UNIVERSIDADE ESTADUAL DE MARINGÁ
CENTRO DE CIÊNCIAS AGRÁRIAS
DEPARTAMENTO DE AGRONOMIA
PROGRAMA DE PÓS-GRADUAÇÃO EM AGRONOMIA

ANGÉLICA ALBUQUERQUE TOMILHERO FRIAS

Interação de vetores e patógenos em dois patossistemas: tristeza dos citros e zebra chip

Maringá
2018

ANGÉLICA ALBUQUERQUE TOMILHERO FRIAS

Interação de vetores e patógenos em dois patossistemas: tristeza dos citros e zebra chip

Tese apresentada ao Programa de Pós-Graduação em Agronomia do Departamento de Agronomia, Centro de Ciências Agrárias da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Doutora em Agronomia.

Área de concentração: Proteção de Plantas.

Orientador: Prof. Dr. William Mário de Carvalho Nunes.

Co-Orientador: Prof[a]. Dr[a]. Cecilia Tamborindeguy.

Maringá
2018

Dados Internacionais de Catalogação na Publicação (CIP)
(Biblioteca Central - UEM, Maringá, PR, Brasil)

F897i

Frias, Angélica Albuquerque Tomilhero
Interação de vetores e patógenos em dois patossistemas: tristeza dos citros e zebra chip / Angélica Albuquerque Tomilhero Frias. -- Maringá, 2018.
87 f. : il. color., figs., tabs.

Orientador: Prof. Dr. William Mário de Carvalho Nunes.
Coorientadora: Prof.ª Dr.ª Cecilia Tamborindeguy.
Tese (doutorado) – Universidade Estadual de Maringá, Centro de Ciências Agrárias, Departamento de Agronomia, Programa de Pós-Graduação em Agronomia, 2018.

1. Virus da Tristeza dos Citrus (CTV). 2. *Candidatus* Liberibacter solanacearum. 3. Fitopatógenos. I. Nunes, William Mário de Carvalho, orient. II. Tamborindeguy, Cecilia, coorient. III. Universidade Estadual de Maringá. Centro de Ciências Agrárias. Departamento de Agronomia. Programa de Pós-Graduação em Agronomia. IV. Título.

CDD 21.ed. 634.3

Mariza Nogami - CRB 9/1569

ANGÉLICA ALBUQUERQUE TOMILHERO FRIAS

INTERAÇÃO DE VETORES E PATÓGENOS EM DOIS PATOSSISTEMAS: TRISTEZA DOS CITROS E ZEBRA CHIP

Tese apresentada à Universidade Estadual de Maringá, como parte das exigências do Programa de Pós-graduação em Agronomia, na área de concentração em Proteção de Plantas, para obtenção do título de Doutor.

APROVADA em 19 de fevereiro de 2018.

Prof. Dr. **William Mário de Carvalho Nunes** _____
Presidente

Prof. Dr. **Dauri José Tessmann** _____
Membro

Profª. Drª. **Cecilia Tamborindeguy Camacho** _____
Membro

Prof. Dr. **Julien Gad Levy** _____
Membro

Profª. Drª. **Rúbia de Oliveira Molina** _____
Membro

DEDICATÓRIAS

À minha mãe, Maria de Albuquerque Tomilhero Frias.

À memória de meu pai, João Tomilhero Frias.

Ao meu irmão Diego de Albuquerque Tomilhero Frias.

Ao Marcos Aurélio Ceron.

Dedico com muito amor.

AGRADECIMENTOS

A Deus, por me amparar nos momentos difíceis, dar-me força interior para superar as dificuldades, mostrar-me o caminho nas horas incertas e suprir-me em todas as minhas necessidades.

À Universidade Estadual de Maringá (UEM), Programa de Pós-Graduação em Agronomia, pela oportunidade concedida à realização deste curso.

À Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior (Capes), pela concessão da bolsa de estudos durante o curso e da bolsa de doutorado sanduiche para o desenvolvimento de parte da minha pesquisa na Texas A&M University- College Station-Texas.

Ao professor doutor William Mário de Carvalho Nunes, pela orientação, ensinamentos, dedicação, incentivo e amizade.

À professora doutora Cecilia Tamborindeguy, pela coorientação, amizade, ensinamentos e por ter me recebido em seu laboratório na Texas A&M University durante meu doutorado sanduiche.

Ao doutor Julien Levy, pelos ensinamentos, amizade e colaboração para concretização da parceria entre o NBA e o laboratório da Doutora Cecilia.

Ao doutor Carlos Alexandre Zanutto, pelos ensinamentos, amizade e ajuda na realização desse trabalho.

À doutora Rubia Molina, pelos ensinamentos, amizade e colaboração no desenvolvimento da presente pesquisa.

Ao doutor Freddy Ibañez, pelos ensinamentos, amizade, estímulo e colaboração no desenvolvimento da minha pesquisa na Texas A&M University.

À doutora Azucena Mendoza, pela amizade, ensinamentos e auxílio na realização da minha pesquisa na Texas A&M University.

Aos funcionários Érika Cristina Sato e Reinaldo Bernardo, pelos inúmeros favores prestados e pela atenção dispensada.

Ao Viveiro de Mudas Pratinha (VMP) e Agro Pratinha pela disponibilidade e fornecimento das mudas.

Aos Srs. José Gilberto Pratinha, Antônio Pratinha (*in memorian*), Waldemar Zanini Junior e Ismael Lopes da Agro Pratinha pelo auxílio e apoio.

Aos amigos Danielle Sayuri Yoshida Nanami, Ana Claudia Mendonça, Gislayne Kelly Coimbra Gonçalves, Daiane de Oliveira Griezer e Elisandro Lupi pela convivência, amizade e presença constante durante o curso.

Aos colegas do Núcelo de Pesquisa em Biotecnologia Aplicada, Paula Thais (*in memorian*), Diego, Bruna, Juliana, Hudson, Camila, Andressa, Nayara e tantos outros que colaboraram na realização desse trabalho.

Aos colegas Leilane Chaves, Nicolly Santos Leite, Wallason Farias, German Irías, Xiaotian Tang, Chloe Hawkings, Zafrullah Khan, pela amizade e agradável convivência durante minha estadia em College Station, Texas.

Aos funcionários da UEM, Edivan Mantovani, José Braz, pela ajuda e amizade.

A todos que, de alguma forma, contribuíram para a realização deste trabalho.

Interação de vetores e patógenos em dois patossistemas: tristeza dos citros e zebra chip

RESUMO

Várias doenças associadas com bactérias e vírus que são transmitidos por insetos vetores emergiram em todo o mundo em diferentes culturas de importância econômica nos últimos anos. Apesar da importância de compreender a interação entre patógeno-vetor na fitopatologia, o conhecimento sobre essas interações ainda é limitado, uma vez que existem poucos estudos que visam compreender os processos biológicos que ocorrem nesta interação biotrófica. Neste estudo, realizado em duas interações vetores-patógenos em dois países da América, mais precisamente a interação entre *Toxoptera citricida* - *Citrus tristeza* virus ocorreu em Maringá-Brasil; e a interação *Bactericera cockerelli* - *Candidatus* Liberibacter solanacearum (Lso) nos Estados Unidos. Nosso primeiro objetivo foi focado na transmissão de três isolados de CTV usando um único pulgão, dois desses isolados são considerados fracos (PIAC e CS1) e comumente utilizados para ensaios de pré-imunização de citros e o isolado severo Forte Rolândia, produz sintomas graves em plantas cítricas no Brasil. Esses isolados foram obtidos de pomares comerciais localizados no Norte e Noroeste do Paraná-Brasil. Após os ensaios de transmissão de pulgões individuais, realizados sob as condições ambientais de Maringá-Brasil, foram caracterizados os sub-isolados PIAC e CS1 detectados nas plantas infectadas com CTV por meio da técnica de SSCP. O segundo objetivo foi compreender a interação *Bactericera cockerelli*- Lso haplótipo B analisando parâmetros populacionais e expressão da transcrição do gene *BcVg1*, o qual está diretamente envolvido na reprodução do inseto. Os resultados obtidos durante a transmissão de um único pulgão mostraram que *T. citricida* transmitiu apenas os sub-isolados de CTV considerados fracos. Os sub-isolados, PIAC e CS1, tiveram uma eficiência de transmissão de 8% e 4%, respectivamente. Enquanto Forte Rolândia não foi transmitido por *T. citricida*. Neste estudo, também encontramos diferenças nos padrões SSCP entre isolados e sub-isolados de CTV, sugerindo que um pulgão pode adquirir apenas um subsolado de CTV. No segundo estudo entre a interação *B. cockerelli*- Lso, observaram-se diferenças estatísticas no número de ovos ovipositados, número de ninfas e adultos quando comparados aos insetos livres de Lso e insetos que carregam o patógeno (Lso). Esses resultados foram associados a uma redução significativa na expressão de transcrição semelhante a *BcVg1* observada nos cruzamentos realizados com fêmeas infectadas com Lso em comparação com insetos livres do patógeno.

Palavras-chave: *Citrus tristeza virus*. *Candidatus* Liberibacter solanacearum. Fitopatógenos.

Interaction vector-pathogen in two pathosystems: citrus tristeza virus and zebra chip

ABSTRACT

Several diseases associated with bacteria and virus that are transmitted by insect-vectors have emerged worldwide in different crops of economic importance during the recent years. Despite the importance of understanding the interaction of vector-pathogen in phytopathology, the knowledge about these interactions are still limited, since there are few studies that had aimed to understand the biological processes occurring in this bi-trophic interaction. This study was performed in two vector-pathogen interactions in two countries of America, more precisely in the interaction *Toxoptera citricida - Citrus tristeza virus* occurred in Maringá-Brasil; and interaction *Bactericera cockerelli* – '*Candidatus* Liberibacter solanacearum' (Lso) in the United States. Our first objective focused in the transmission of three CTV isolates using one-single aphid, two of these isolates are considered as mild (PIAC and CS1), and are commonly used for citrus-preimmunization assays. The third isolate, Forte Rolândia produces severe symptoms on Brazilian citrus plants. These isolates were obtained from commercial groves located in the northern regions of the state of Paraná-Brazil. After the one-single aphid transmission assays, performed under the environmental conditions of Maringá, Brazil, we characterized the subisolates PIAC and CS1 existing on the CTV-infected plants using the SSCP technique. The second objective was to understand the interaction *Bactericera cockerelli*- Lso haplotype B analyzing life-history parameters and the expression of the *BcVg1-like* transcript, which is directly involved in the reproduction of the insect. The results obtained during the one-single aphid transmission showed that *T. citricida* transmitted only the CTV subisolates considered as weak. The subisolates, PIAC and CS1, had a transmission efficiency of 8% and 4%, respectively. However, Forte Rolândia was not transmitted by *T. citricida*. In this study, we also found differences in the SSCP patterns between CTV isolates and CTV subisolates, suggesting that an aphid can acquire only one CTV subisolate. In the second study between the interaction *B. cockerelli*- Lso, statistical differences in the number of eggs oviposited, number of nymphs and adults were observed between Lso-free insects and insects that carry the pathogen (Lso), these results were associated with a significant reduction in the *BcVg1-like* transcript expression observed in those crosses performed with Lso-infected females compared to pathogen-free insects.

