3.3. Spotting assay

LB 1.5% agar medium was prepared and autoclaved at 121°C, 1 atm for 20 minutes, then cooled and poured into 100 × 15mm sterile Petri dishes. *R. solanacearum* grown to exponential phase at 28°C, 190 rpm, was diluted to 10^7 colony forming units (CFU)/mL. Ten microliters of the bacterial suspension were mixed with 90µL of liquid medium and spread on the pre-made agar plates to create a confluent lawn of bacteria. The bacterium was given an hour to set at room temperature. Ten microliters of each peptide concentration was spotted onto a plate of agar preseeded with a layer of bacterium. After spotting, the plates were incubated at 28°C for two days until zones of clearance (haloes devoid of bacterial cells) were clearly visible and the plates were scored for the minimum inhibitory concentration (MIC) as that beyond which no visible clearance was observed. Data presented is representative of three rounds of independent plating, each with three plates.

5.3.4. Kill-curves

R. solanacearum was grown overnight and adjusted to 10^6 CFU/mL with LB broth. Selected peptides (PPC20, CCR25, STK20, CHITI25 Cit, CHITI25 Vit, and ACX23), chosen due to their lower MIC values compared to those of CecB (data obtained from the spotting assay), were added to the bacterial suspension at a final concentration of 50% of previously determined MIC, and incubated in a rotary shaker at 190 rpm 28°C. Aliquots were taken at 30 minute-intervals up to 2 hours, serially diluted with LB broth and plated. The number of colony forming units (CFU) was used to determine the efficiency of each peptide in clearing the pathogen. Three replicates for each treatment were performed.

5.3.5. Electron microscopy

R. solanacearum cells were fixed to bristles in 2.5% (v/v) glutaraldehyde for an hour and rinsed four times (10 minutes each) with cacodylate buffer 0,1M pH 7.2. Cells were secondary fixed in 1% (w/v) osmium tetroxide for ca. 1 hour at room temperature,

treated with 1% (v/v) tannic acid during 30 minutes, and rinsed twice with distilled water before being dehydrated in increasing concentrations of ethanol (50-100% [v/v]) for 10 minutes each. Samples were then washed twice in neat hexamethyldisilazane (Sigma-Aldrich, USA) for 15 minutes each. Bristles were attached to a scanning electron microscope stub using an adhesive carbon disc, and samples were gold-coated (≈25nm) before being examined using a Quanta FEG 250 scanning electron microscope (FEI, Amsterdam, The Netherlands).

5.3.6. Cytotoxic assay

In order to determine the peptides' toxicity, MTT cell viability assay [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] using the human intestinal epithelial cell line SK-CO15 (Sigma-Aldrich) was carried out with PPC20, CCR25, STK20, CHITI25 Vit, CHITI25 Cit, ACX23, CecB, CATH12, and PBS treatments. Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 0.1% streptomycin, and 10mg/mL penicillin (GIBCO) in 5% CO_2 atmosphere at 37°C. After reaching 80% confluence, cells were added to 96-well plates at a density of 1×10^6 cells/well and cultured in DMEM medium without FBS for 24h at 37°C, 5% CO_2 with each peptide in its minimum inhibitory concentration, determined in the spotting assay for *R. solanacearum*. After incubation, 10µL of MTT solution (5mg/mL) were added and cells were re-incubated for 4h. After this period, 50µL of a solution containing 20% SDS (sodium dodecyl sulfate) and 50% N,N-dimethyl formamide (pH 4.7) was added and incubated in the dark overnight. The amount of viable cells in each well was determined by the absorbance of solubilized formazan. Absorbance was measured in a wavelength of 570nm (Thermo Plate, TP-Reader).

5.3.7. Statistical analysis

All assumptions required for the analysis of variance (ANOVA) were confirmed. The error normality was evaluated by Shapiro-Wilk and the variance of homogeneity by Levene, both at 0.05 significance level. Subsequently, the data set was submitted to the ANOVA. When significant differences were detected, averages of peptides were compared by the Tukey test, and differences between treatment and control were analyzed using the Dunnett test. All analyses were performed at 0.01 significance level.

5.4. Results and discussion

Antimicrobial peptides have been considered powerful compounds for plant protection due to their antiviral, antifungal, and antibacterial activities (BROGDEN, 2005; MONTESINOS, 2007; KEYMANESH; SOLTANI; SARDARI, 2009). Among known AH-AMPs, CecB has been intensively studied. Results from *in vitro* and leaf disk assays show that growth of bacterial organisms was retarded or completely inhibited by low concentrations of this lytic peptide (ALAN; EARLE, 2002; LI; GRAY, 2003; JAN; HUANG; CHEN, 2010; DANDEKAR et al., 2012). Furthermore, grapevines, tomato, tobacco, and potato plants engineered to express cecropins and cecropin derivatives and chimeras (JAYNES et al., 1993; HUANG et al., 1997; ARCE et al., 1999; OSUSKY et al., 2000; JAN; HUANG; CHEN, 2010; VUTTO et al., 2010; DANDEKAR et al., 2012) suggest their use as transgenes to generate plant lines with enhanced resistance to bacterial and fungal diseases. However, information is scarce regarding the efficacy of such peptides on xylem-restricted pathogens.

R. solanacearum is a worrisome vascular bacterium capable of infection of hundreds of vegetable species, causing bacterial wilt. Although some studies report the efficacy of CecB in controlling *R. solanacearum* (JAYNES et al., 1993; JAN; HUANG; CHEN, 2010), results are conflicting (ALAN; EARLE, 2002). These authors found that CecB treatment led to a delay or complete inhibition of the growth of several bacterial organisms (belonging to the genera *Pseudomonas*, *Xanthomonas*, *Pectobacterium*, and *Dickeya*), but showed no effect in the growth rate of *R. solanacearum*.

Regarding transgenic plants, resistance to bacterial wilt due to a protein of insect origin is potentially controversial to groups opposed to GMOs. Therefore, substituting CecB by plant-derived components could help alleviate this potential concern. This study resorted to a validated methodology (SCALPEL) (CHAKRABORTY et al., 2015) to identify α-helical AMPs that mirror the function of CecB in order to select promising candidates derived from plant proteins to control the bacterial pathogen *R. solanacearum*.

5.4.1. Antimicrobial activity

To determine whether the selected peptides (Table 1) inhibit bacterial growth, different concentrations of each were spotted on the surface of LB plates previously seeded with *R. solanacearum* (Figure 1). Inhibition haloes indicating no bacterial growth, making the minimum inhibitory concentration (MIC), were scored. MIC values varied

among the tested peptides, from 25µM for PPC20 until 100µM for PPC20 Mut and BCR16. The negative control ISS15 did not show any effect even at higher concentrations neither did GDS17, CATH12, and PBS (Table 2).

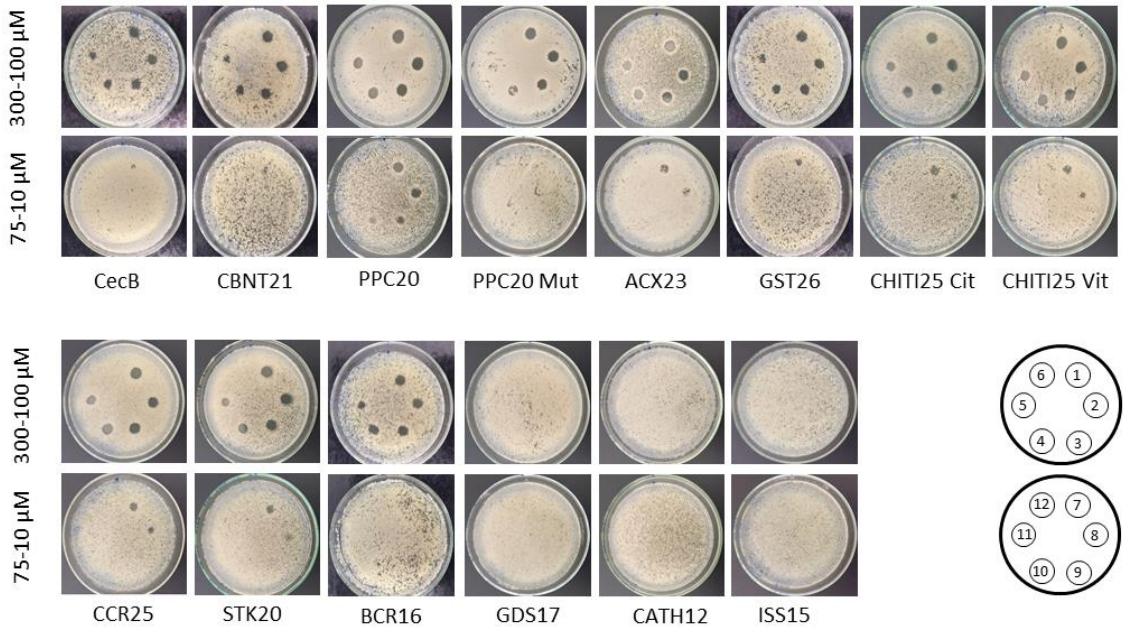

Figure 1. Plating assay to determine minimum inhibitory concentration (MIC) of SCALPEL identified peptides for *Ralstonia solanacearum* (GMI1000). Clockwise: (1) 300µM, (2) 250µM, (3) 200µM, (4) 150µM, (5) 100µM, (6) PBS; (7) 75µM, (8) 50µM, (9) 30µM, (10) 25µM, (11) 10µM, (12) PBS.

Table 2. Minimum inhibitory concentration (MIC) values of AMPs. PPC20, ACX23, CHITI25 Cit, CHITI25 Vit, CCR25, and STK20 showed lower MIC than CecB. GDS17 and CATH12, in contrast, failed to inhibit *R. solanacearum* growth even at higher concentrations – like the anionic ISS15, which had no effect on this pathogen until the concentration of 300µM. Data were identical across triplicates.

Peptide	MIC value (µM)	Peptide	MIC value (µM)
CecB	75	CHITI25 Vit	50
CBNT21	75	CCR25	50
PPC20	25	STK20	50
PPC20 Mut	100	BCR16	100
ACX23	50	GDS17	ND
GST26	75	CATH12	ND
CHITI25 Cit	50	ISS15	ND

ND: not determined.

The efficacy of CecB and its analog (Shiva-1) in controlling *R. solanacearum* has already been reported (JAYNES et al., 1993; JAN; HUANG; CHEN, 2010). However, the concentration required to inhibit this bacterium was higher than the needed for other

phytobacteria (ALAN; EARLE, 2002) and even higher than the concentration of ampicillin and kanamycin (JAN; HUANG; CHEN, 2010). Here, the identification of plant-derived peptides showing MIC values lower than CecB can potentially indicate new therapeutical options in the control of bacterial wilt disease.

Compared to CecB, a well-studied α-helical AMP used as the positive control in this study, six peptides were more efficient in inhibiting *Ralstonia* growth. All of them were subsequently tested in a kill-curve assay at half MIC to confirm their ability to clear the pathogen *in vitro* (Figure 2). Control treatment consisted of the bacterium growing in the presence of PBS. At different time points, during a 2-hour assay, a dilution of *R. solanacearum*/peptide mix was plated on LB agar plates and the number of colony forming units was recorded.