Keywords: *Citrus tristeza virus*. *Candidatus* Liberibacter solanacearum. Phytopathogens.

LISTA DE TABELAS

CHAPTER 1

Citrus tristeza virus transmission by one single *Toxoptera citricida* vector under Brazilian conditions

CHAPTER 2

Effects of *'Candidatus* Liberibacter solanacearum' (haplotype B) on *Bactericera cockerelli (Šulc)* fitness and vitellogenesis

LISTA DE FIGURAS

CHAPTER 1

Citrus tristeza virus transmission by one single *Toxoptera citricida* vector under Brazilian conditions

CHAPTER 2

Effects of *'Candidatus* Liberibacter solanacearum*'* (haplotype B) on *Bactericera cockerell*i (Šulc) fitness and vitellogenesis

SUMÁRIO

CHAPTER 2

Effects of *'Candidatus* Liberibacter solanacearum*'* (haplotype B) on *Bactericera cockerell*i (Šulc) fitness and vitellogenesis

INTRODUÇÃO GERAL

Várias doenças associadas a bactérias e vírus transmitidos por insetos vetores emergiram em distintas culturas de importância econômica nos últimos anos em todo o mundo (DAWSON et al., 2013; TAMBORINDEGUY et al., 2017). Nas Américas, em particular na cultura dos citros e das solanáceas, o clima associado com a capacidade de evolução gênica dos patógenos e consequente quebra da resistência das culturas, tem favorecido a propagação e disseminação de várias doenças (BAR-JOSEPH, 1989; SECOR e RIVERA-VARAS, 2004; MORENO et al., 2008). Algumas dessas enfermidades são causadas pelo vírus do gênero Closterovirus: *Citrus tristeza virus* e por bactérias pertencentes ao gênero Liberibacter, incluindo, *Candidatus* Liberibacter americanus e *Candidatus* Liberibacter solanacearum (TEIXEIRA, 2005; MUNYANEZA, 2012).

A ordem Hemiptera possui aproximadamente 82.000 espécies descritas, e se classifica como a 5º maior ordem de insetos catalogados possuindo as três maiores subordens (Auchenorrhyncha, Sternorrhyncha e Heteroptera) (CRYAN e URBAN, 2012). Os insetos pertencentes à Subordem Sternorrhyncha (mosca branca, afídeos, cochonilha e psilídeos) caracterizam-se como os principais vetores de patógenos de plantas. Na cultura dos citros em especial, o afídeo *Toxoptera citricida* destaca-se como o principal vetor do vírus *Citrus tristeza virus* agente causal da tristeza dos citros (FAGOAGA et al., 2006; COSTA et al., 2010). Na década de 40, um surto ocasionado por essa doença atingiu as zonas citrícolas do Brasil destruindo milhões de arvóres de pomares comerciais (BENNETT e COSTA, 1949; MORENO et al., 2008). Com uma transmissão do tipo semi-persistente, a aquisição e transmissão do vírus pelo vetor se dá em até 30 minutos de contato direto com floema da planta hospedeira (MÜLLER, 1996; ZANUTTO et al., 2013).

Outro importante representante da mesma subordem está o psilídeo *Bactericera cockerelli* (Šulc), também conhecido como psilídeo da batata. Psilídeos são hemípteras que se alimentam por meio do floema das plantas e nos últimos anos têm ganhado destaque como maiores vetores de fitobactérias (IBANEZ et al., 2017). O vetor polífago *B. cockerelli*, embora possua preferência pelas plantas da família Solanaceae (como tomate, batata e pimenta) também pode completar seu ciclo de vida em outra família de plantas (Convolvulaceae) (ALFRADO-FERNÁNDEZ et al., 2017). *B. cockerelli* transmite a α-proteobacteria *Candidatus* Liberibacter solanacearum, a qual ocasiona na cultura da batata uma doença denominada "zebra chip" gerando perdas de milhões de dólares para produtores nas Américas (SECOR e RIVERA-VARAS, 2004; MUNYANEZA, 2012).

Atualmente, é consenso que os insetos sugadores e os patógenos transmitidos pelos mesmos, podem reduzir substancialmente o rendimento e a qualidade das culturas de importância econômica (ALEXANDER et al., 2014; TRĘBICKI et al., 2017). A interação existente entre vetor-fitopatógeno é diversificada e constituem um fascinante campo de investigação (PIETERSE e DICKE, 2007). Apesar da importância do entendimento da transmissão vetorial para fitopatologia, o conhecimento acerca da interação fitopatógeno-vetor ainda é limitada, uma vez que há poucos estudos que visam compreender a especificidade biológica existente nessa interação bitrófica.

Dessa forma, o presente estudo teve como objetivo compreender as interações entre os vetores *Toxoptera citricida* e *Bactericera cockerelli,* e seus respectivos fitopatógenos *Citrus tristeza* virus e *Candidatus* Liberibacter solanacerarum, agentes causais da tristeza dos citros e "zebra chip". Ambas as enfermidades ocorrem nas Américas.

REVISÃO DE LITERATURA

1. Afídeos como vetores virais

Doenças em plantas causadas por vírus representam 47% das patologias emergentes, sendo uma grande ameaça para agricultura (ANDERSON et al., 2004). Atualmente, existem mais de 2000 espécies de vírus pertencentes à cerca de 20 famílias que afetam diferentes culturas de plantas cultivadas pelo homem (WHITFIELD et al., 2015).

Uma das características biológicas importantes compartilhadas por muitos vírus infectantes de plantas e animais é o seu eficiente movimento no vegetal hospedeiro, a fim de garantir sua disseminação por meio dos vetores específicos (insetos) durante o processo de alimentação (NG e FALK, 2006). Determinantes codificados pelos vírus interagem especificamente com os receptores presentes nas células e tecidos do vetor, auxiliando sua presença na natureza (WATSON e ROBERTS, 1940).

As plantas como organismos sésseis, não são significativas transmissoras de vírus, exceto em alguns casos de transmissão por semente ou pólen, assim a maioria dos vírus é transmitida de hospedeiro para hospedeiro por meio de um vetor especifico, o qual é capaz de transportá-lo de forma eficaz (BLANC et al., 2014; ROOSSINCK, 2015). Embora sejam reconhecidos diversos organismos como vetores de vírus (fungos, nematoides, artrópodes), os mais frequentes são os pertencentes ao filo Arthropoda, sendo a grande maioria membros da ordem Hemiptera (WHITFIELD et al., 2015).

A ordem Hemiptera está representada por insetos especializados tais como: afídeos, cochonilhas, mosca-branca e cigarrinhas. Estas espécies de insetos alimentam-se via floema, xilema ou por meio de células do mesófilo e estão entre os principais propagadores/ transmissores de fitopatógenos (PAN et al., 2017). A maioria das espécies de vírus de plantas transmitidos por hemípteras são majoritariamente limitados ao floema, enquanto outras possuem a capacidade de explorar quase todos os tecidos da planta hospedeira (HOGENHOUT et al., 2008). Dentro da ordem hemíptera, representantes da família Aphididae atuam como eficientes agentes de dispersão para fitopatógenos. Sua importância é maior na transmissão de vírus, sendo os pulgões responsáveis pela transmissão de 50% dos vírus de plantas (NAULT, 1997; NG e PERRY, 2004). No meio ambiente, os pulgões estão presentes em número elevado graças a predominante reprodução parternorgênica. Esse fator genético somado as condições ambientais propicias, possibilitam o rápido crescimento

exponencial da espécie (BRAGARD et al., 2013). Além dessas, outras características contribuem para o sucesso dos pulgões como vetores de vírus de plantas. Estes incluem: (I) natureza polífaga presente em algumas espécies de pulgões permitindo a colonização de uma ampla gama de plantas promovendo e garantindo a perpetuação dos vírus fitopatogênicos e (II) presença de um estilete tipo agulha com capacidade de perfurar e adentrar a parede celular das plantas favorecendo a transferência da partícula viral para uma célula hospedeira sem causar danos (NG e PERRY, 2004).

1.1.Vírus-vetor: mecanismos de interação e transmissão

A transmissão do vírus por meio dos vetores depende de alguns fatores como os seguintes: o tempo que o vetor necessita para alimentar-se e adquirir o vírus, tempo que o vírus permanece virulífero, tempo de alimentação para transmissão do vírus e capacidade de movimentar-se e/ ou propagar-se no intestino do vetor (BRAGARD et al., 2013).

O estudo da transmissão de vírus de plantas por insetos vetores iniciou-se há mais de um século por N. Takami (GUTIERREZ et al., 2013). Após vários estudos desenvolvidos, dois sistemas de classificação foram propostos e aceitos para descrever o processo de transmissão vírus-vetor. O primeiro sistema baseia-se no tempo de retenção/aquisição do vírus no interior do inseto; o segundo fundamenta-se na relação de transporte vírus-vetor (HARRIS, 1977).

Watson e Roberts (1939) propuseram inicialmente uma classificação de retenção/transmissão vírus-vetor baseada em dois grupos: não persistente (Figura 1a) e persistente. O primeiro, designado como não persistente, o vírus é adquirido pelo vetor durante a "picada de prova" nas células da epiderme de uma planta infectada e imediatamente deve ser inoculado em novo hospedeiro, uma vez que sua capacidade infectiva é perdida se este se mantiver retido no interior do vetor por poucos minutos. Em contraste, na transmissão persistente o vírus sobrevive no interior do seu hospedeiro por um longo período, o qual pode variar entre horas ou dias e o tempo de aquisição e inoculação possui incubação indefinida com latência necessária para uma inoculação eficaz.

Logo, reconheceu-se a existência de um grupo intermediário, os vírus semi-persistentes (Figura 1b); estes possuem um período de aquisição e inoculação que pode variar de minutos a horas e não necessitam de período de latência antes da inoculação no novo

hospedeiro (SYLVESTER, 1990; HOGENHOUT et al., 2008). Além disso, aquisição e inoculação das partículas virais ocorre principalmente por meio dos tecidos do floema (NG e FALK, 2006).