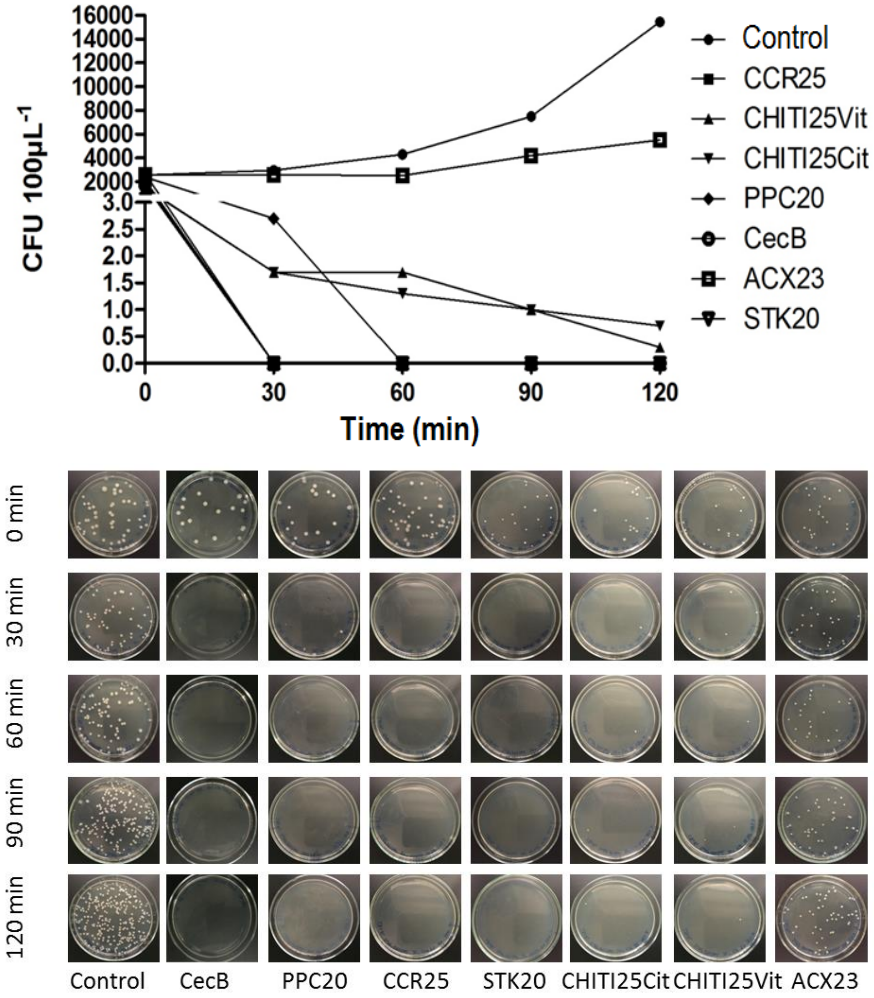

Figure 2. Kill-curves of selected peptides on *R. solanacearum*. Bacterial cells were incubated with 50% of peptides' MIC. CecB: 37.5µM; PPC20: 12.5µM; CCR25: 25µM; STK20: 25µM; CHITI25 Cit: 25µM; CHITI25 Vit: 25µM; ACX23: 25µM. CecB and PBS were considered positive and negative controls, respectively. Three replicates for each treatment were performed.

Within 30 minutes, CecB, CCR25, and STK20 demonstrated their efficacy for bacterial clearance as no colonies were formed. Despite a few colonies still being seen with 30-minute incubation, PPC20 completely cleared bacterial growth within 1 hour incubation at the lowest concentration tested (12.5µM). CHITI25 Cit and CHITI25 Vit had similar killing ability with a few resistant colonies growing on the plates. In contrast, ACX23 seemed to have a bacteriostatic effect over *R. solanacearum*, expressing a mortality percentage of 64.21 at the end of the experiment.

The α-helix structures of the peptides are essential for binding to and/or forming pore-like structures in targeted cell membranes (HRISTOVA; DEMPSEY; WHITE, 2001; FERRE et al., 2006; GLÄTTLI; CHANDRASEKHAR; VAN GUNSTEREN, 2006; OH et al., 2007; WANG et al., 2007; JI et al., 2010). Moreover, the cationic and hydrophobic characteristics of the antimicrobial peptides determine their mode of action and efficacy. Except for ISS15, all peptides tested have a positive net charge at physiological pH, as it is the case for most of the natural occurring AMPs (ZEITLER et al., 2013).

The varied efficacy of an AMP towards different prokaryotic pathogens possibly comes from differences in the phospholipid stoichiometry and architecture across different genera and species (YEAMAN; YOUNT, 2003). The increased potency of PPC20 (and decreased potency of CecB) towards *R. solanacearum* as compared to other plant pathogens (*X. fastidiosa*, *X. arboricola*, and *L. crescens*) (CHAKRABORTY et al., 2015) could be further studied by comparing the membrane composition and specific cell wall modifications in these pathogens. Subsequent to membrane binding and translocation, peptides diffuse into the cytoplasm to reach intra-cellular targets (FJELL et al., 2011). The differences in these targets could be another plausible reason for the variable killing ability of AMPs. Interestingly, smaller peptides had low (BCR16) or no activity (CATH12 and GDS17) on *R. solanacearum*. Known AMPs differ dramatically in size (from 12 to over 50 amino acids), sequence, and structure and share only amphipathicity and positive charge (HANCOCK, 1999; ZASLOFF, 2002). This lack of sequence or structural homology makes it challenging to design potent antimicrobial peptides with the desired activities or to predict their activity *in vitro* and *in vivo*. Potency and selectivity of an AMP can be enhanced by increasing peptide length (VOGEL et al., 2002), to a maximun of 24 residues beyond which no substantial increase in antimicrobial activity is observed (DESLOUCHES et al., 2005). However, it does not always hold true for all AMPs (NIIDOME et al., 2005; SUN et al., 2014).

Among tested peptides, PPC20 showed a MIC value three times lower than CecB. Therefore, their killing ability was compared at the same concentration, standardized to 50% of the MIC of PPC20 (12.5µM). This standardization made it possible to highlight the efficacy of PPC20 in controlling *R. solanacearum*. Under the same condition, a few bacterial colonies survived the CecB treatment whereas 100% mortality was achieved when cells were incubated with PPC20 (Figure 3).

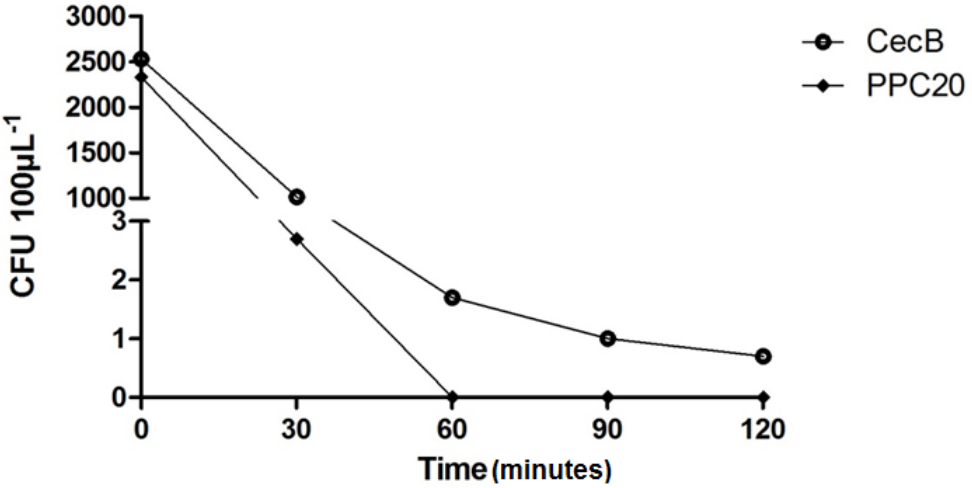

Figure 3. Comparison of antibacterial activity between CecB and PPC20 peptides. Kill-curves were standardized to 50% of the lowest MIC value, which corresponds to that of PPC20 (12.5µM).

PPC20 is a linear 20-amino acid α-helical AMP within the existing structure of phosphoenolpyruvate carboxylase from sunflower (PDBid:3ZGBA.α11) (PAULUS; SCHLIEPER; GROTH, 2013) (Figure 4a). It has hydrophobic residues aligned on one surface (characterized by a large hydrophobic moment) and a high proportion of positively charged residues, which is critical for its ability to permeabilize bacterial membranes (BROGDEN, 2005; HUANG; HUANG; CHEN, 2010) (Figure 4b). The Pymol rendered AH surfaces of PPC20 shows that Asp259 stands out as a negative residue in an otherwise positive surface (Figures 4b and 4c). The mutation of Asp259 to Asn259 generated PPC20Mut. However, this mutation had a negative effect on the antimicrobial efficacy (Table 2). The effect of PPC20 on lysing *R. solanacearum* and enabling it to clear the pathogen is shown in Figure 5. Pored cells with leaked intracellular content are formed, eliminating viable cells depending on PPC20 concentration.

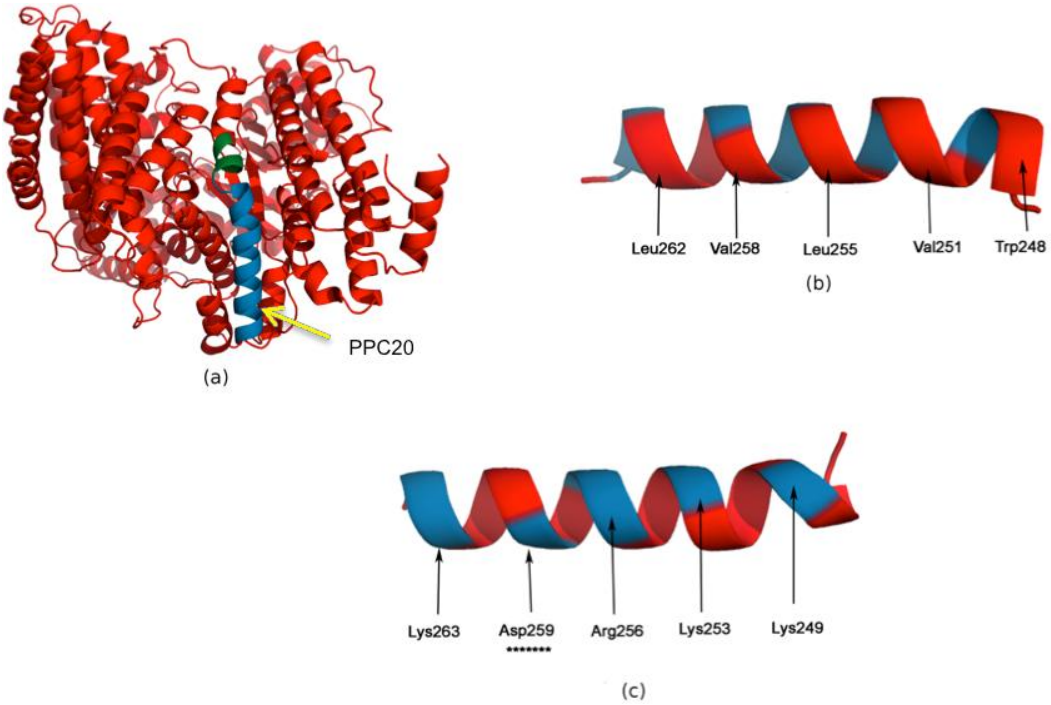

Figure 4. Peptide PPC20 from phosphoenolpyruvate carboxylase in sunflower (PDBid:3ZGBA.α11). **(a)** 3ZGBA.α11 is marked in green and blue. The π AH was ignored, and so was the small AH preceding it (marked in green). PPC20 is marked in blue. **(b)** hydrophobic surface of PPC20. **(c)** charged surface of PPC20. Asp259 stands out as a negative residue in an otherwise positive surface. Asp259 was mutated to Asn259 in order to generate PPC20Mut. Source: Chakraborty et al. (2015).

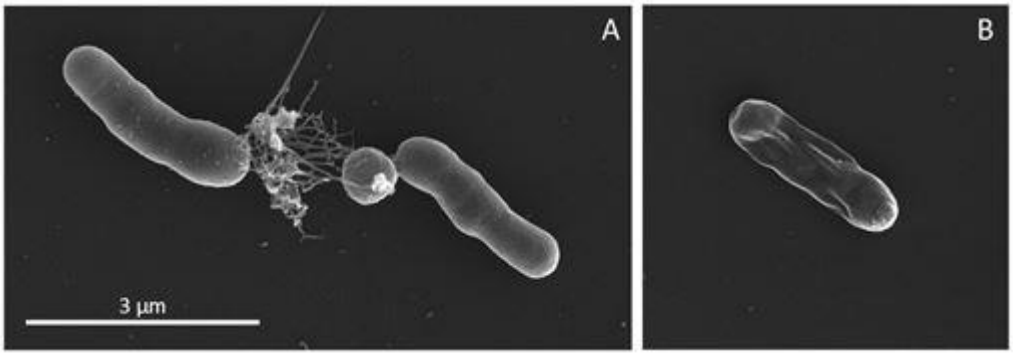

Figure 5. Bacteriolytic effect of PPC20 peptide on *Ralstonia solanacearum*. Scanning electron microscopy of bacterial cells in PBS with associated extracellular matrix and membrane vesicle (A), and cells treated with 25µM of PPC20 (MIC value) for 1 hour (B).

5.4.2. Human cell viability assay

Since humans would be exposed to these peptides during agricultural applications or by transgenic plants, low toxicity of AMPs against human cells is an initial barrier to further applications. Therefore, the cytotoxic activity of the elected peptides was tested in human intestinal epithelial cells incubated with each peptide at 100% of their MIC. CCR25 and CHITI25 Cit were lytic to human cells, whereas PPC20 retained high levels

of cell viability (80.33%), exhibiting lower toxicity even compared to CecB (52.33%) (Figure 6). Peptide length may be associated with toxicity, as demonstrated by Liu et al. (2007), who found that the antimicrobial activity of the peptides increased with chain length as did the hemolysis of red blood cells.