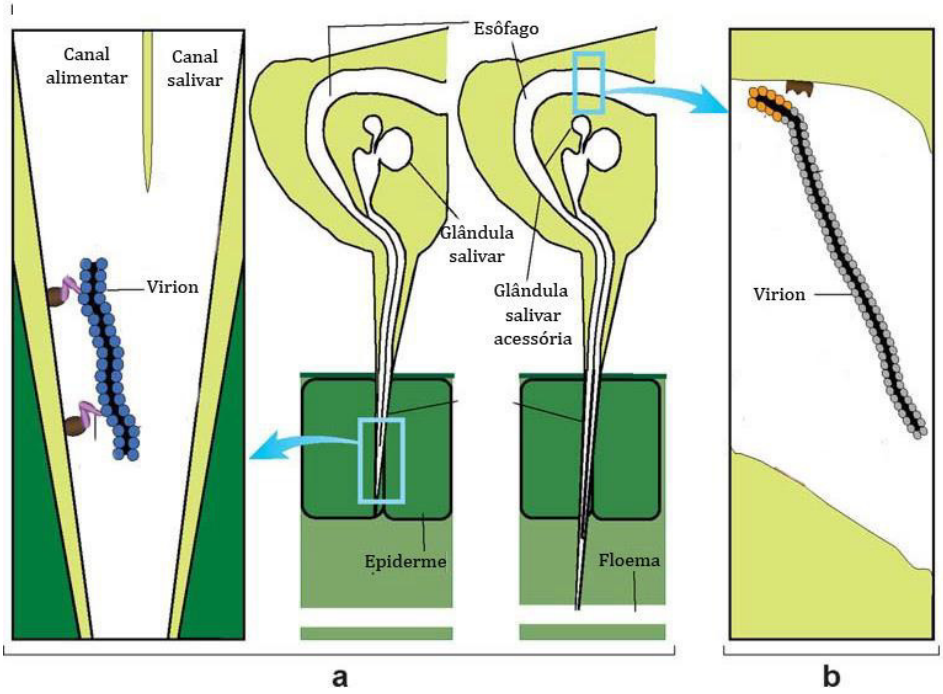

Figura 1. Representação esquemática de dois modelos de transmissão: (a) transmissão não persistente. (b) transmissão semi-persistente. Aquisição de estirpes virais (dois quadros esquerdos) e alimentação (quadro direito) de um vetor hemíptera. O quadro esquerdo dá uma visão ampliada da ponta do feixe de estilete, mostrando que, ao penetrar a epiderme de uma planta infectada, virions de transmissão não-persistente podem ser vinculados a locais dentro do estilete do inseto. (b) Visualização ampliada mostrando as interações entre as regiões dentro do intestino do inseto e interação com vírus transmitidos de forma semi-persistente. Os pontos castanhos ligados ao estilete e à parede do trato superior do sistema digestório representam os locais de ligação para proteínas do cápsidio ou proteínas auxiliares codificadas pelo virions. Adaptado de NG e FALK (2006).

Keneddy et al. (1962) propuseram a segunda teoria, a qual foi subdividida em três categorias de interação vírus-vetor: não circulatória, circulatória e propagativa. A primeira categoria, interação não circulatória (Figura 2), o vírus é absorvido pelo inseto vetor durante a alimentação e este pode anexar-se imediatamente ao revestimento cuticular e/ou aos canais salivares no interior do aparelho bucal do vetor. Minutos após aquisição, o vírus pode ser inoculado em plantas hospedeiras, pois este é retido de forma transitória no interior dos insetos. Dessa forma, classificam-se como uma das interações mais eficientes (NG e FALK 2006; GUTIERREZ et al., 2013).

A segunda categoria de transmissão viral foi classificada como circulatória (Figura 2). Nesse caso, a partícula viral é absorvida pelo inseto em conjunto com o conteúdo celular da célula vegetal, percorrendo o epitélio intestinal, difundindo-se na hemolinfa e alcançando as glândulas salivares sem efetuar replicação. Após esse ciclo, o vírus está pronto para ser inoculado em novas plantas hospedeiras em conjunto com o conteúdo salivar do vetor durante o processo de alimentação (HOGENHOUT et al., 2008).

Na terceira categoria de interação vírus-vetor designada como propagativa, o vírus completa seu ciclo de forma semelhante à segunda categoria descrita anteriormente, porém replica-se no interior do intestino e/ou em outros tecidos do inseto. Muitas vezes, a taxa de transmissão de partículas virais para o interior das plantas é menor quando comparada a aquisição pelo vetor, uma vez que o movimento do vírus através do intestino é uma barreira significativa para sua transmissão (NAGATA et al., 2002; AMMAR e HOGENHOUT, 2008).

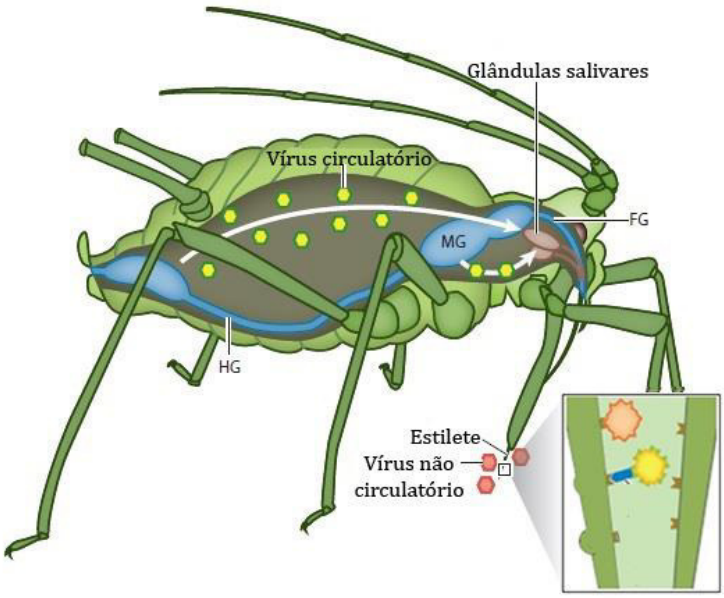

Figura 2. Diferentes rotas de vírus de plantas em seus afídeos vetores. No desenho ilustrativo do pulgão, o intestino está representado em azul e as glândulas salivares e o ducto salivar em marrom. As setas brancas representam o ciclo dos vírus circulatórios (hexágonos amarelos) dentro do corpo do pulgão, através do epitélio intestinal para a hemolinfa e / ou outros órgãos, e, finalmente, para as glândulas salivares. Os vírus não circulatórios aparecem em seus locais de anexo na ponta dos estiletes como hexágonos vermelhos. O quadro ampliado na parte inferior direita demonstra o canal salivar localizado na ponta do estilete da maxila do pulgão. Os vírus não circulatórios interagem com receptores presentes na cutícula do estilete. Abreviações: FG (parte anterior do trato digestivo); HG (parte posterior do trato digestivo); MG (intestino médio). Adaptado de Blanc et al. (2014).

1.2.*Citrus tristeza virus*: genoma, replicação e movimentação no hospedeiro

A espécie *Citrus tristeza virus*, membro da família *Closteroviridae*, gênero *Closterovirus*, é um vírus de partículas flexuosas e longas em formato helicoidal medindo cerca de 11 x 2.000 nm de tamanho com material genético de fita simples do tipo RNA de polaridade positiva (KITAJIMA et al., 1964; BAR- JOSEPH et al., 1989).

As partículas virais do CTV são bipolares e encapsuladas por duas proteínas de revestimento de 25 kDa (p25) e 27 KDa (p27). A principal proteína de revestimento (CP) recobre entre 95 a 97% do genoma viral, enquanto a segunda e menor proteína de revestimento (CPm) encapsula 3 a 5% da porção terminal, formando a cobertura viral (SATYANARAYANA et al., 2004).

O CTV possui um genoma de tamanho estimado de 20 quilobases (Kb), o maior relatado de vírus vegetal, organizado em 12 Open Reading Frames (ORFs) codificando pelo menos 17 proteínas com duas regiões não traduzidas (UTRs) na extremidade 5' 3', respectivamente (BAR-JOSEPH, 1985; KARASEV et al., 1995; FLORES et al., 2013), ver Figura 2. A replicação das cadeias genômicas de RNA do CTV depende diretamente da síntese de cadeias de polaridade negativa que servem de molde para a geração de novas fitas positivas (SATYANARAYANA et al., 2002).

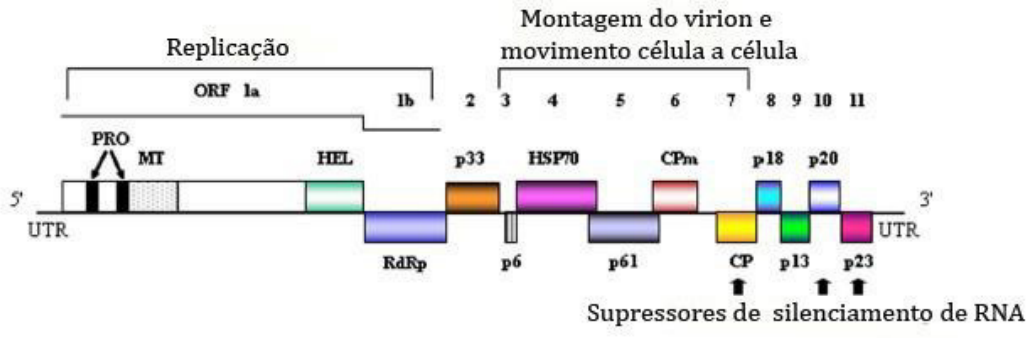

Figura 3. Organização genômica do *Citrus tristeza virus*. Representação esquemática do RNA genômico do CTV em blocos que denotam quadros de leitura aberta (ORFs) flanqueados pelas regiões não traduzidas (UTRs). ORFs 1a e 1b contêm vários domínios: PRO, protease tipo papaína; MT, metiltransferase; HEL, helicase; RdRp, RNA polimerase - dependente da RNA. O papel funcional de alguns desses produtos proteicos está indicado na figura (Replicação, Movimentação do vírus e Supressores de silenciamento de RNA). Adaptado de Flores et al. (2013).

Na porção terminal 5’ estão presentes as ORFs 1a e 1b os quais são responsáveis por codificarem proteínas relacionadas à replicação viral. Já na porção 3’ estão as ORFs 2-11 as quais codificam proteínas p33, p6, p65, p61, p27, p25, p18, p13, p20 e p23 relacionadas na interação com hospedeiro, montagem viral, especificidade vetorial e translocação no interior da planta (PAPPU et al., 1994; KARASEV et al., 1995; DOLJA et al., 2006).

A ORF 1a codifica uma poliproteina de aproximadamente 349 KDa, a qual possui dois domínios semelhantes a papaina-like-protease (PRO): metiltransferase (MT) e helicase (HEL). Enquanto a ORF 1b, via “frameshift”, codifica uma proteína de 54 kDa, que contem domínios de RNA polimerase dependende de RNA (KARASEV et al., 1995; BAK E FOLIMINOVA, 2015).

As regiões do genoma importantes para a replicação e montagem do virion no interior do hospedeiro foram estudadas, sendo mapeadas e descritas as funções de cada proteína que compõe o genoma do CTV (MORENO et al., 2008). A ação das proteínas CP, CPm, p65 (homóloga HSP70) e p61 estão envolvidas na montagem dos virions no interior do hospedeiro (SATYANARAYANA et al., 2002); a proteína hidrofóbica p6 possui papel no movimento do vírus no interior da planta (DOLJA et al., 2006; SATYANARAYANA et al., 2004); p20 e p23 juntamente com CP são supressores do silenciamento do RNA; p33, p18 e p13, possuíam funções desconhecidas até recentemente, porém estudos demonstraram que estas proteínas são dispensáveis para a infecção sistêmica em diferentes variedades cítricas (LU et al., 2004; FOLIMINOVA et al., 2008; TATINENI et al., 2008). Pesquisadores observaram que a presença do gene p33 no genoma do CTV permite a infeção da variedade laranja azeda, limão, toranja e calamondin, enquanto p13 e p18 demonstraram funções similares nos dois últimos hospedeiros. Por outro lado, a ausência do p33 e p18 permite a infeção por CTV em plantas cítricas de toranja, enquanto p13 permite a infeção em calamondin (TATINENI et al., 2011).