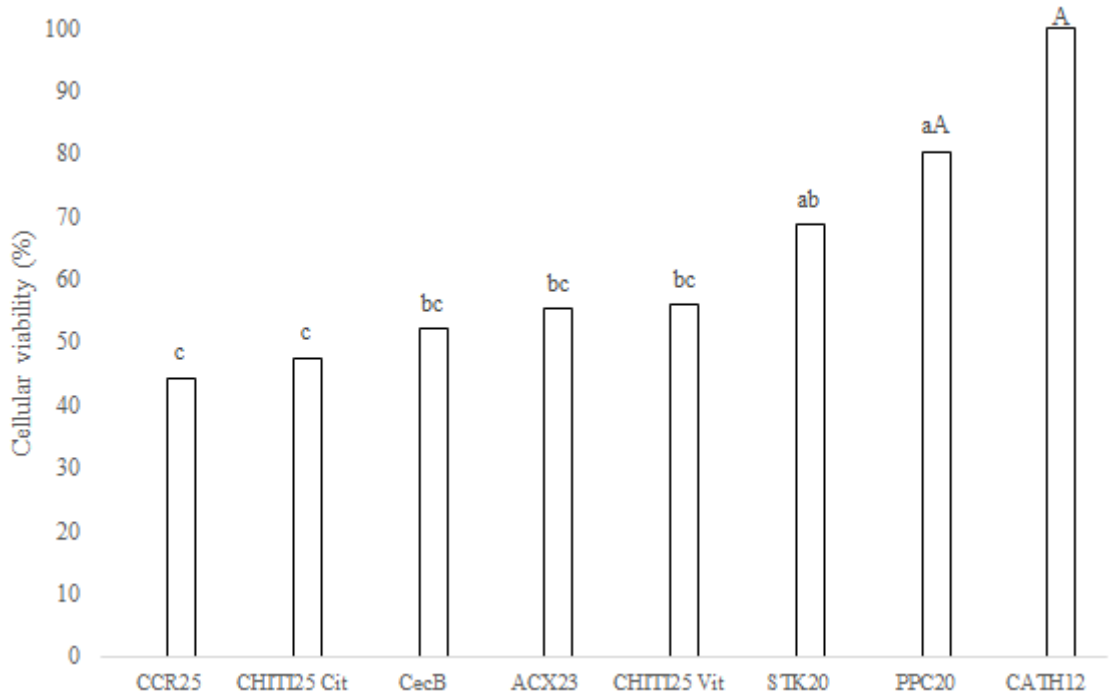

Figure 6. Human cell viability assay to determine cytotoxic activity of selected peptides. Different lowercase letters, for each peptide, are statistically different by the Tukey test ($P \leq 0.01$). Uppercase letters show no statistical difference between PPC20 and CATH12 by the Dunnett test ($P \leq 0.01$).

CATH12 was derived from the human cathelicidin protein, comprising the residues 18 to 29 of LL-37. Cathelicidins are a group of antimicrobial peptides that, besides antibacterial, antifungal, and antiviral functions, feature chemotactic and immunostimulatory/modulatory effects (VANDAMME et al., 2012). It was expected that this peptide would not jeopardize the viability of the cell line SK-CO15. Indeed, cells reached maximum viability (100%) under this treatment. Since CATH12 did not inhibit *R. solanacearum* growth, as hypothesized due to its short length, MTT cell viability assay was performed using the same MIC of PPC20. This standardized condition implied the effect of PPC20 peptide on human cells does not differ from that of CATH12, reinforcing its potential use in transgenic plants.

The reasons why certain AMPs have greater antimicrobial properties, show different MICs for a given pathogen, and feature varied efficacy across different

pathogens, remain obscure and controversial even after decades of research (WIMLEY; HRISTOVA, 2011), especially concerning aspects of their mechanism of action. Practical AMP design is tailored to maximize the action of AMPs on a certain pathogen and minimize it on human (mammalian) cells. The fundamental premise of the action of cationic amphipathic peptides, the focus of this study, is their affinity to outwardly oriented anionic phospholipids of bacterial membranes, absent in mammalian membranes (EPAND; VOGEL, 1999). Thus, expectedly ISS15 shows no effect on the tested pathogen (CHAKRABORTY et al., 2015). At the same time, it was surprising to find CCR25 having significant cytotoxicity, since it was a human-derived peptide, although longer peptides are reported to stimulate toxicity to mammalian cells (DONG et al., 2012).

5.5. Conclusions

In plant protection, bacterial infections are hard to overcome, considering that plant disease control is mainly based on the application of chemical pesticides, which are under strong restrictions and regulatory requirements. As an alternative, AMPs have been proposed in agriculture as a new avenue to control microbial diseases that are still challenging to combat, such as bacterial wilt caused by *Ralstonia solanacearum*.

The characteristic properties of a peptide like CecB that endows its antimicrobial properties is not encoded in the linear sequence or its α-helical structure, but can be extracted from the Edmundson wheel (SCHIER; EDMUNDSON, 1967). SCALPEL is tailored to incorporate the quantifiable properties of Ahs – amphipathicity and hydrophobicity – in the search for such peptides. The native structures from the same organism, as chosen through SCALPEL, could possibly ensure that administration of such peptides will be better tolerated and not elicit an adverse immune response. However, this aspect is yet to be demonstrated.

The most promising candidate selected in this study is an alpha-helix derived from a plant protein, phosphoenolpyruvate carboxylase (PPC20). PPC20 was more potent than CecB against *R. solanacearum*, simultaneously showing lower toxicity to human cells. Besides being a possible compound for use in pesticides, PPC20 displays a promising alternative for practical use of antimicrobial peptides in plant protection by generation of transgenic crops resistant to bacterial wilt.

Acknowledgments

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CAPÍTULO 5

EXPRESSION OF A CHIMERIC ANTIMICROBIAL PROTEIN IN TRANSGENIC TOMATO CONFERS RESISTANCE TO THE PHYTOPATHOGEN *Ralstonia solanacearum*

6 EXPRESSION OF A CHIMERIC ANTIMICROBIAL PROTEIN IN TRANSGENIC TOMATO CONFERS RESISTANCE TO THE PHYTOPATHOGEN *Ralstonia solanacearum*

6.1. Abstract

Plant biotechnology offers the possibility to improve field yield and safety of economically important crops without altering cultivar identity. Recently, research interest on antimicrobial peptides has increased because of their broad range activity, resulting in several biotechnological applications addressed to plant protection. The present study taps into the *in vitro* characterization of a chimeric protein and its potential use for development of transgenic tomato plants with resistance to a bacterial pathogen. The chimera was designed based on the NE-CecB antimicrobial protein, which has been previously validated on the plant pathogen *Xylella fastidiosa*. Each domain was substituted by homologous genes found in plant genomes, comprising a pathogenesis-related protein (SlP14a) joined to a plant-derived cecropin B-like peptide (an alpha-helix from phosphoenolpyruvate carboxylase – PPC20). *In vitro* antibacterial activities of SlP14a and SlP14a-PPC20 were confirmed in kill-curves assays against the bacterial wilt pathogen *Ralstonia solanacearum*, suggesting their use as promising candidates in plant protection. Later, tomato plants were engineered to express SlP14a-PPC20 chimera and challenged with *R. solanacearum* in an attempt to increase disease resistance. Transgenic and control plants reacted differently when inoculated with the pathogen. Within control plants (wild-type *Solanum lycopersicum* cv. MoneyMaker), disease evolved from wilting symptoms to plant death in two weeks. SlP14a-PPC20-transgenic plants, however, showed no symptoms or reduced disease severity. Bacterial multiplication in stems of transgenic plants was suppressed more than 2-fold compared to control plants, and absence of disease symptoms development could be associated with this growth suppression. In conclusion, SlP14a-PPC20 has an *in vivo* antibacterial activity representing an alternative strategy for the development of resistant tomato varieties.

Keywords: genetic engineering, disease resistant plants, cecropin B, therapeutic antimicrobial protein, agricultural biotechnology, genetically modified organism (GMO).

6.2. Introduction

Bacterial wilt, caused *by Ralstonia solanacearum*, is considered one of the world's most destructive plant vascular disease. The bacterium is a quarantine pathogen in many European countries (OEPP/EPPO, 2004) and a Bioterrorism Agent in the United States (USDA, 2012). In Brazil, *R. solanacearum* occurs in all states (MORAIS et al., 2015), compromising yields of agriculturally important crops and condemning growing fields, especially those dedicated to the certification of potato seeds.

Control of bacterial wilt is difficult, since the pathogen can survive for several years in infested soil and weeds. Plant breeding for resistant cultivars, considered an important control strategy for this bacteriosis, is troublesome due to the lack of good resistance sources among the vegetable species and the genetic diversity of the pathogen (LOPES, 2005; REMENANT et al., 2010). Hence, exploring the inherited ability of plants to overcome biotic stresses, combined to genetic engineering, may provide a promising alternative for bacterial wilt control.

Antimicrobial peptides (AMPs) and proteins are part of the host resistance response, leading to constitutive as well as induced resistance against diverse infections. These proteins can be delivered rapidly after infection with a limited input of energy and can efficiently repel pathogenic invaders (HANCOCK; DIAMOND, 2000; HANCOCK, 2001). Introduction of genes encoding small antimicrobial proteins into plants has been proved effective in enhancing resistance to both bacterial and fungal pathogens (MONTESINOS, 2007; RAMADEVI; RAO; REDDY, 2011; BREEN et al., 2015; HOLÁSKOVÁ et al., 2015).

The synergistic combination of two innate immune functions has already been demonstrated, namely: 1) pathogen surface recognition and 2) pathogen lysis, in a single protein, provide a robust class of antimicrobial therapeutics (DANDEKAR et al., 2012). In support of this idea, expression of a chimeric antimicrobial protein that links two bioactive protein domains, one from human neutrophil elastase (NE; bacterial surface recognition domain) and Cecropin B from insects (CecB; lytic domain) linked by a flexible hinge, has been shown to confer resistance to Pierce's Disease (PD) in grapevine (DANDEKAR et al., 2012). Transgenic grapevine lines expressing the NE-CecB chimeric protein has shown intensive reduction or no PD symptoms: less xylem blockage and leaf scorching. However, a major concern is that the presence of a protein of human and insect origin in plants is potentially controversial to groups opposed to GMOs.

Therefore, substituting NE and CecB by plant-derived components could help alleviate this potential aversion.

This study seeks to swap the human NE domain and the CecB lytic domain with equivalent proteins from plant sources and confirm whether the new chimera functions as effectively as the NE-CecB. The validation of this novel antimicrobial chimera as a biocontrol agent was accomplished by using bioassays, introducing it into tomato plants by transgenesis, and assessing the level of pest resistance it entailed.

6.3. Materials and methods

6.3.1. Synthesis and construction of SlP14a and PPC20 genes

Using bioinformatic tools (CHAKRABORTY et al., 2011; 2013; CHAKRABORTY; RAO, 2012), a putative plant elastase candidate protein was identified from tomato that had a similar active site configuration as NE. The search for the precise active site motif was created from the human NE protein PDBid:1B0F (Figure 1a). The active site residues consist of the following residues: Ser195, His57, Asp102, Ser214, and Gly193 (CHAKRABORTY et al., 2013). Preliminary results yielded a significant match in a member of the PR-1 group of pathogenesis-related proteins in *Solanum lycopersicum* (tomato) (Figures 1b and 1c), the protein P14a (PDBid:1CFE), a protease associated with the pathogenesis-related proteins (MILNE et al., 2003). Furthermore, a striking structural homology was found to be shared between P14a and a protein found in snake venom, which has been demonstrated to be an elastase (BERNICK; SIMPSON, 1976). Acronym SlP14a was used to denominate this putative plant elastase candidate.