A expressão do genoma de CTV inclui pelo menos três mecanismos comumente utilizados por vírus de RNA de cadeia positiva, sendo estes: processamento proteolítico, alterações nos quadros de leitura nas sequências dos ribossomas e formação de uma sequência de RNA subgenômico localizado na região coterminal (KARASEV et al., 1995; GOWDA et al., 2001; SATYANARAYANA et al., 2002; GOWDA et al., 2003). Os dez genes (p33, p6, p65, p61, p27, p25, p18, p13, p20 e p23) localizados na parte terminal 3' da cadeia genômica de CTV são expressos por síntese de RNA subgenômico da região 3’ que atua como RNAs mensageiros do ORF localizado na parte terminal 5', para ser traduzido (HILF et al., 1995).

Um grupo semelhante e adicional de RNA subgenômico (sgRNA) de cadeia negativa também é produzido nas células infectadas, mas estas acumulam 40-50 vezes menos do que as produzidas pelas cadeias positivas do RNA subgenômico (SATYANARAYANA et al., 2002; VIVES et al., 2005; DOLJA et al., 2006).

De forma similar, fitas positivas 5' de sgRNA–coterminal são produzidas por meio da terminação prematura da síntese de gRNA. Embora a função biológica dessas fitas seja atualmente desconhecida, a coexistência de fitas positivas e negativas de múltiplas espécies de RNAs em células infectadas sugerem que estas possam auxiliar na formação de moléculas de RNA recombinante (GOWDA et al., 2001; DOLJA et al., 2006).

O CTV por ser um vírus RNA de fita simples, está mais propenso a mutações na região menos conservada do seu genoma (LAI, 1992). Estudos demonstraram que a variação genética ocorre de forma desigual no genoma do CTV, sendo a região 5'-UTR a menos conservada (LÓPEZ et al., 2000). As variações nas sequências constatadas e posterior análise filogenética de distintos isolados permitiu o agrupamento destes em seis distintos subgrupos, classificados como T36, VT, T30, B165, HA16-5 e T68 (KARASEV et al., 1995; MAWASSI et al., 1996; MELZER et al., 2010; BISWAS et al., 2012).

Dessa forma, a recombinação entre os isolados divergentes é uma das principais forças geradoras de diversidade genética na formação dos complexos virais, uma vez que um isolado viral de CTV é composto por dois ou mais haplótipos distintos a nível molecular (BAR-JOSEPH et al., 2002).

A maioria dos vírus de plantas utilizam o sistema de movimentação especifico, por meio da codificação de proteínas, assim como proteínas do hospedeiro que facilitam sua translocação através dos canais do plasmodesma (BENETIZ-ALFONSO et al., 2010). Esse sistema é bem conhecido e compreendido, no entanto, o mecanismo de transporte à longa distância via floema, como dos vírus pertencentes ao gênero *Closterovirus,* ainda não está bem elucidado (BAK e FOLIMINOVA, 2015).

O CTV segue geralmente o padrão de movimentação via floema, porém o grau de entendimento da ocorrência desse processo é limitado, uma vez que há diferenças em relação a cada espécie de citros (DAWSON et al., 2013). Em geral, acredita-se que o CTV movimenta-se no interior da planta em forma de partículas virais (PRICE, 1966), sendo sugerido que as proteinas (HSP70h, p61, CP e COM) envolvidas na montagem dos virions, além da hidrofóbica p6, estão envolvidas na movimentação desse (TATINENI et al.,2008).

1.3.Origem da tristeza dos citros

O centro de origem da tristeza dos citros é incerto, porém pesquisas sugerem que essa doença teve início em países do Continente Asiático e arquipélago Malaio, uma vez que nessas áreas foram descritas as primeiras espécies cítricas sendo possivelmente propagada para outros países por meio de borbulhas infectadas (MOREIRA, 1954; MORENO et al., 2008).

O século 19 pode ser considerado como um marco para a citricultura mundial, iniciando-se a expansão da cultura para todo o mundo. Associado a esta teve origem o registro de moléstias que causaram perdas significativas em pomares de citros (COSTA, 1949; BAR-JOSEPH, 1989).

O primeiro e mais expressivo registro ocorreu nas ilhas de Açores, onde uma grave doença que atacava as raízes dos citros foi registrada, sendo esta atribuída ao fungo *Phytophthora* (BORDIGNON et al., 2003). Diante de tal fato, os produtores foram forçados a utilizar porta enxerto de laranja Azeda (*Citrus aurantium* L.) resistente ao fungo em seus pomares, porém, logo notou-se o insucesso do uso deste porta enxerto em países como Austrália, Sul da África e Java (MENEGHINI, 1946; BENNET e COSTA, 1949; MÜLLER et al., 2005).

Apesar da ocorrência deste fenômeno observado nas cultivares cítricas ser incipiente em regiões como Java, Austrália e África do Sul (BORDIGNON et al., 2003), a primeira grande dizimação de pomares ocorreu no ano de 1930, em Corrientes – Argentina, a qual ocasionou a perda de cerca de 10 milhões de árvores em 15 anos (BAR-JOSEPH, 1989).

A primeira hipótese levantada como possível causa dessa moléstia fundamentava-se em uma provável incompatibilidade varietal entre copa e porta enxerto, anos mais tarde essa hipótese foi descartada e atribuída a um patógeno de origem viral (BORDIGNON et al., 2003).

No Brasil, após o primeiro relato da doença no ano de 1937, iniciou-se vários estudos na tentiva de elucidar o agente causal da enfermidade em citros (BENNETT e COSTA, 1949; BAR-JOSEPH et al., 1989). Dentre várias pesquisas e hipóteses sugeridas, Meneghini (1946) foi o pioneiro em executar e descrever a teoria mais concreta. O pesquisador sugeriu que a patologia observada era causada por um vírus, a qual foi evidenciada por meio de transmissão afidial pelo vetor *Toxoptera citricida* (Kirkaldy). Sua teoria foi comprovada por Bennett e

Costa (1949) ao repetirem seus ensaios e atestarem a efetiva transmissão do vírus pelo vetor além de descreverem outro meio de disseminação, através de borbulhas infectadas.

A partir de então, o objetivo dos pesquisadores era efetuar a caracterização do vírus em estudo. Após anos de investigação, os pesquisadores do Departamento de Virologia do Instituto Agronômico de Campinas- BR confirmaram por meio da microscopia eletrônica a natureza do patógeno através da observação das partículas virais presentes no floema de plantas infectadas com a tristeza dos citros (KITAJIMA et al., 1964).

A expressão dos sintomas induzidos pelo CTV pode variar de acordo com a severidade das estirpes virais, combinação das espécies copa-porta enxerto e variação ambiental (MORENO et al., 2008). Os sintomas típicos desencadeados pelo CTV são declínio rápido das plantas, porte reduzido, caneluras, amarelecimento do pé franco e diminuição no tamanho dos frutos (DAWSON et al., 2013). Alguns desses sintomas podem ser observados na Figura 4.

Figura 4. Sintomas fenotípicos induzidos pelo vírus *Citrus tristeza virus*. (A) Caneluras (depressões no lenho); (B) Planta de laranja doce da variedade Pêra infectada com uma estirpe severa do vírus, apresentando sintomas de porte reduzido; (C) Diminuição no tamanho dos frutos (seta branca) quando comparados ao fruto obtido de planta não infectada (seta preta). (Fotos 1A e 1C foram cedidas gentilmente pelo Prof. Dr. Gerd Walter Müller).

1.4.*Toxopetra citricida*: descrição, ciclo de vida e biologia

O CTV pode ser transmitido por várias espécies de afídeos, entre as quais o pulgão preto (*Toxoptera citricida* Kirkaldy) (Hemiptera: Aphididae) é o vetor mais eficiente na transmissão (YOKOMI et al., 1994). De possível origem Asiática (MICHAUD, 1998), acredita-se que sua dispersão para América do Sul tenha ocorrido a partir do Brasil ou Argentina durante a década de 20, quando se iniciou a expansão da cultura dos citros e importação de material vegetal de distintos países (ROCHA-PEÑA et al., 1995).

O pulgão preto dos cítros na fase adulta (sem asas) apresenta dimensões de 1,5 a 2,4 mm, coloração marrom escura a preta brilhante e as ninfas possuem coloração castanha escura (Figura 5). A forma alada do pulgão distingue-se pelos segmentos escuros presentes em sua antena, portanto alados são menos comuns de serem encontrados na natureza, pois tendem a deixar a colônia logo após emergirem em busca de novos hospedeiros (HALBERT e BROWN, 1996).

Figura 5. Forma adulta (sem asa) e ninfas do *Toxoptera citricida* em brotos de laranja (*Citrus sinensis*), variedade Pêra.

O pulgão se alimenta de brotos, folhas e botões de flores recém-expandidos de seus hospedeiros. O inseto possui dois tipos de reprodução: sexuada em regiões frias, por exemplo, Japão, e assexuada em regiões tropicais e subtropicais, como Brasil podendo alcançar até 30 gerações anuais dependendo da disponibilidade de hospedeiros (MICHAUD, 1998). O tempo de desenvolvimento varia de acordo com a temperatura; a 20ºC o *T. citricida* possui um período de desenvolvimento ninfal de 6-8 dias, com período pré-reprodutivo de 8 dias e longevidade de 28 dias. Já sob temperatura abaixo de 9ºC, o ciclo se completa em até 125 dias (YOKOMI, 2009).

Com transmissão do tipo semi-persistente, o pulgão pode adquirir e transmitir o vírus de plantas infectadas com tempo de alimentação entre 5 a 10 minutos, porém a eficiência da transmissão pode ser aumentada com alimentação de até 24 horas (COSTA et al.,1951). Após aquisição do vírus pelo inseto, este pode ser transmitido em até 30 minutos para novo hospedeiro ou permanecer virulífero por até 48 horas, não sendo necessário o período de latência, uma vez que a partícula viral não se multiplica ou circula no corpo do inseto (YOKOMI, 2009). Esse tipo de transmissão bimodal é favorecida por intermédio das interações entre proteínas capsidiais e cuticulares do vírus e do vetor, respectivamente (HERRON et al., 2006).

Dessa forma, a combinação da propagação afidial associada ao tipo de transmissão semi-persistente, têm contribuído para disseminação da tristeza dos citros por meio do *T. citricida* (MORENO et al., 2008). Porém, vários fatores influenciam na disseminação do vírus, incluindo a espécie e densidade dos pulgões associados, natureza da estirpe viral, variedade da planta doadora-receptora e condições ambientais (HERRERA-ISIDRON et al., 2009) .

2. Pisilídeos como vetores de bactérias

Nos últimos 30 anos, fitobactérias associadas a insetos vetores têm ocasionado perdas econômicas significativas em diversas culturas em todo o mundo (NADARASAH e STAVRINIDES, 2011). Os Phytoplasmas (originalmente chamados de organismos semelhantes à micoplasma) e bactérias fitopatogênicas (Ex. *Liberibacter* spp.), são importantes agentes que causam mais de 700 doenças em distintas espécies de plantas, muitas das quais são vetoriadas por insetos pertencentes à ordem Hemiptera (SECOR e RIVERA-

VARAS, 2004), das famílias Cicadellidae, Cixidae, Psyllidae, Cercopidae, Delphacidae, Derbidae, Menopilidae e Flatidae (WEINTRAUB et al., 2006).