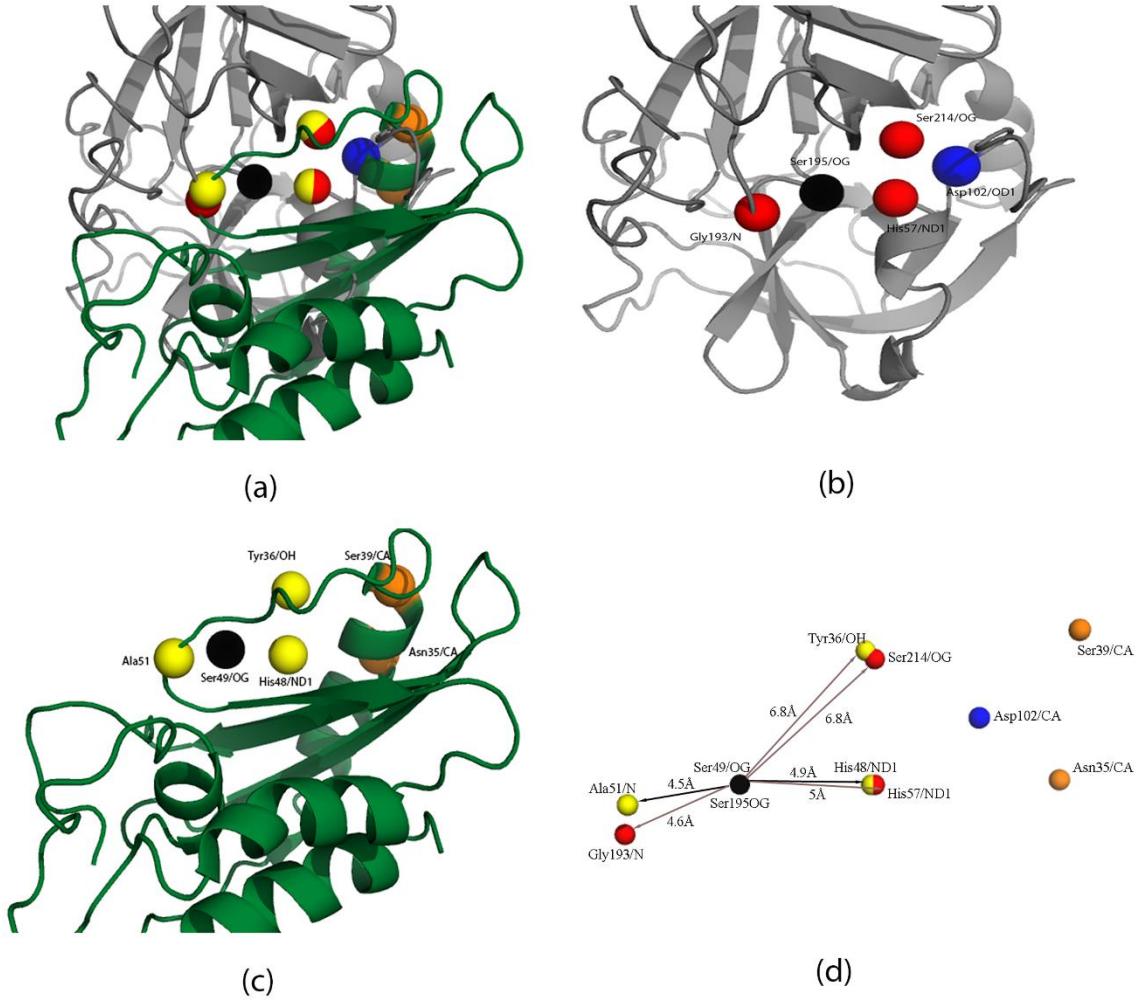

Figure 1. Superimposing proteins based on partial matches (Ser195, His57, and Ser214 from PDBid:1B0F) and (Ser49, His48, and Tyr36 from PDBid:1CFE). **(a) s**uperimposition of proteins based on partial matches: NE is in grey and P14a is in green. **(b)** human neutrophil elastase (NE) (PDBid:1B0F) with active site atoms – Ser195/OG, His57/ND1, Ser214/OG, Gly193/N. **(c)** P14a protein (PDBid:1CFE) with predicted active site residues – Ser49/OG, His48/ND1, Tyr36/OH, Ala51/N. **(d)** distance between pairs of residues in the partial matches. Asp102 in PDBid:1B0F is close to Asn35 and Ser39 in PDBid:1CFE. Source: Chakraborty (2012).

A similar approach using the mentioned bioinformatic tools was conducted to identify an appropriate plant component that had the same 3D structure and biochemical activity as CecB. This methodology has been previously detailed in Chakraborty et al. (2015). Briefly, a choice was made to limit the study to the structural motifs Lys10, Lys11, Lys16, and Lys29 from CecB (PDBid:2IGR) (Figure 2). The CLASP (CataLytic Active Site Prediction) analysis yielded a list of significant matches. Among all candidates listed from this search, an alpha-helix derived from phosphoenolpyruvate carboxylase was elected, named PPC20 (Figure 3). This peptide is fully conserved (100% identity in the 20 residues) in tomato (Accession id:XP_004248242). PPC20 has

previously shown *in vitro* antibacterial activity against plant pathogens *Xylella fastidiosa*, *Xanthomonas arboricola*, and *Liberibacter crescens* (CHAKRABORTY et al., 2015). It was considered a promising antimicrobial peptide in the control of *R. solanacearum* (data unpublished).

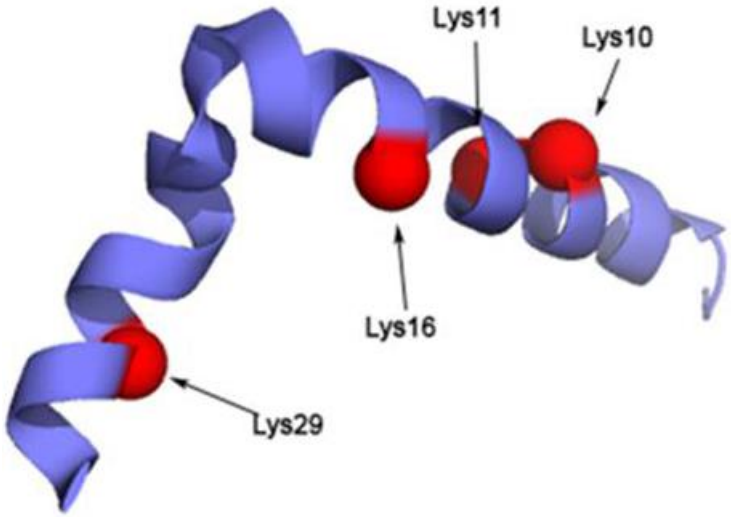

Figure 2. Cecropin B structure (CecB; PDBid:2IGR) showing chosen motifs (Lys10, Lys11, Lys16, and Lys29).

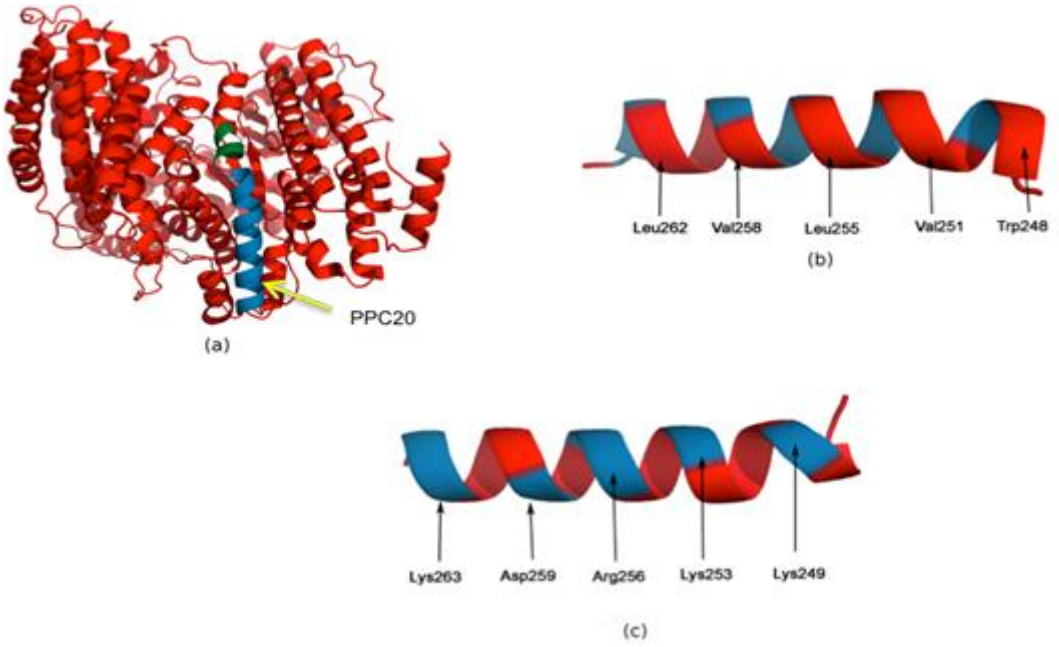

Figure 3. Peptide PPC20 from phosphoenolpyruvate carboxylase (PDBid:3ZGBA.α11). **(a)** 3ZGBA.α11 is marked in green and blue. The π alpha-helix (AH) was ignored, and so was the small AH preceding it (marked in green). PPC20 is marked in blue. **(b)** hydrophobic surface of PPC20. **(c)** charged surface of PPC20. Source: Chakraborty et al. (2015).

Selected candidates (SlP14a and PPC20) were screened through an EPA (US Environmental Protection Agency) regulatory search tool to ensure that they were not classified as a toxin or an allergen. Later, they were chemically synthesized (GenScript USA, Inc).

Cloning was performed according to In-Fusion® HD Cloning protocol (Clontech® Laboratories, Inc., Takara Bio Company, USA). The T-DNA portion of the expression construct is shown in Figure 4. The coding sequences (Figure 5) were downstream from the *Cauliflower mosaic virus* (CaMV) 35S promoter and upstream from an octopine synthase gene (*ocs*) 3'-UTR regulatory region required for proper polyadenylation. The expression cassette held an antibiotic resistant gene as the selection marker for plant transformation.

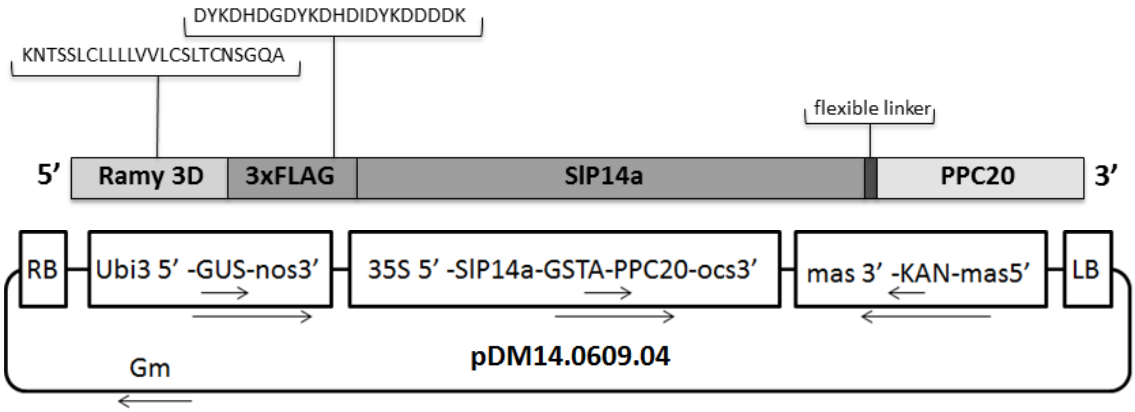

Figure 4. Gene layout for the chimera SlP14-PPC20. Components are linked using a flexible linker (glycine, serine, threonine, and alanine – GSTA), which sustains the correct folding of each domain. A 3xFlag enterokinase cleavable tag is added to enable easy detection and purification. Below, schematic diagram of the expression construct used for plant transformation. RB and LB: right and left borders, respectively; Ubi3: ubiquitin promoter; GUS: β-glucuronidase gene reporter; nos: nopaline synthase terminator; 35S: *Cauliflower mosaic virus* promoter; GSTA: flexible peptide linker; ocs: octopine synthase terminator; mas3' and mas5': manopine synthase promoter and terminator, respectively; KAN: kanamycin resistance gene.

(A)	**SlP14a**	MSWDANLASRAQNYANSRAGDCNLIHSGAGENLAKGGGDFTGR AAVQLWVSERPSYNYATNQCVGGKKCRHYTQVVWRNSVRLGCG RARCNNGWWFISCNYDPVGNWIGQRPY
(B)	**PPC20**	TIWKGVPKFLRRVDTALKNI

Figure 5. Amino acid sequence of selected candidates for (A) a putative plant elastase (SlP14a) and (B) a CecB plant homologue (PPC20).

The pDM14.0609.04 plasmid was introduced into *Rhizobium radiobacter* EHA105 by high voltage electroporation (WEN-JUN; FORDE, 1989) for plant transformation.