A ordem Hemiptera, o grupo mais bem sucedido de insetos vetores de fitobactérias e phytoplasmas, possuem várias características morfológicas e fisiológicas que tornam seus membros eficientes vetores, estas foram descritas por Weintraub e Beanland (2006): (I) são hemimetábolos, dessa forma as ninfas e os adultos se alimentam de forma similar, muitas vezes tanto imaturas quanto adultos podem transmitir phytoplasmas e fitobactérias; (II) se alimentam especificamente e seletivamente em certos tecidos vegetais, os que o torna vetores especializados e eficientes de agentes patogênicos que residem nesses tecidos vegetais; (III) possuem aparelho bucal especializado promovendo sucesso na inoculação dos patógenos no sistema vascular da planta, sem danificar seus tecidos condutores e provocar respostas defensivas; (IV) possuem relação propagativa e persistente com patógenos.

Os psilídeos, pertencentes à superfamília Psylloidea são importantes componentes da fauna dos hemípteras, os quais constituem um pequeno grupo de aproximadamente 3800 espécies (BURCKHARDT et al., 2014). São conhecidos por apresentarem alimentação altamente especializada, uma vez que se alimentam de uma restrita gama de plantas hospedeiras (BONANI et al., 2010). Sua nutrição é obtida por meio dos tecidos do floema e dependem de endossimbiontes primários para que sintetizem e complementem sua dieta com aminoácidos essenciais (TAMBORINDEGUY et al., 2017).

Recentemente, vários estudos têm descrito as espécies *Trioza erytrea; Diaphorina citri* Kuwayama*, Bactericera cockerelli* (Sulc) como vetores das bactérias do gênero Liberibacter, incluindo *Candidatus* Liberibacter africanus (Laf), *Candidatus* Liberibacter asiaticus (Las*), Candidatus* Liberibacter americanus (Lam) e *Candidatus* Liberibacter solanacearum (Lso), dentre as quais Laf, Las, Lam estão associadas com o Citrus Greening e Lso está associada com enfermidades em solanáceas (GRAFTON-CARDWELL et al., 2013; TAMBORINDEGUY et al., 2017).

2.1.Bactéria-Vetor: Mecanismos de interação e transmissão

Os agentes fitopatogênicos das plantas estão onipresentes em ecossistemas gerenciados e naturais. A epidemiologia desses patógenos depende da dinâmica de sua interação com seus vetores. Essa dependência gera interações complexas envolvendo

microrganismo- inseto- planta-ambiente (EIGENBRODE et al., 2017). A associação entre os microrganismos e insetos pode resultar em distintas interações simbióticas as quais podem afetar na aptidão do inseto. Essas associações estão bem descritas na literatura como mutualismo, parasitismo e comensalismo (LEUNG e POULIN, 2008, SU et al., 2013, SOLOMON et al., 2015).

O mutualismo pode ser definido como a interação entre dois organismos de espécies distintas em que a aptidão de cada indivíduo é aumentada pela ação do outro (JANZEN, 1985). Este pode ser de natureza defensiva, nas condições onde o microrganismo confere proteção ao inseto hospedeiro, ou nutricional, onde o microrganismo complementa a dieta do inseto com nutrientes-chave (WILKINSON et al., 2000; NADARASAH e STAVRINIDES, 2011).

As interações mutualísticas são prevalentes em todos os tipos de ecossistemas e desempenham papel essencial para o equilíbrio das comunidades (TRAVESET E RICHARDSON, 2014). Em virtude disso, a interação mutualística pode ser observada entre diversos organismos. Por exemplo, a relação *Pseudomonas* sp.-*Paederus riparus* na qual a bactéria ajuda a proteger o inseto contra predação (PIEL, 2002) e *Wolbachia sp.-Drosophila melanogaster* evitando parasitismo por vírus (TEIXEIRA et al., 2008). Porém, em casos extremos, as bactérias mutualistas podem tornar-se patógenos oportunistas uma vez que estas encontrem condições ambientais favoráveis, como ocorre com a espécie *Photorhabdus luminescens,* uma vez que a mesma pode ser mutualista ou patogênica dependendo se ela habita o intestino do seu hospedeiro nematoide ou a hemolinfa (DILLON e DILLON, 2004).

Sobretudo, o sucesso das associações mutualísticas entre inseto-patógeno é frequentemente desafiada pela invasão de microrganismos que prejudicam o inseto ou seus parceiros microbianos benéficos, sendo essa classificada como uma associação parasitária (BIEDERMANN e ROHLFS, 2017). O parasitismo pode também ocorrer entre microrganismos e insetos, porém nessa interação apenas o microrganismo se beneficia com a retirada de nutrientes do seu hospedeiro (NADARASAH et al., 2011).

Os estudos das interações hospedeiro-parasita fundamentam-se principalmente nas duas vertentes: virulência e patogenicidade. A virulência é definida como a capacidade do parasita se multiplicar a fim de garantir o sucesso da sua transmissão, e a patogenicidade refere-se a aspectos da associação entre hospedeiro-parasita resultando em danos apenas ao hospedeiro (ARAÚJO et al., 2003; ALIZON et al., 2009). Assim definida, exemplos de parasitismos foram descritos entre as seguintes interações: *Arsenophonus nasoniae* e espécies

de Diptera (TAYLOR et al., 2011); fungo *Gregarina cuneata -Tribolium castaneum* (GIGLIOLLI et al., 2016).

Diferente do parasitismo, o reconhecimento de que a interação entre patógeno-hospedeiro nem sempre ocasiona em danos e/ou doença resultou na inclusão da associação comensalista, a qual caracteriza-se como uma associação simbiótica entre hospedeiro e microrganismo em que o microrganismo é beneficiado, mas o inseto não é afetado (CASADEVALL e PIROFSKI, 2000). Por todas essas razões, as associações comensais podem variar de altamente específicas a inespecíficas. As interações específicas são favorecidas nos casos em que o microrganismo especializado encontra insetos também especializados repetidas vezes durante longos períodos, contudo, as interações mais gerais (inespecíficas) ocorrem quando o inseto transitório encontra microrganismos especializados (ou vice-versa) (NADARASAH et al., 2011).

Alguns patógenos de plantas completam seu ciclo de vida no inseto vetor ou na planta hospedeira, mas não em ambos, enquanto outros se multiplicam tanto no inseto como no interior do vegetal hospedeiro, possuindo assim a capacidade de infectar dois reinos evolutivamente divergentes (HOGENHOUT et al., 2003; CICERO et al., 2016).

Os dois principais modos pelos quais insetos transmitem agentes patogênicos bacterianos para as plantas são circulante e não circulante, enquanto a capacidade da bactéria mover-se no interior no inseto classifica-se como propagador e não propagador (NAULT, 1997; NG e FALK, 2006). Em resumo, na transmissão circulante não propagativa, a bactéria é ingerida pelo inseto vetor durante sua alimentação na planta hospedeira, em seguida ela migra para o trato digestivo superior mantendo-se nesse tecido para em seguida ser regurgitada durante o processo de alimentação (BACKUS et al., 2015). Não ocorrendo replicação do microrganismo no interior do inseto vetor (LIST, 1939). Em contraste, fitobactérias transmitidas de modo circulante/propagativa, a bactéria possui capacidade de replicar-se no interior do vetor e espalhar-se para outros órgãos dentro do inseto (KILLINY et al., 2010). As bactérias são translocadas através do trato digestivo até atingir a hemolinfa, de onde transitam e entram nas glândulas salivares, antes da inoculação na planta hospedeira (KWON, 1999). Por fim, na transmissão não circulante/não propagativa ocorre uma associação física entre inseto/bactéria, onde posteriormente microrganismos são transmitidos mecanicamente para planta hospedeira, ou seja o microrganismo se mantém no aparelho bucal (estilete) (NG e FALK, 2006; UZEST et al., 2007).

2.2.*Candidatus* Liberibacter solanacearum: genoma, replicação e movimentação na planta hospedeira

O gênero Liberobacter foi inicialmente proposto por Jagoueix et al. (1994) com a descrição e inclusão de duas espécies: *Candidatus* Liberobacter asiaticum e *Candidatus* Liberobacter africanum com base nas sequências do gene rplKAJL–rpoBC operon (β operon). Anos depois, a nomenclatura das duas espécies foi revisada e renomeada *Candidatus* Liberibacter asiaticus e *Candidatus* Liberibacter africanus para se adequar com o Código Internacional de Nomenclatura de Bactérias (GARNIER et al., 2000).

No ano de 2004, a terceira espécie foi descrita e denominada *Candidatus* Liberibacter americanus, referindo-se ao fato de que a primeira detecção e ocorrência da espécie foram no continente americano, mais precisamente no Brasil (TEIXEIRA et al., 2005). Posteriormente, análises filogenéticas baseadas nas sequências do gene 16S rRNA de isolados bacterianos obtidos a partir de plantas de *Capsicum annuum* (pimenta), *Physalis peruviana* (tomatillo), *Solanum betaceum* (tamarillo), *Solanum lycopersicum* (tomate) e *Solanum tuberosum* (batata), demonstraram se tratar de uma nova espécie do gênero Liberibacter, sendo intitulada *Candidatus* Liberibacter solanacearum (LIEFTING et al., 2009).

As bactérias pertencentes ao gênero Liberibacter são gram-negativas, não cultiváveis e limitadas ao floema das plantas hospedeiras (TEIXEIRA et al., 2005; BOVÉ, 2006; HANSEN et al., 2008). Imagens obtidas por meio de microscopia eletrônica de varredura revelaram que *Candidatus* Liberibacter solanacearum possuem morfologia em formato baciliforme e alongado, 4 μm de comprimento com extremidades arredondadas, 0,2 μm de largura e com uma superfície externa lisa (TANAKA et al., 2007; LIEFTING, 2009a).

O genoma de *Candidatus* Liberibacter solanacearum (Figura 6) foi descrito por Lin et al. (2011) com as seguintes características: composto por 35,24% G + C, com um cromossoma de 1,26 Mpb que possuem 1.192 regiões codificantes. Destas 848 foram determinadas usando a base de dados do Cluster Ortólogo e aproximadamente 35% das sequências codificam proteínas hipotéticas. Os cientistas também identificaram três operons completos de RNA ribosomal (16S, 23S e 5S) e 45 genes que codificam RNAs de transferência e pelo menos 35 prováveis pseudogenes dentro do genoma de *Candidatus* Liberibacter solanacearum. Ao efetuarem a comparação dos genomas de *Candidatus* Liberibacter solanacearum e *Candidatus* Liberibacter asiaticum detectaram 884 sequências codificadoras de proteínas comuns a ambos os organismos. Também foram identificados

genes derivados de fago, incluindo uma lisozima relacionada ao fago e uma proteína da família primase.

Os eventos fundamentais do ciclo celular de uma célula procariótica são similares ao de uma célula eucariótica: replicação do genoma, duplicação do DNA, separação da célula em duas por meio da citocinese e posterior crescimento celular (GOLEY et al., 2007). Em estudos realizados em *Candidatus* Liberibacter solanacearum, demonstraram que as proteínas *Cell cycle transcriptional regulator* (CtrA), *Chromosomal replication initiator protein* (GcrA) e *Cell cycle regulator* (DnaA) codificadas pelo genoma da bactéria são as principais reguladoras do ciclo celular bacteriano e podem ser alvo de pequenas moléculas inibidoras as quais podem influenciar no crescimento e /ou replicação da bactéria (CHENG et al., 2007).