6.3.2. Bacterial strains and growth conditions

Plasmid cloning and amplification of SlP14a and PPC20 genes were performed in *Escherichia coli* strain DH5α. Plant pathogen, *R. solanacearum* strain GMI1000 (kindly provided by C. A. Lopes, Embrapa Hortaliças, Brazil), was cultured in Luria-Bertani (LB) medium (5g yeast extract, 10g tryptone, 10g NaCl, 15g agar per liter) at 28°C. *R. solanacearum* GMI1000 is a highly pathogenic strain classified as phylotype I, biovar 3, originally isolated from tomato plants from French Guyana. For plant transformation, *Rhizobium radiobacter* strain EHA105pCH32 was grown in LB medium supplemented with the antibiotics gentamicin, tetracycline, and rifampicin.

6.3.3. Protein expression in *E. coli*

For expression of recombinant proteins (SlP14a and SlP14a-PPC20), *E. coli* cells were cultivated overnight in LB medium containing 50µg/mL kanamycin at 37°C with shaking at 180 rpm. The induction of recombinant protein synthesis was performed at OD_{600nm} of 0.8 with 1mM IPTG. The recombinant synthesis was continued for three hours at 30°C with 180-rpm rotation.

Bacterial cells in LB medium were centrifuged at 8,000 rpm for 30 minutes. Cell pellets were stored at -80^{o}C before processing. After thawing, pellets obtained from 50mL of initial suspension were resuspended in 2mL of lysis buffer (Tris-HCl 1M pH 7.5, NaCl 5M, Lysozyme 10mg/mL, Glycerol, protease inhibitor cocktail [Thermo Scientific, USA]) and incubated for 10 minutes at room temperature on a shaking platform at high speed, followed by 20-minute incubation on ice with constant vortex. After lysis, cell lysates were cleared by centrifugation at 8,000 rpm for 30 minutes at 4°C. Supernatant was collected, and proteins were purified using Anti-Flag M2 Magnetic Beads (Sigma-Aldrich, USA). Western blot and a kill-curve assay were performed to confirm the presence of proteins and their antimicrobial activity, respectively.

6.3.4. Transient expression by agro-infiltration of tobacco leaves

R. radiobacter strains carrying plasmid constructs (SlP14a, SlP14a-PPC20, and Empty Vector – EV-pDU97.1005) were streaked on LB agar plates containing 20µg/mL

gentamicin, 10µg/mL tetracycline, and 50µg/mL rifampicin and incubated at 28°C for two days. For each construct, a single colony was then inoculated into LB broth with mentioned antibiotics and grown overnight at 28°C, 220 rpm. The cells were harvested in sterile microcentrifuge tubes by centrifugation (8,000 rpm for 2 min), washed twice in infiltration medium (10mM MES monohydrate, 10mM $MgCl_2$, pH 5.6 [KOH]) and resuspended in the same solution to a final OD_{600nm} = 0.6. Prior to infiltration, 100mM acetosyringone was added at a rate of 1:1,000.

Agro-infiltration was conducted by infiltrating agrobacterial suspensions (0.6 OD_{600nm}) into intercellular spaces of greenhouse-grown *Nicotiana benthamiana* leaves. A needleless plastic syringe was used to infiltrate bacterial suspensions into the abaxial side of leaves (three plants per construct). After infiltration, *N. benthamiana* plants were kept in room temperature.

Infiltrated leaves were harvested five days after infiltration and total protein was extracted. Briefly, leaves were frozen in liquid nitrogen and homogenized using a mortar and a pestle. The resulting powder was then resuspended in 50mM Tris-HCl pH 7.5, 75mM NaCl, 2mM EDTA, 1% Triton X-100, 5% Glycerol, protease inhibitor cocktail (Thermo Scientific, USA), and homogenized in a vortex. The homogenate was clarified by centrifugation at 8,000 rpm for 30 minutes at 4°C. Using Anti-Flag M2 Magnetic Beads (Sigma-Aldrich, USA), proteins were purified and their functional activity evaluated in an *in vitro* mortality assay.

6.3.5. *In vitro* antibacterial activities of SlP14a and SlP14a-PPC20: kill-curve

R. solanacearum was grown overnight and adjusted to 10^5 CFU/mL with LB medium. Purified proteins (SlP14a, SlP14a-PPC20 and Empty Vector, previously isolated from *E. coli* and from leaves of *N. benthamiana*) were added to the bacterial suspension and incubated in a rotary shaker at 28°C, 190 rpm. Aliquots were taken at 30-minute intervals up to 2 hours and serially diluted with LB broth. The titers were determined by counting the number of visible colonies per plate. Three replicates were performed for each treatment.

6.3.6. Measurement of protein concentrations

The concentration of total proteins was measured according to Bradford assay method, which involves reacting the samples with a dye that binds proteins. To measure protein concentration, standard solutions (Bovine Serum Albumin, Merck, Germany) and

protein samples were prepared and Bradford reagent was added according to manufacturer's instructions. The absorbance of samples and standard solutions were measured at 595nm after 10-minute incubation at room temperature. Protein extraction buffer was used as blank. A standard curve was prepared using the standard solutions absorbance, and the protein concentrations of samples were estimated.

6.3.7. Protease assay

Pierce Fluorescent Protease Assay Kit (Thermo Scientific, USA) was used to determine protease activity, following the instructions provided. Briefly, a fluorescein-labeled casein solution was prepared and incubated with the samples (1:1 vol/vol) at room temperature for 10 minutes. The fluorescence was measured in a microplate reader using a fluorescein excitation/emission filter set (485/538nm). The protease activities were compared to a standard curve and reported as picograms of trypsin per mL.

6.3.8. Western blot analysis

Proteins extracted and purified from *E. coli* cells and *N. benthamiana* leaves (Empty vector, SlP14a, and SlP14a-PPC20) were used for Western blot analysis. Protein samples were separated by 12.5% (wt/vol) SDS-PAGE and electro-transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corp., Burlington, MA). Immunodetection was performed using polyclonal anti-Flag antibody conjugated with peroxidase at 1:1,000 dilution for 3 hours. Antigen-antibody complexes were detected using ECL Plus Western Blotting Detection Reagents (GE Life Sciences, USA), and the images were recorded on X-ray film.

6.3.9. Plant transformation (tomato)

Tomato seeds (*Solanum lycopersicum* cv. MoneyMaker) were surface sterilized with a 0.05% (wt/vol) NaClO + 0.1% (vol/vol) Tween solution for 10 minutes and rinsed five times with sterile deionized water in order to prevent any growth of microorganisms while in culture. The sterilized seeds were germinated and grown on MSSV medium containing Murashige and Skoog (MS) salts, 3% (wt/vol) sucrose, Nitsch vitamins (THOMAS; PRATT, 1981), and 0.3% (wt/vol) phytagel, and maintained in a growth chamber with cool white fluorescent light (150µmol m^{-2} s^{-1}) under a 16/8h (light/dark) photoperiod at 25±2°C with a relative humidity of 55%. The pH of the MSSV medium was adjusted to 5.7 with potassium hydroxide prior to autoclaving for 20 minutes at

121°C. Cotyledon leaves from 7-day-old seedlings were excised. To stimulate the initial growth, the explants were preconditioned overnight with preculture medium (MS salt, 2% [wt/vol] sucrose, Gamborg B5 basal salt mixture with vitamins [GAMBORG; MILLER; OJIMA, 1968], 2mg/L benzylaminopurine, pH 5.7, 0.3% [wt/vol] phytagel, 0.25mg/L indole-3-acetic acid) at 25±2°C. The explants were then cocultivated with the overnight culture of *R. radiobacter* EHA105 containing the pDM14.0609.04 plasmid for 48 hours at 25±2°C in the dark. After being washed three times with sterile deionized water containing 50mg/mL kanamycin, the explants were incubated in regeneration medium (MS salt, Nitsch vitamins [NITSCH; NITSCH, 1969], 3% [wt/vol] sucrose, 100mg/L myo-inositol, pH 5.7, 2mg/L zeatin, 2mg/L kinetin, 0.3% [wt/vol] phytagel, 50mg/mL kanamycin) under a 16/8h (light/dark) photoperiod at 25±2°C and 55% humidity for the purpose of shoot induction.

6.3.10. Regeneration and selection of transgenic tomato plants

To select transformants, the explants were subcultured once a week in regeneration medium supplemented with 50mg/mL of kanamycin for a period of several weeks. The initial *callus* was observed at the site of wounding on the explants. When shoots appeared from the *calli* and were approximately 1-2cm long, they were separated and transferred into shoot formation medium (MSSV) supplemented with 50mg/mL kanamycin. The regenerated shoots were then transferred and grown in Magenta boxes for root induction (1/2 MS salt, LS vitamin [LINSMAIER; SKOOG, 1965], 3% [wt/vol] sucrose, 0.7g/L 2-n-morpholino-ethanesulfonic acid, pH 5.7, 0.3% [wt/vol] phytagel, supplemented with 50mg/mL kanamycin). The rooted plants were then transplanted into boxes containing commercial substrate. The tomato plants (T0) were grown in a growth cabinet (16h at 28°C and 8 hours in the dark at 23°C) in a mixture of peat-vermiculite-perlite (10:1:2 [vol/vol/vol]).

6.3.11. Confirmation of transgenic plants: DNA isolation and PCR analysis

Genomic DNA was isolated from leaf tissue of T0 and T1 tomato plants (obtained after T0 self-pollination) using a genomic DNA purification kit (Epicentre, Madison, WI, USA). Integration of the SlP14a-PPC20 gene into the plant genome was confirmed by PCR with the forward primer 5'-TGATGAGTCCTGCTTTAATGAG-3' and the reverse primer 5'-GGCTTCTTCCTTTCGACTGTAA-3', which amplified a 2.8Kb-fragment corresponding to the region from the 35S promoter to the ocs terminator. The following

program was run: initial denaturation for 2 minutes at 94°C, followed by 30 cycles of 1 minute at 94°C, 3 minutes at 66°C, and 1 minute at 72°C. The final extension step lasted 5 minutes at 72°C. All reactions had a final volume of 25µL and contained 1X Taq polymerase buffer (50mM KCl and 10mM Tris, pH 8.5), 1.5mM $MgCl_2$, 0.2mM of each dNTP, 0.5µM of each primer, 1 unit of Taq polymerase (Invitrogen Platinum Taq DNA Polymerase, Thermo Scientific), and 100ng of template. PCR products were analyzed on 1% (wt/vol) agarose gels, stained with ethidium bromide, and visualized under UV light.

6.3.12. *In vivo* plant bioassay for resistance to bacterial wilt

R. solanacearum GMI1000 was grown for 48 hours at 28°C on LB agar medium. The bacterial suspension for inoculation was prepared with 0.85% (wt/vol) NaCl and adjusted to 10^8 CFU/mL. Four-week-old tomato plants (wild-type and T1 transgenic plants) were inoculated by wounding the stems with an entomological needle which passed through a 10µL-drop of the bacterial suspension. Inoculated plants were kept in a growth chamber at 25±2°C. Disease progress and symptoms were then recorded after infection over a 14-day period. Disease readings were made according to the following numerical grades: 1: no symptoms; 2: leaf at the point of inoculation wilted; 3: two or three leaves wilted; 4: all except for the top leaves wilted; 5: completely wilted plants (WINSTEAD; KELMAN, 1952).

To evaluate bacterial multiplication in infected plants, stems were removed, weighed, surface sterilized, and macerated after 14 days of infection. Extracts were dispersed on LB agar medium. After incubation for 48 to 72 hours, bacterial growth was monitored by counting viable CFUs. The experiment was repeated twice and had each plate in triplicate.

6.3.13. Statistical analysis

All assumptions required for the analysis of variance (ANOVA) were confirmed. The error normality and the variance of homogeneity were evaluated by Shapiro-Wilk and Levene tests, respectively, both at 0.05 significance level. Subsequently, the data set was submitted to the ANOVA. Kill-curves assays were analyzed in a split-plot arrangement in which the main plot was the protein and the split-plot was the incubation time. When significant interaction was observed, complexes variances were applied. Averages of protein treatments, averages of incubation time, and differences between control and transgenic tomato plants were compared by the Tukey test, polynomial

regression, and the Dunnett test, respectively. All analyses were carried out at 0.05 significance level.

6.4. Results and discussion

6.4.1. Effectiveness of SlP14a and SlP14a-PPC20 against *R. solanacearum*

Diseases caused by viruses, bacteria, and fungi adversely affect the productivity of various crop plants, resulting in huge yield losses and decreased quality and safety of agricultural products. Among plant diseases, bacterial wilt, caused by the vascular pathogen *Ralstonia solanacearum*, is considered one of the most destructive (DENNY, 2006). Although many plant pathogens are narrowly adapted to one or a few related plant hosts, *R. solanacearum* has an unusually broad host range that includes monocotyledonous and dicotyledonous plants (HAYWARD, 1991). Means to control this disease are limited.

Antimicrobial peptides (AMPs) have been considered powerful compounds for plant protection in agriculture due to their activity against a broad range of pathogenic organisms (BROGDEN, 2005; MONTESINOS, 2007; KEYMANESH; SOLTANI; SARDARI, 2009). Over 1,700 natural AMPs have been identified, and thousands of derivatives and analogues have been computationally designed, engineered or synthetically generated using natural AMPs as templates (HOLÁSKOVÁ et al., 2015). The identification of AMPs for plant protection has the potential not only to improve resistance for better crop productivity, but also minimize the use of agrochemicals.

CecB has long been reported to possess *in vitro* lytic activity against several Gram-negative phytopathogens, such as *Rhizobium radiobacter*, *Xylella fastidiosa*, *Xanthomonas vesicatoria*, *X. arboricola*, *Pseudomonas syringae* (three patovars), *Pectobacterium carotovorum* subsp. *carotovorum*, *Dickeya chrysanthemi*, *Liberibacter crescens*, and *Ralstonia solanacearum* (ALAN; EARLE, 2002; LI; GRAY, 2003; JAN; HUANG; CHEN, 2010; DANDEKAR et al., 2012; CHAKRABORTY et al., 2015). The design of cecropin combined with other peptides as chimeras has made it possible to avoid cellular degradation by plant peptidases and to promote accumulation of sufficient levels of peptides in plants to resist pathogens (JAYNES et al., 1993; HUANG et al., 1997; OWENS; HEUTTE, 1997; OSUSKY et al., 2000). A NE-CecB chimeric protein, consisting of two bioactive protein domains – one from human neutrophil elastase (NE; surface recognition domain) and the other from insect (CecB; lytic domain) linked by a

flexible linker – has previously exhibited antimicrobial activity against the phytobacterium *X. fastidiosa*, providing transgenic grapevines resistance to this pathogen (DANDEKAR et al., 2012).

To confirm whether homologues of CecB and NE derived from plants would provide protection against a worrisome bacterial disease of tomato, the effectiveness of SlP14a and SlP14a-PPC20 expressed in *E. coli* and *N. benthamiana* against *R. solanacearum* (Tables 1 and 2, respectively) was investigated by a broth culture inhibition assay. Protein synthesis in both sources was previously confirmed by Western blot (Figure 6).

Table 1. Colony forming units (CFU) in 100µL^{-1} of *Ralstonia solanacearum* (GMI1000) after incubation of bacterial cells with antimicrobial proteins expressed in *E. coli*. Proteins were purified using Anti-Flag M2 Magnetic Beads (Sigma-Aldrich, USA).

Protein	**Time (minutes)[1]**				
	0	**30**	**60**	**90**	**120**
Empty Vector	4433.33 a	5033.33 a	4666.67 a	12000.00 a	19000.00 a
SlP14a	4466.67 a	5713.33 a	6486.67 a	7520.00 b	11080.00 b
SlP14a-PPC20	4666.67 a	5200.00 a	7466.67 a	9213.33 ab	11960.00 b

[1] averages followed by different letters, in each column, are statistically different by the Tukey test at 0.05 significance level.

Table 2. Colony forming units (CFU) in 100µL^{-1} of *Ralstonia solanacearum* (GMI1000) after incubation of bacterial cells with antimicrobial proteins expressed in *N. benthamiana*. Total protein was extracted from agro-infiltrated leaves five days after infiltration. Proteins were purified using Anti-Flag M2 Magnetic Beads (Sigma-Aldrich, USA).

Protein[1]	**CFU 100µL^{-1}**
Empty Vector	5786.67 a
SlP14a	4265.33 b
SlP14a-PPC20	4624.67 b

[1] averages followed by different letters are statistically different by the Tukey test at 0.05 significance level.

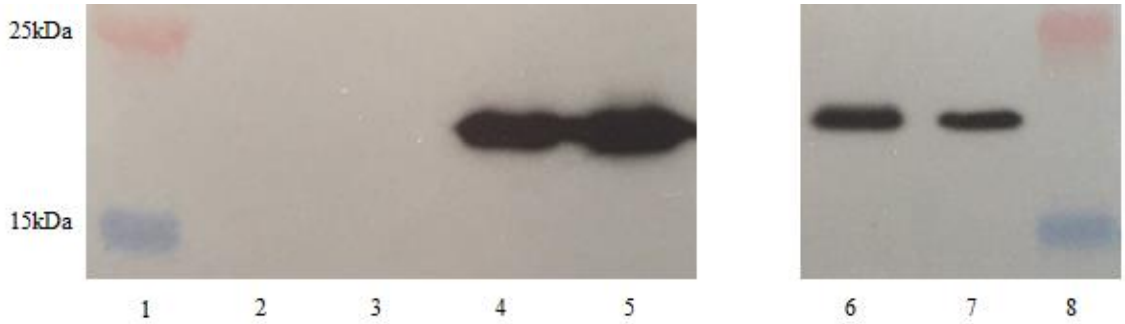

Figure 6. Analysis of SlP14a and SlP14a-PPC20 proteins expressed in heterologous system (*E. coli*) and in *N. benthamiana* (transient expression). Lanes 1 and 8: protein

ladder (Thermo Scientific Page Ruler Plus Prestained Protein Ladder, 10-250kDa); Lane 2: Empty Vector (pJexpress401:95086); Lane 3: Empty Vector (pDU97.1005); Lanes 4 and 5: SlP14a, from *E. coli* and *N. benthamiana*, respectively; Lanes 6 and 7: SlP14a-PPC20, from *E. coli* and *N. benthamiana*, respectively. As shown, SlP14a-PPC20 has a slightly higher molecular weight than SlP14a.

Antimicrobial activities of recombinant SlP14a and the chimera (SlP14a-PPC20) were verified 90 minutes after incubation of bacterial cells with the proteins, clearing almost half CFUs by the end of the assay. Interestingly, both proteins altered the growth pattern of the tested pathogen. Bacterial titer increased at a rate of 5.0 and 6.2 x 10^2 CFUs per 10 minutes due to incubation with SlP14a and SlP14a-PPC20, respectively, whereas without the proteins, *Ralstonia* followed a polynomial growth (Figure 7). In contrast, expression of those proteins in eukaryotic cells exhibited killing efficacy of up to 26.3%, regardless of the time of incubation. Other research groups testing the effectiveness of CecB-like peptides in killing bacteria, despite assaying in different ways, have reported similar levels of killing efficacy (JAYNES et al., 1993; JAN; HUANG; CHEN, 2010).

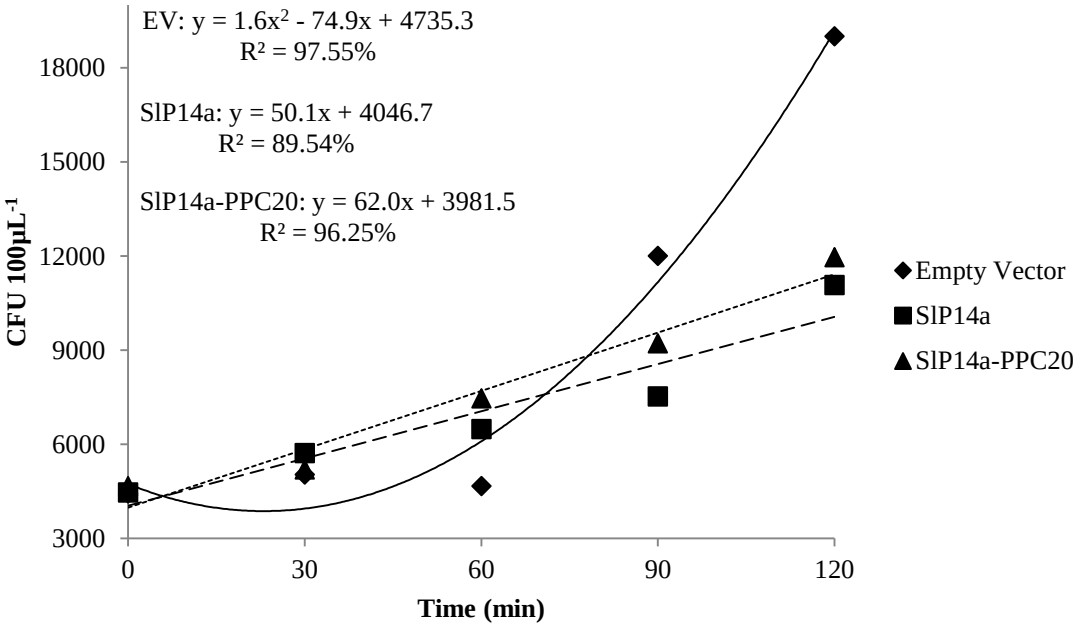

Figure 7. Time-kill curves of *Ralstonia solanacearum* (GMI1000). Bacterial growth was inhibited by the presence of SlP14a and SlP14a-PPC20 purified proteins previously expressed in *E. coli*.

The SlP14a protein is an acidic PR-1. Acidic PR-1 genes do not contain any known targeting peptide sequences for vacuolar destination (VIDHYASEKARAN, 2002), but have been detected in extracellular spaces of the xylem elements of TMV-

infected tobacco leaves using immunogold labeling (CORNELISSEN et al., 1986). The delivery of this protein to the plant xylem, the site of *Ralstonia* colonization, can facilitate targeting of bacterial cells, which may be an appealing strategy for plant protection. The structure of tomato P14a (PR-1b) was solved by nuclear magnetic resonance and found to represent a unique molecular architecture (FERNANDÉZ et al., 1997): four β-strands arranged in antiparallel with four α-helices forming a compact structure stabilized by hydrophobic interactions and multiple hydrogen bonds making it more stable and insensitive to proteases. Besides being involved in plant immune defense responses, PR-1 proteins have shown to directly inhibit oomycetes (ALEXANDER et al., 1993; NIDERMAN et al., 1995; RIVIÈRE et al., 2008). Although antimicrobial mechanism(s) of PR-1 proteins has(have) not been completely elucidated (SUDISHA et al., 2012), SlP14a is proposed to be a protease (Table 3) since it was selected based on the 3D structure and active sites of a human elastase. In addition, a protease from the venom of *Conus textile*, Tex31, also displays similarity to members of the PR-1 protein superfamily (MILNE et al., 2003), suggesting an enzymatic activity for SlP14a.

Table 3. Protease activity of SlP14a and SlP14a-PPC20 proteins. Assays were performed with total protein extracted from recombinant *E. coli* and purified protein from SlP14a-PPC20-expressing tomato[1].

Protein extracted from *E. coli*	**Protease Activity[2]**
Empty Vector (pJexpress)	28.74 b
SlP14a	160.33 a
SlP14a-PPC20	100.30 ab
Protein extracted from tomato plants	**Protease Activity[2]**
MoneyMaker (control)	2.12 c
91.004	3.29 b
91.003	11.28 a

[1] averages followed by different letters, in each expression system, are statistically different by the Tukey test at 0.05 significance level;
[2] expressed in picograms of trypsin mL^{-1}.

The hypothesis of the mechanism of action of SlP14a and SlP14a-PPC20 is that the surface recognition domain recognizes components in the bacterial outer membrane binding the protein to the targeted-pathogen cell. This domain, as a pathogenesis-related protein, also has antimicrobial effect, although less active than the cecropin-derived domain. Later, the lytic activity of PPC20 disrupts the membrane by pore formation, leading to cell death. Combined, both domains work in synergy, enhancing lytic potency. This synergism has been proposed to explain the higher cytotoxicity effect of lytic

peptides against pathogenic bacteria in the presence of lysozyme (JAYNES et al., 1993), in which loss of integrity of the peptidoglycan bacterial cell wall, combined with the lytic activity of the peptides, creates a synergistic interaction similar to the concerted action that has been reported for the humoral immune system of the cecropia moth.

The results obtained in the *in vitro* experiments pointed to the antibacterial activity of the proteins, which made them promising candidates for use in plant protection. Therefore, tomato plants were engineered to express SlP14a-PPC20 chimera and challenged with *R. solanacearum* cells in an attempt to increase disease resistance.