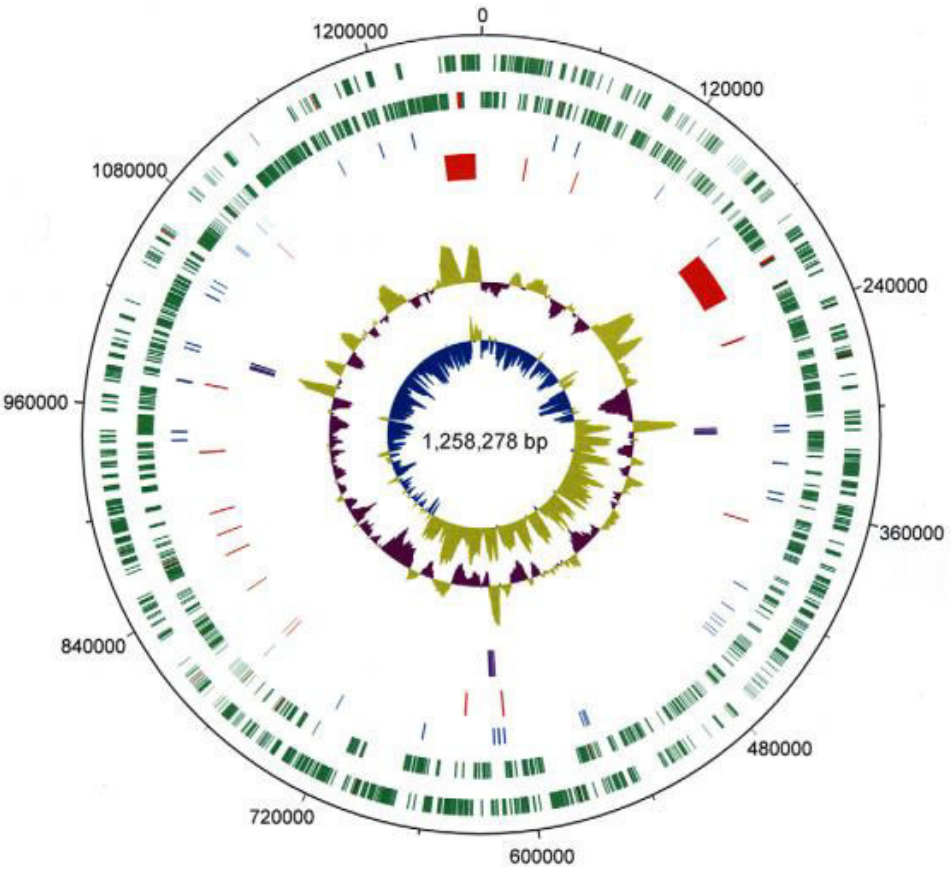

Figura 6. Representação esquemática do genoma *Candidatus* Liberibacter solanacearum. Representação circular do genoma de 1,26 Mbp. As faixas dos círculos externos representam (1) ORFS senso (verde) e (2) ORFS antisenso (verde) com pseudogenes em vermelho; (3) tRNA (azul); (4) regiões derivadas do bacteriófago e remanescentes prováveis de fago (vermelho); (5) três cópias do operon rRNA (16S, 23S e 5S) (roxo); (6)% de conteúdo de G + C. Adaptado de Lin et al.(2011).

Além disso, pesquisadores também constataram que apenas uma parte dos genes envolvidos na divisão celular foram mantidos em *Candidatus* Liberibacter solanacearum ao

longo da evolução, porém o significado dessas perdas de genes ainda permanece desconhecido (LIN et al., 2011). Por outro lado, aos estudarem os genes relacionados à locomoção bacteriana no interior na planta hospedeira, não foi possível identificar genes codificadores para formação de flagelos, portanto o que se sabe é que sua movimentação no interior do vegetal depende do fluxo dos tecidos do floema para que a bactéria alcance diferentes regiões como raíz e parte aérea (NELSON et al., 2013).

Recentemente, cinco diferentes haplótipos de *Candidatus* Liberibacter solanacearum (Lso) foram identificados: LsoA, LsoB, LsoC, LsoD e LsoE (NELSON et al., 2011, NELSON et al., 2013, TERESANI et al., 2014). Os haplótipos A e B estão associados com psilídeo da batata, *Bactericera cockerelli* (Sulc.) (NELSON et al., 2011) nas Américas e Nova Zelândia, enquanto os haplótipos C, D e E estão associados com *Trioza apicalis* e *Bactericera trigonica* no velho mundo (MUNYANEZA et al., 2010, TERESANI et al., 2014).

2.3.Origem da doença “zebra chip”

Por volta de 1990, uma moléstia de causa desconhecida começou a chamar a atenção dos produtores de batata (*Solanum tuberosum* L.) do México e partes da América Central (SECOR e RIVERA-VARAS, 2004; MUNYANEZA et al., 2007a; LIEFTING et al., 2008). Os sintomas observados se assemelhavam com aqueles causados por Phytoplasmas, incluindo enrolamento ascendente das folhas, clorose, tubérculos aéreos, proliferação de gemas axilares, nanismo, escurecimento do sistema vascular, senescência precoce e coloração escura do anel vascular ao longo do tubérculo (CROSSLIN et al., 2010). Os tubérculos frescos apresentavam descoloração marrom quando cortados e depois de fritos as manchas tornavam-se mais proeminentes (MUNYANEZA, 2012), ver Figura 7. Devido aos sintomas característicos observados nos tubérculos, o termo "zebra chip" (ZC) foi usado para descrever a doença e essa designação tornou-se bem estabelecida na literatura (CROSSLIN et al., 2010).

No ano 2000, sintomas similares foram observados em campos comercias de batatas no Sul do Texas, sendo este o primeiro registro da moléstia nos Estados Unidos (MUNYANEZA et al., 2007a, b; SECOR et al., 2009). Desde então, ZC foi relatada em todo o sudoeste dos Estados Unidos, México, América Central e mais recentemente, em Nova Zelândia (MUNYANEZA et al. 2007a,b, 2009b; LIEFTING et al., 2008; SECOR et al., 2009; CROSSLIN et al., 2010; REHMAN et al., 2010).

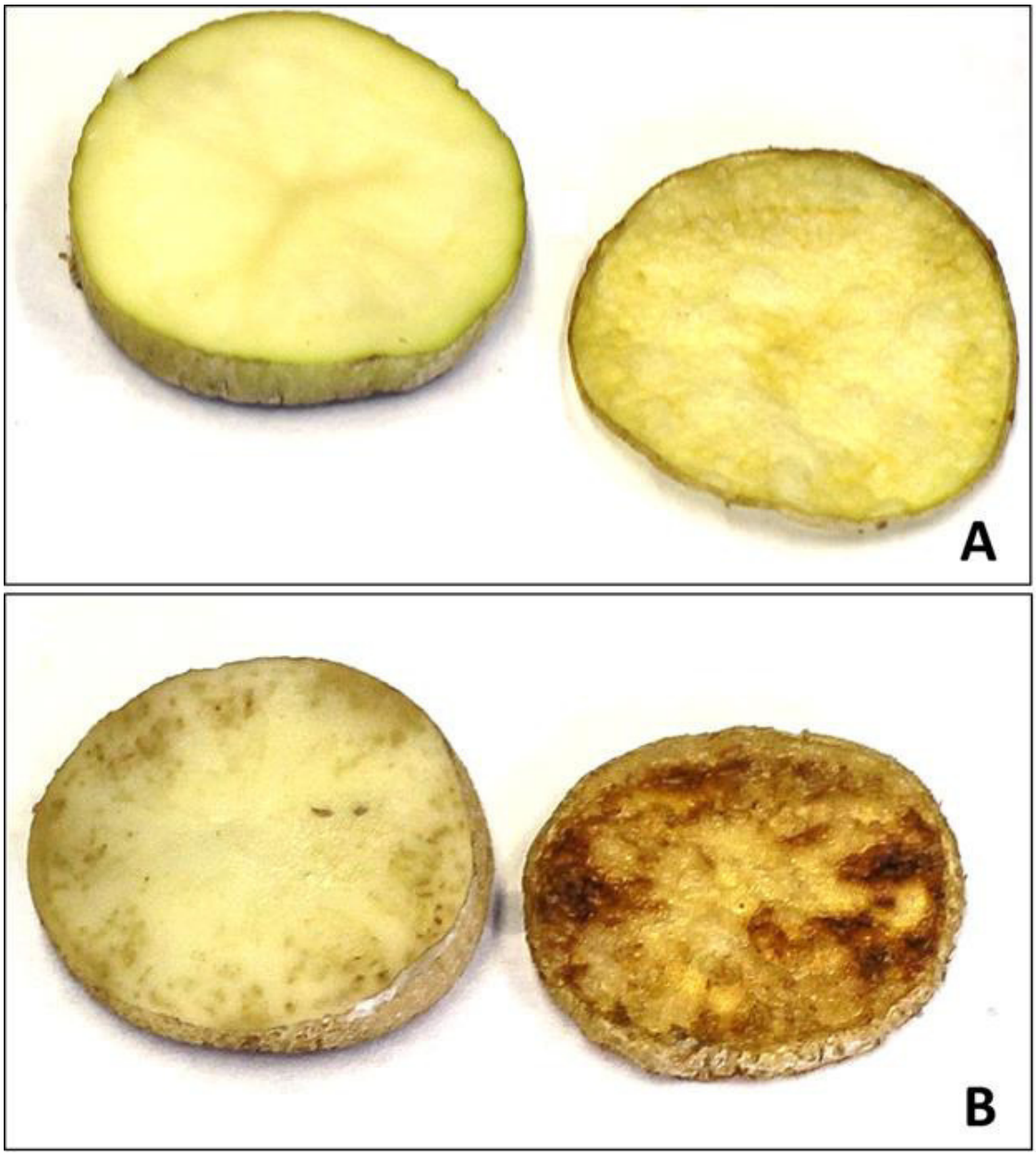

Figura 7. Sintomas de "Zebra chip" em tubérculos de batatas frescos (esquerda) e fritos (direita). (A) Tubérculos de batata obtidos de plantas livres de infeção com Lso (B) Tubérculos de batata obtidos de plantas infectadas com Lso. (Fonte: fotos cedidas gentilmente pelo Dr. Julien Levy - Tamborindeguy Laboratory- Texas A&M University).

Devido a grande disseminação da enfermidade observada nos campos de batata, os fitopatologistas iniciaram vários estudos para elucidar o agente causal da doença "Zebra chip" (SECOR et al., 2006; SECOR et al., 2009; NAVARRE et al., 2011). Inicialmente, suspeitou-se tratar-se da já conhecida **"**potato purple top wilt" causada pelo patógeno '*Candidatus* Phytoplasma americanum', uma vez que os sintomas observados se assemelhavam a essa

doença já descrita (LEE et al., 2004). Portanto, por meio de marcadores moleculares essa hipótese foi descartada e proseguiu-se a analisar outros fitopatógenos: *Tomato spotted wilt virus*, *Tobacco rattle virus*, *Alfalfa mosaic virus*, *Potato leafroll virus*, *Potato mop top virus*, *Potato virus Y*, *Xylella fastidiosa* e *Serratia marescens,* mas novamente os resultados demonstraram que nenhum desses patógenos era o agente causal da enfermidade estudada (NAVARR E et al., 2011).