6.4.2. Selection of transgenic tomato plants and their progeny (PCR analysis)

Transgenic tomato plants carrying the pDM14.0609.04 construct were generated and grown on medium containing 50mg/mL kanamycin, and three independent kanamycin-resistant T0 (original) transgenic lines were selected for further analysis (91.002, 91.003, and 91.004). Among them, line 91.002 was PCR-negative. Therefore, seed multiplication was continued only with lines 91.003 and 91.004. Integration of the SlP14a-PPC20 gene into the tomato genome of progenies (T1, or first generation) was confirmed by PCR (Figure 8). The following control experiments were carried out: PCR amplification in the absence of a template (lane 29, as a negative control), genomic DNA isolated from wild-type tomatoes as a template (lanes 19 to 28), and plasmid pDM14.0609.04 DNA as a template (lanes 30 and 31, as positive controls). Out of 12 T1 transgenic plants (91.004 progeny) (lanes 6 to 16, and lane 18), only three plants (lanes 6, 16, and 18) gave negative PCR results. Regarding the progeny of line 91.003, one tomato plant out of four did not incorporate the transgene (lane 5). The presence of the transgene did not have any obvious detrimental effect on the PCR-positive plants, since they had an indistinguishable phenotype from non-transformed controls.

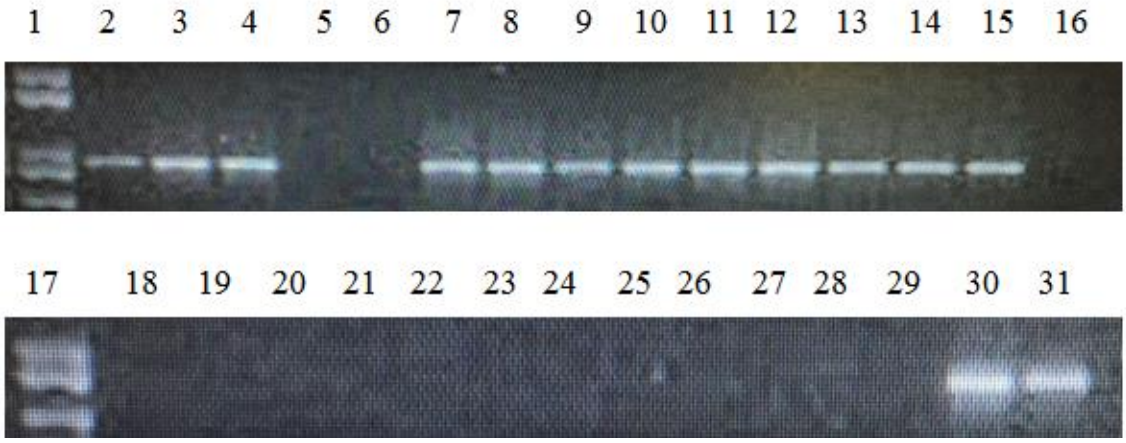

Figure 8. PCR analysis of the SlP14a-PPC20 gene in transgenic tomatoes. Genomic DNA isolated from 50mg of fresh leaves from transgenic tomatoes (T1 plants, lanes 2 to 16 and lane 18) and non-transgenic control plants (lanes 19 to 28) were used as templates for PCRs. The PCR products were analyzed on 1% (wt/vol) agarose gels. Lanes 1 and 17: 1Kb Plus DNA ladder (Thermo Scientific, USA); Lane 29: no template (negative control); Lanes 30 and 31: PCR product amplified from plasmid pDM14.0609.04 (positive control); Lanes 2 to 5: progenies of line 91.003; Lanes 6 to 16 and lane 18: progenies of line 91.004.

Transgenic tomato plants, including T0 and T1 generations, carrying the SlP14a-PPC20 gene were analyzed for protein expression by Western hybridization assay. An anti-Flag antibody conjugated with peroxidase was used to detect the protein. In none of the transgenic plants could the SlP14a-PPC20 protein be detected. This can be due to breakdown of the protein by plant endogenous proteases (OWENS, 1995; OWENS; HEUTTE, 1997; SHARMA et al., 2000) or is most likely caused by low concentration of SlP14a-PPC20 in the samples.

6.4.3. Plant pathogen resistance of SlP14a-PPC20-expressing tomato

The chimera SlP14a-PPC20 was designed under the CaMV 35S promoter, a strong and constitutive promoter that is frequently employed to drive AMP expression for plant protection (JAN; HUANG; CHEN, 2010; JUNG et al., 2012; ZAKHARCHENKO et al., 2013a, b; COMPANY et al., 2014). To enhance stability, an auxiliary secretion signal sequence from rice (RAmy3D) was included to target the chimera to extracellular space (HUANG et al., 2015). This approach aimed to improve protein-pathogen interaction in transgenic plants, preventing xylem colonization by *Ralstonia* cells. The SlP14a signal peptide was predicted using the software SignalP 4.0 (http://www.cbs.dtu.dk/services/SignalP/) and then replaced by RAmy3D. The use of signal peptides for subcellular targeting of AMPs was reported in transgenic sweet orange

plants resistant to *Xanthomonas axonopodis* (BOSCARIOL et al., 2006) and tomato plants resistant to bacterial wilt and bacterial spot (JAN; HUANG; CHEN, 2010). Additionally, the transgene constructed for the chimera production was subjected to codon optimization for high level expression in tomato (OptimumGene^{TM}, GenScript, USA, Inc), by upgrading the Codon Adaptation Index to 0.90.

To evaluate the resistance of transgenic plants to the phytopathogen *R. solanacearum*, control (wild-type) and transgenic T1 tomato plants were challenged with 10µL of an inoculum concentration of 10^8 CFU/mL. The symptoms of bacterial wilt disease were evaluated after infection until the 14th-day post-inoculation (DPI). By the 14th DPI, three wild-type plants had not shown disease symptoms, probably due to an escape during stem infection with the bacterium, as no *Ralstonia* cells could be recovered from those plants in a platting test. Still, all leaves of 53.8% of wild-type plants wilted (Figure 9), leading to plant death. T1 transgenic tomato plants expressing SlP14a-PPC20 were healthier and showed reduced disease severity. Line 91.003 stood out as the most resistant one (Figure 9).

Figure 9. Enhanced resistance to bacterial wilt disease in SlP14a-PPC20-transgenic tomatoes. Four-week-old tomato plants, including wild-type and transgenic, were challenged with 10µL of the pathogen *Ralstonia solanacearum* GMI1000 (10^8 CFU/mL) by stem inoculation. Disease development and symptoms in wild-type (right) and transgenic (left) tomatoes were recorded on different days. The photograph was taken on the 14th day post-inoculation.

Bacterial challenge results indicated that progeny 91.003 exhibited a delayed appearance of symptoms, which were less severe than those shown by the control plants. This delay can be expressed in incubation period (defined as the number of days required for the development of visible symptoms), which was of 7 days vs. 4 days for the control. Furthermore, there was a dramatic difference in the mortality of transgenic plants when compared to control plants two weeks after infection, namely 7.7 and 53.8%, respectively. Among the transgenic, no 91.003-line plants died. The disease development was recorded on individual plants by a rating scale varying from 1 (no symptoms) to 5 (completely wilted plants) (WINSTEAD; KELMAN, 1952), scored by the 4th to the 14th DPI (Figure 10). Although wilting also appeared on transgenic plants, the score attributed at the end of the experiment was significantly lower than the one of wild-type (Table 4), and plants wilted more slowly.

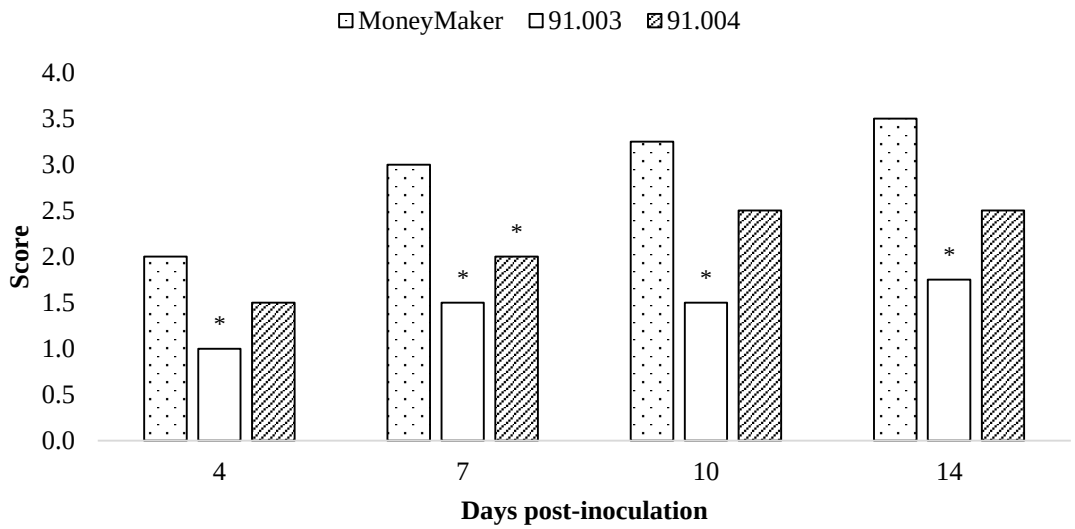

Figure 10. Average progression of *Ralstonia solanacearum* infection in transgenic (91.003 and 91.004) and control (MoneyMaker) tomato plants. Disease development was scored based on an index using a five-point scale. Asterisks denote significant differences ($P < 0.05$) with respect to non-transgenic plants.

A bacterial wilt index – BWI – was calculated based on the rating scale (EMPIG et al., 1962), according to the formula: $BWI = [\sum(S*P)]/N$ (S: score attributed due to the symptoms; P: number of plants grouped within the same score; and N: total number of inoculated plants). Based on this index, wild-type and transgenic tomato plants were classified as to their resistance against the pathogen (MORGADO; LOPES; TAKATSU, 1992). Wild-type plants were classified as moderately susceptible (BWI = 3.3), whereas transgenic lines 91.004 and 91.003 were moderately resistant (BWI = 2.4) and resistant

(BWI = 1.6), respectively. Therefore, when tested with a stem-inoculation assay, lines 91.003 and 91.004 were markedly less susceptible to the bacterial pathogen. Both lines showed significantly fewer wilted leaves in individual plants, and overall fewer plants wilted by the end of the experiment (Figure 10 and Table 4). Still, whether the levels of disease resistance are correlated with SlP14a-PPC20 protein expression levels in transgenic lines calls for further analysis to determine whether the observed resistance is due to a direct or indirect effect. Research into the chimera mRNA accumulation levels in leaves and stems needs to be done to clarify this point.

Plants engineered to express cecropins and cecropin derivatives and chimeras (JAYNES et al., 1993; HUANG et al., 1997; ARCE et al., 1999; OSUSKY et al., 2000; JAN; HUANG; CHEN, 2010; VUTTO et al., 2010; DANDEKAR et al., 2012) suggest the use of CecB-like proteins in plant protection. Furthermore, the hypothesis of a defense role of P14a has been supported by different reports of homologous genes. PR1 has inhibitory effect on *Phytophthora infestans* and *Uromyces fabae* in tomato and broad bean (*Vicia faba* L.) (NIDERMAN et al., 1995; RAUSCHER et al., 1999). Constitutive expression of PR1a gene in transgenic tobacco confers resistance to *Peronospora tabacina* and *P. parasitica* var. *nicotianae* (ALEXANDER et al., 1993). Conversely, silencing of PR1 in barley and tobacco resulted in an increase in susceptibility to *Blumeria graminis* f. sp. *hordei* (SCHULTHEISS et al., 2003) and *P. parasitica* (RIVIÈRE et al., 2008), respectively. These results point to an important role of P14a in plant defense against pathogens. Here, the increased resistance to *R. solanacearum* in tomato plants expressing the chimeric protein SlP14a-PPC20 suggests that PR1 does have activity on bacterial pathogens, as previously proposed (SAROWAR et al., 2005; LI et al., 2011).

After 14 days of inoculation, the infected stems were ground with a solution of NaCl 0.85% and the extract was plated in order to evaluate bacterial multiplication in plants. The number of CFUs in transgenic tomato plants of line 91.003 was 56% lower than that of the wild-type plants. This result implies an association between disease symptoms (wilting score) and pathogen quantity (the number of *R. solanacearum* cells per gram of stem) (Table 4).

Table 4. Colony forming units (CFU) of *Ralstonia solanacearum* (GMI1000) per gram of stem recovered 14 days after inoculation of tomato plants with the bacterium. Score attributed to disease symptoms previously to stem removal.

Protein[1]	CFU (10^5) g^{-1}	SCORE
MoneyMaker	2434.91 a	3.31 a
91.003	1069.03 b	1.56 b
91.004	1626.96 ab	2.38 ab

[1] averages followed by different letters, in each column, are statistically different by the Tukey test at 0.05 significance level.

These findings are encouraging in a scenario of a vast range of bacteria causing significant crop loss, since introduction of genes for antimicrobial peptides into plants may result in an enhanced resistance similar to that found for the tomato plants in this study. Indeed, several groups have reported enhanced levels of resistance in plants expressing antimicrobial peptides (MONTESINOS, 2007; RAMADEVI; RAO; REDDY, 2011; BREEN et al., 2015; HOLÁSKOVÁ et al., 2015).

The approach presented in this study may be a proof of concept for the use of plant-derived peptides to render different plants less susceptible to bacterial diseases in general. Also, it may be more difficult for the pathogen to circumvent the lytic activity of the peptide, synergistically combined with the surface recognition domain, since a dramatic modification of the bacterial membrane would seem to be necessary to permit pathogen resistance (STEINBERG et al., 1997).

6.4.4. Antibacterial activities of transgenic tomato plant extracts

The ability of SlP14a-PPC20 protein extracted from the leaves of transgenic tomato plants to inhibit the growth of *R. solanacearum* was determined by a liquid growth inhibition assay. Incubation with purified protein isolated from 200mg of leaves of transgenic tomatoes (line 91.003) showed bacterial growth inhibition ranging from 71 to 84% (Table 5). Protein extracts from line 91.004 did not display any antimicrobial activity compared to those of wild-type plants, although growth rate was 1.13-fold slower (Figure 11). In a similar study, according to optical density recordings after a 17-hour incubation, growth inhibition of 16-35% was determined for different bacteria (*Escherichia coli*, *Salmonella enteritidis*, and *Pectobacterium carotovorum*) treated with extracts of transgenic tomato expressing solely CecB compared to wild-type plants (JAN; HUANG; CHEN, 2010). Although pathogens' susceptibility may vary, the noteworthy effectiveness can be attributed to the combination of the CecB plant homologue to the pathogenesis-related protein domain.

Table 5. Colony forming units (CFU) in 100µL^{-1} of *Ralstonia solanacearum* (GMI1000) after incubation of bacterial cells with transgenic tomato plant extracts. Proteins were purified using Anti-Flag M2 Magnetic Beads (Sigma-Aldrich, USA).

Protein	Extract incubation time (minutes)[1]				
	0	30	60	90	120
MoneyMaker	2533.33 a	1700.00 a	2266.67 a	8000.00 a	6333.33 a
91.003	1466.67 a	833.33 a	1233.33 a	2333.33 b	1010.00 b
91.004	1533.33 a	2000.00 a	1533.33 a	6000.00 a	5666.67 a

[1] averages followed by different letters, in each column, are statistically different by the Tukey test at 0.05 significance level.

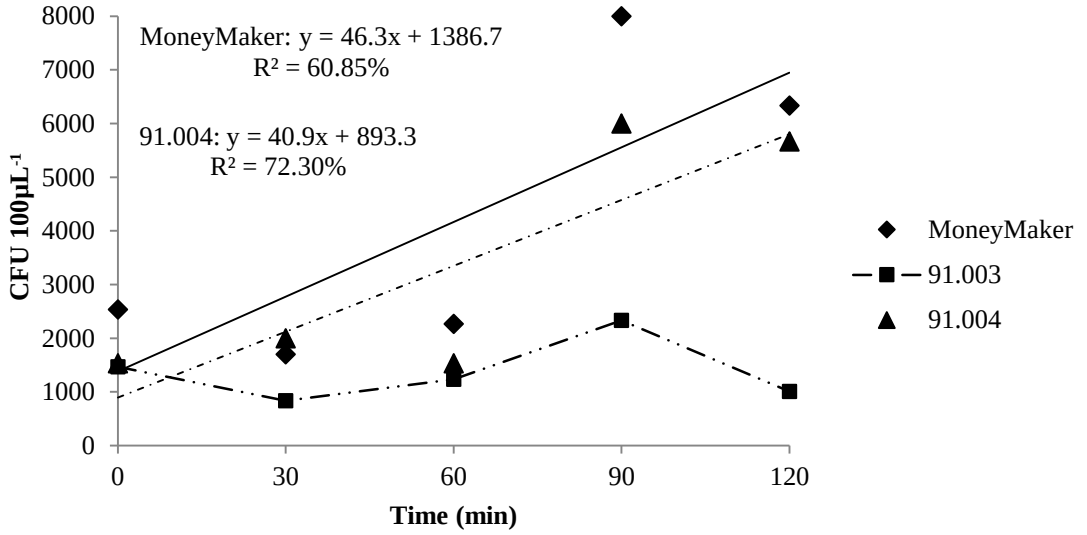

Figure 11. Time-kill curves of *Ralstonia solanacearum* (GMI1000). Bacterial growth was inhibited by the presence of SlP14a-PPC20 purified protein previously extracted from transgenic tomato plants.

6.5. Conclusions

In this study, transgenic tomato plants constitutively expressing the SlP14a-PPC20 gene were generated by *Rhizobium*-mediated transformation. *In vitro*, extracts of transgenic tomatoes showed antimicrobial activity inhibiting the growth of *R. solanacearum*. In *in vivo* challenge studies, transgenic tomatoes showed improved resistance to bacterial wilt disease, resulting in a delay of symptoms and a significant reduction of plant mortality, thus showing the potential of SlP14a-PPC20 as a promising tool for the development of resistant tomato varieties.

One of the most serious concerns regarding the use of lytic peptides in enhancing plant defense against invading pathogens is the possible toxicity to the plant. The present investigation showed that the SlP14a-PPC20 gene expressed in tomato plants had no

deleterious effects on the transgenic plants: plant morphology, plant growth, and yields of fruits and seeds were normal (data not shown). In most cases, the minimum lethal concentration of cecropin derivatives required for toxicity to plant protoplast, intact cells, and tissues is much higher than that required to kill bacterial cells (NORDEEN et al., 1992; MILLS et al., 1994). Therefore, the expression of the chimera in tomato by the method described here is considered safe for the plant, as expression levels of the chimeric protein are so low as not to be detected by Western blot.

As the proposed SlP14a-PPC20 chimera is plant-derived, negative public perception may be reduced. Furthermore, despite its effectiveness in protecting tomato plants against bacterial wilt disease, resistance breakdown is less probable to occur since pathogen will have to overcome both modes of action of the protein.

Currently, genes encoding newly designed, more active peptides, are frequently introduced into different plant species to test their protection ability against a broad spectrum of phytopathogens. Some of these novel peptides also possess high *in vitro* cytotoxic activity against fungi, nematodes, and insects (JANG et al., 2004; PARK et al., 2004; VAN DER WEERDEN; LAY; ANDERSON, 2008; CHEN et al., 2014; ZHAO et al., 2014; SCHUBERT et al., 2015), so their future applications remain a challenge and a promise. These broader effects should be further assessed to SlP14a-PPC20 protein and its derivatives.

Acknowledgments

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7 CONCLUSÕES

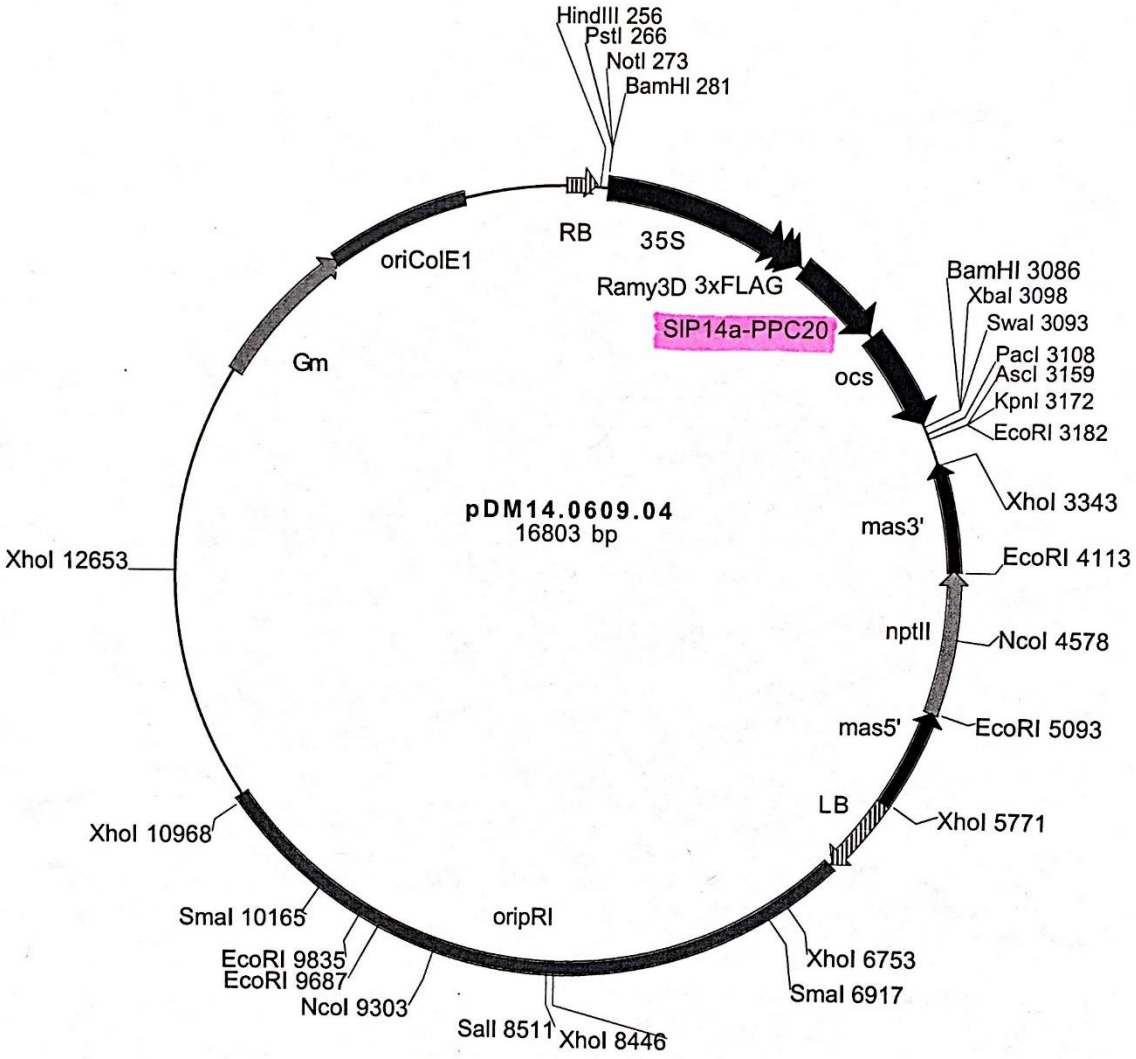
A caracterização de uma proteína quimérica com atividade antimicrobiana à *Ralstonia solanacearum* foi realizada com sucesso. Os resultados obtidos demonstraram que a proteína, denominada SlP14a-PPC20, foi eficaz em controlar a bactéria em ensaios conduzidos *in vitro* e que sua expressão em plantas de tomate configurou em resistência à murcha-bacteriana. Estes resultados sugerem que esta nova proteína pode ser incluída como uma alternativa ao manejo da murcha-bacteriana, mediante o desenvolvimento de cultivares resistentes.

As metodologias usadas permitiram a seleção de domínios bioativos totalmente derivados de plantas para composição da proteína quimérica. A validação da metodologia computacional SCALPEL permitiu a seleção de um domínio lítico antibacteriano pouco tóxico a células humanas, e a análise de similiridade de tríades catalíticas pelo CLASP resultou em um domínio de reconhecimento com atividade enzimática (protease). Essa abordagem é interessante para amenizar a aversão pública a transgênicos, reduzir riscos decorrentes do consumo humano e evitar respostas adversas do hospedeiro à quimera. De fato, a expressão do transgene não apresentou efeitos deletérios nas plantas de tomate transformadas.

Conclui-se que a proteína proposta neste trabalho apresenta potencial para aplicação na defesa de plantas. Em estudos futuros, sua incorporação poderá ser realizada em diferentes culturas de importância econômica afetadas pela murcha-bacteriana. Ainda, a eficiência da quimera SlP14a-PPC20 poderá ser testada contra outros fitopatógenos, como fungos, vírus, nematoides, outras bactérias e mesmo contra insetos, para constatar seu espectro de ação.

ANEXOS

ANEXO A: PLASMID MAP – pDM14.0609.04



ANEXO B: PLASMID MAP – pJexpress401:502431-1