Simultâneo as análises moleculares, testes de enxertia entre plantas sintomáticas de batata e plantas livres da doença foram realizados a fim de determinar se havia um agente infeccioso associado com a doença "zebra chip". Os pesquisadores observaram que as plantas receptoras começaram a exibir sintomas semelhantes às plantas originais (infectadas). Esses resultados estabeleceram fortes indícios de que um microrganismo estava envolvido na doença (CROSSLIN e MUNYANEZA, 2009; SECOR et al., 2009).

O primeiro avanço para o real entendimento do patógeno associado com a doença "Zebra chip" ocorreu a partir da observação por meio da microscopia eletrônica de organismos similares a bactérias em tecidos vasculares de plantas infectadas (BOER et al., 2007). Posteriormente, com uso de técnicas moleculares, pesquisadores confirmaram que a doença estava associada a uma bactéria pertencente ao gênero Liberibacter (LIEFITING et al., 2008). Baseando-se em sequências do gene 16S rRNA de isolados bacterianos de plantas de batata sintomáticas, se identificou o agente causal da doença "zebra chip", *Candidatus* Liberibacter solanacearum (LIEFITING et al., 2009). Em locais onde se encontraram uma alta incidência de 'Zebra chip', também eram observados a presença de populações do psilídeo da batata, *Bactericera cockerelli* (LIEFITING et al., 2008).

2.4.*Bactericera cokerelli*: descrição, ciclo de vida e biologia

O psilídeo da batata, *Bactericera cockerelli* (Sulc) (Psylloidea: Triozidae) é vetor da bactéria *Candidatus* Liberibacter solanacearum, uma das fitobactérias mais destrutivas da cultura da batata no hemisfério ocidental (MUNYANEZA et al., 2007a; HANSEN et al., 2008; WU et al., 2016). A espécie *Bactericera cockerelli* descrita por Sulc (1909) é nativa da América do Norte e atualmente encontra-se disseminada por vários países incluindo Canadá, El Salvador, Guatemala, Honduras, México, Nicaragua, Estados Unidos, Austrália e Nova Zelândia (EPPO, 2017).

De natureza polífaga, *B.cockerelli* possui uma ampla gama de hospedeiros que excede 20 famílias de plantas, com forte preferência por espécies das solanáceas (BUTLER e TRUMBLE, 2012). O clima é um elemento importante para o desenvolvimento do psilídeo da batata. O vetor é adaptado a temperaturas quentes, mas não muito elevadas (MUNYANEZA, 2012). O Clima frio durante as migrações, ou pelo menos a ausência de temperaturas elevadas, têm sido correlacionadas com aumento populacional deste inseto. O desenvolvimento do psilídeo é favorecido em temperaturas de aproximadamente 27ºC enquanto a ovoposição, incubação e a sobrevivência são reduzidas a 32ºC cessando a 35ºC (ABDULLAH, 2008).

O ciclo de vida do psílido da batata é caracterizado por ser hemimetábolo, possuindo três estágios de vida: ovo, ninfa e adulto (Figura 8). Os ovos são de cor amarelo-alaranjada, de forma oblonga com um comprimento médio e largura de 0,3 mm e 0,1 mm, respectivamente (BUTLER e TRUMBLE, 2012). Os ovos podem ser depositados na parte abaxial e adaxial da folha da planta hospedeira e anexados com sua haste de 0,2 mm de comprimento (BUTLER e TRUMBLE, 2012). As ninfas de *B. cockerelli* passam por cinco estádios ninfais e a conclusão do desenvolvimento ninfal leva em média 15 dias (com um intervalo de 12 a 44 dias) (YANG e LIU, 2009, NACHAPPA et al., 2012). Os primeiros quatro estádios exigem uma média de 2,4-2,8 dias para completar seu desenvolvimento, enquanto o quinto estádio pode se completar em até 4 dias (BUTLER e TRUMBLE, 2012). As ninfas do primeiro estádio possuem coloração pálida com cabeça e abdômen de cor laranja, ao decorrer do seu desenvolvimento a cor pode alterar-se para verde-amarelada (KNOWLTON e JANES, 1931). Após a última muda ninfal, os adultos (recentemente emergidos) podem ser de cores diferentes, como o verde pálido ou a âmbar claro, tornando-se mais escuros ao terceiro dia, momento no qual linhas brancas ou amarelas são encontradas na cabeça e no tórax. O comprimento do corpo dos adultos pode variar de 1,3-1,9 mm e sua sobrevivência pode variar de 16 a 97 dias (LIU e TRUMBLE, 2007). Tanto as fêmeas virgens como acasaladas podem ovipositar após um período de pré-oviposição que varia de 3 a 25 dias (ABDULLAH, 2008). O acasalamento pode ocorrer várias vezes ao longo da vida e as fêmeas acasaladas podem ovipositar em média 300 ovos durante seu ciclo adulto (YANG e LIU, 2009).

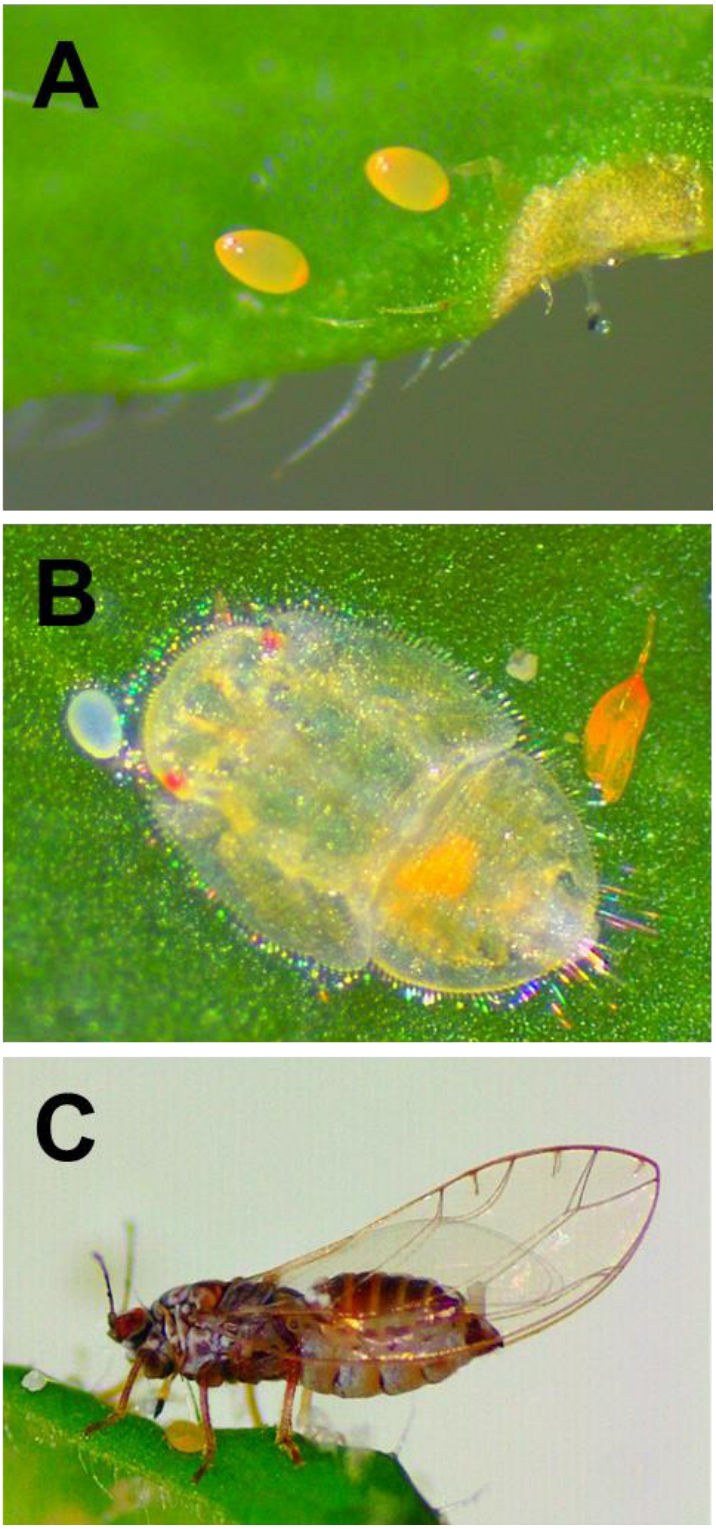

Figura 8. Estágios de vida do psilídeo da batata, *Bactericera cockerelli* (Hemiptera: Triozidae). A, ovos, B, ninfa e C, adulto (fêmea). (Fonte: fotos cedidas gentilmente pelo Dr. Freddy Ibanez- Tamborindeguy Laboratory- Texas A&M University).

REFERÊNCIAS

ABDULLAH, N.M. Life history of the potato psyllid *Bactericera cockerelli* (Homoptera: Psyllidae) in controlled environment agriculture in Arizona. **African Journal of Agricultural Research**, v.3, n.1, p.060-067, 2008.

ALEXANDER, H.M.; MAUCK, K.E.; WHITFIELD, E.; GARRET, K.A.; MALMSTROOM, C.M. Plant-virus interactions and the agro-ecological interface. **European Journal of Plant Pathology**, n.138, p.529-547, 2014.

ALFRADO-FERNÁNDEZ, A.; HERNÁNDEZZ-LOPIS, D.; FONT, M.I. Haplotypes of '*Candidatus* Liberibacter solanacearum' identified in Umbeliferous crops in Spain. **European Journal of Plant Pathology**, v.149, p. 1-5, 2017.

ALIZON, S.; HURFORD, A.; MIDEO, N.; BAALEN, V. Virulence evolution and the trade-off hypothesis: history, current state of affairs and the future. **J. Evol. Biol**., v. 22, p.245–259, 2009.

AMMAR, E.D.; HOGENHOUT, S.A. A neurotropic route for maize mosaic virus (Rhabdoviridae) in its planthopper vector *Peregrinus maidis*. **Virus Res**, n.131, p.77–85, 2008.

ANDERSON, P.K.; CUNNINGHAM, A.A.; PATEL, N.G.; MORALES, F.J.; EPSTEIN, P.R.; DASZAK, P. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. **Trends Ecol. Evol**., n.19, p.535–544, 2004.

ARAUJO, A.; JANSEN, A.M.; BOUCHET, F. REUNHARD, K.; FERREIRA, L.F. Parasitism, the diversity of life, and paleoparasitology. **Mem Inst Oswaldo Cruz**, v. 98, p. 5-11, 2003.

BACKUS, E.A.; SHUGART, H.J.; Rogers, E.E.; Morgan, J.K.; SHATTERS, R. Direct evidence of egestion and salivation of Xylella fastidiosa suggests sharpshooters can be "flying syringes**". Phytopathology**, v.105, n.5, p. 608-620, 2015.

BAK, A.; FOLIMINOVA, S.Y. The conundrum of a unique protein encoded by *Citrus tristeza virus* that is dispensable for infection of most hosts yet shows characteristics of a viral movement protein. **Virology**, n.485, p.86–95, 2015.

BAR-JOSEPH, M.; CHE, X.; MAWASSI, M.; GOWDA, S.; SATYANARAYANA, T.; AYLLÓN, M.A.; ALBIACH-MARTÍ, M.R.; GARNSEY, S.M.; DAWSON, W.O. The continuous challenge of *Citrus tristeza virus* molecular research. In: **Proceedings of the 15th Conference of the International Organization of Citrus Virologis**ts - IOCV, p.1-7. IOCV, Riverside, CA. 2002.

BAR-JOSEPH, M.; GUMPF, D.J.; DODDS, J.A.; GINZBERG, I. A Simple Purification method for *Citrus tristeza virus* and estimation of its genome size. **Phytopathology**, v.75, p.195-198, 1985.

BAR-JOSEPH, M.; MARCUS, R.; LEE, R.F. The continuous challenge of *Citrus tristeza virus* control. **Annual Review of Phytopathology**, v.27, p. 291-316, 1989.

BENETIZ-AFONSO, Y.; FAULKNER, C.; RITZENTHALER, C.; MAULE, A.J. Plasmodesmata: gateways to local and systemic virus infection. **American Phytopathological Society**, v. 23, n. 11, p. 1403-1412, 2010.

BENNETT, C.W. e COSTA, A.S. Tristeza disease of citrus. **Journal of Agricultural Research**, v.78, n.8, p.207-237, 1949.

BIEDERMANN, PETER HW, AND MARKO ROHLFS. "Evolutionary feedbacks between insect sociality and microbial management". **Current Opinion in Insect Science**, v.22, p.92-100, 2017.

BISWAS, K.K.; TARAFDAR, A.; SHARMA, S.K. Complete genome sequence of mandarin decline *Citrus tristeza virus* of the Northeastern Himalayan hill region of India: comparative analyses determine recombinant. **Arch Virol**., v.157, p.579–583, 2012.

BLANC, S.; DRUCKER, M.; UZEST, M. Localizing viruses in their insect vectors. **Annu. Rev. Phytopathol**., n. 52, p.403–25, 2014.

BOER, S.H.; SECOR, G.; LI, X.; GOURLEY, J.; ROSS, P.; RIVERA, V. Preliminary characterization of the etiologic agent causing zebra chip symptoms in potato. **In New and old pathogens of potato in changing climate: Proceedings of the EAPR Pathology Section seminar.** Hattula, Finland/Asko Hannukkala and Marjo Segerstedt (eds.). p.30, 2007.

BONANI, J.P.; FERERES, A.; GARZO, E.; MIRANDA, M.P.; GLORIA, A.; LOPS, J.R.S. Characterization of electrical penetration graphs of the Asian citrus psyllid, Diaphorina citri, in sweet orange seedlings. **Entomologia Experimentalis et Applicata**, v.134, n.1, p.35-49, 2010.

BORDIGNON, R.; MEDINA FILHO,H.P.; MÜLLER,G.W.; SIQUEIRA, W.J. **A tristeza dos citros e suas implicações no melhoramento genético de porta enxertos**. Bragantia, Campinas, v. 62, n.3, p.345-355, 2003.

BOVÉ, J.M. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. **Journal of Plant Pathology**, v.1, p.7-37, 2006.

BRAGARD, C.; CACIAGLI, P.; LEMAIRE, O.; LOPEZ-MOYA, J.J.; MACFARLANE, S.; PETERS,D.; SUSI, P., TORRANCES, L. Status and prospects of plant virus control through interference with vector transmission. **Annu. Rev. Phytopathol**., n.51, p. 177–201, 2013.

BURCKHARDT, D.; OUVRARD, D.; QUEIROZ, D.; PERCY, D. Psyllid host-plants (hemiptera: psylloidea): resolving a semantic problem. **Florida Entomologist**, v.97, n.1, p.242-246, 2014.

BUTLER, C.D.; TRUMBLE, J.T. The potato psyllid, *Bactericera cockerelli* (Sulc) (Hemiptera: Triozidae): life history, relationship to plant diseases, and management strategies. **Terrestrial Arthropod Reviews**, v.5, n.2, p.87-111, 2012.

CASADEVALL, A.; PIROFSKI, L. Host-Pathogen Interactions: Basic concepts of microbial commensalism, colonization, infection, and disease. **Infection and Immunity**, v.68, n.12, p. 6511–6518, 2000.

CHENG, J.; SIBLEY, C.D.; ZAHEER, R.; FINAN, T.M. A Sinorhizobium meliloti minE mutant has an altered morphology and exhibits defects in legume symbiosis. **Microbiology**, v.153, n.2, p.375-387, 2007.

CICERO, J.M.; FISHER, T.W.; QURESHI, J.A.; STANSLY, P.A.; BROWN, J. K. Colonization and intrusive invasion of potato psyllid by '*Candidatus* Liberibacter solanacearum'. **Phytopathology**, v. 107, n.1, p. 36-49, 2016.

.

COSTA, A.S.; GRANT, T.J. Studies on transmission of the tristeza virus by the vector, *Aphis citricidus*. **Phytopathology**, v.41, n.2, 1951.

COSTA, A.S. **Investigações sobre a tristeza dos citrus. Bragantia**, Campinas, v.9, p.1-26, 1949.

COSTA, A.T.; CARVALHO-NUNES, W.M.; ZANUTTO, C.A.; MÜLLER, G.W. Stability of *Citrus tristeza vírus* protective isolates in field conditions. **Pesq. agropec. Bras**, v.45, n.7, p.693-700, 2010.

CROSSLIN, J.M.; MUNYANEZA, J.E. Evidence that the zebra chip disease and the putative causal agent can be maintained in potatoes by grafting and in vitro. **American Journal of Potato Research**, v.86, n.3, p.183-187, 2009.

CROSSLIN, J.M.; MUNYANEZA, J.E.; BROWN, J.K.; LIEFTING, L.W. Potato zebra chip disease: a phytopathological tale. **Plant Health Progress**, n.17, p.1-9, 2010.

CRYAN, J.R.; URBAN, J.M. Higher-level phylogeny of the insect order Hemiptera: is Auchenorrhyncha really paraphyletic, **Systematic Entomology**, n.37, p.7-21, 2012.

DAWSON, W.O.; GARNSEY, S.M.; TATIENE, S.; FOLIMINOVA, S.Y.; HARPER, S.J.; GOWDA, S. *Citrus tristeza virus*-host interactions. **Frontiers in Microbiology**, v.4, n.88, p.1-10, 2013.

DILLON. R.J.; DILLON, V.M. The gut bacteria of insects: nonpathogenic interactions. **Annu. Rev. Entomol**., v. 49, p.71–92, 2004.

DOJLA, V.V.; KREUZE, J.F.; VALKONEN, J.P.T. Comparative and functional genomics of closteroviruses. **Virus Research**, v.117, p.38–51, 2006.

EIGENBRODE, S.D.; BOSQUE-PÉREZ, N.A.; DAVIS, T.S. Insect-Borne Plant Pathogens and Their Vectors: Ecology, Evolution, and Complex Interactions. **Annual Review of Entomology**, v.63, p.169-191, 2017.

EPPO- European and Mediterranean Plant Protection Organization. Global database (available online). Paris, France. Disponível em: https://www.eppo.int/. Acesso em: 31/10/2017, 10:18.

FAGOAGA, C.; LÓPES, C.; MENDOZA, A.H.; MORENO, P.; NAVARRO, L.; FLORES, R., PEÑA, L. Post-transcriptional gene silencing of the p23 silencing suppressor of *Citrus tristeza virus* confers resistance to the virus in transgenic Mexican lime. **Plant Molecular Biology**, n.60, p.153-165, 2006.

FLORES, R.; RUIZ-RUIZ, S.; SOLER, N.; SANCHEZ-NAVARRO, J.; FAGOAGA, C.; LÓPEZ, C.; NAVARRO, L.; MORENO, P.; PEÑA, L. *Citrus tristeza virus* p23: a unique protein mediating key virus–host interactions. **Frontiers in Microbiology**, v.4, p.1-9, 2013.

FOLIMINOVA, S.Y.; FOLIMONV, A.D.; TATINENI, S.; DAWSON, W. *Citrus tristeza virus*: Survival at the edge of the movement continuum. **Journal of Virology**, v.82, n.13, p. 6546–6556, 2008.

GARNIER, M.; JAGOUEIX-EVEILLARD, S.; CRONJE, P.R., LE ROUX, H.F.; BOVE, J.M. Genomic characterization of a liberibacter present in an ornamental rutaceous tree, *Calodendrum capense*, in the Western Cape Province of South Africa. Proposal of *Candidatus* Liberibacter africanus subsp. capensis. **International Journal of Systematic and Evolutionary Microbiology**, v.50, n.6, p.2119-2125, 2000.

GIGLIOLLI, A.A.S.; JULIO, A.H.F.; CONTE, H. The life cycle of *Gregarina cuneat*a in the midgut of *Tribolium castaneum* and the effects of parasitism on the development of insects. **Bulletin of Entomological Research**, v.106, p. 258–267, 2016.

GOLEY, E.D.; INIESTA, A.A.; SHAPIRO, L. Cell cycle regulation in *Caulobacter*: location, location, location. **Journal of Cell Science**, v.120, n.20, p.3501-3507, 2007.

GOWDA, S.; AYLLÓN, M.A.; SATYANARAYANA, T.; BAR-JOSEPH, M.; DAWSON, W.O. Transcription Strategy in a *Closterovirus:* a Novel 5'Proximal Controller Element of *Citrus tristeza virus* Produces 5' and 3'Terminal Subgenomic RNAs and Differs from 3'OpenReading Frame Controller Elements. **Journal of Virology**, v.11, n.1, p.340–352, 2003.

GOWDA, S.; Satyanarayana, T.; AYLLÓN, M.A.; ALBIACHI-MARTI, M.R.; MAWASSI, M.; RABINDRAN, S.; GARNSEY, S.M.; DAWSON, W.O. Characterization of the *cis*-Acting Elements Controlling Subgenomic mRNAs of *Citrus tristeza virus:* Production of Positive- and Negative-Stranded 39-Terminal and Positive-Stranded 59-Terminal RNAs. **Virology**, v.286, p.134-151, 2001.

GRAFTON-CARDWELL, E.E.; STELINSKI, L.L.; STANSLY, P. Biology and management of Asian citrus Psyllid, vector of the Huanglongbing pathogens. **Annual Review of Entomology**, v.58, p. 413-432, 2013.

GUTIERREZ, S.; MICHALAKIS, Y.; MUNSTER, M.V.; BLANC, S. Plant feeding by insect vectors can affect life cycle, population genetics and evolution of plant viruses. **Functional Ecology**, n.27, 610–622, 2013.

HALBERT, S.E.; BROWN, L.G. *Toxoptera citricida*, brown citrus aphid-Identification, biology and management strategies. **Entomology**, n.374, p.1-6, 1996.

HANSEN, A.K.; TRUMBLE, J.T.; STOUTHAMER, R.; PAINE, T.D. A new huanglongbing species,"*Candidatus* Liberibacter psyllaurous," found to infect tomato and potato, is vectored by the psyllid Bactericera cockerelli (Sulc). **Applied and environmental microbiology**, v.74, n.18, p.5862-5865, 2008.

HARRIS K.F. **An ingestion-egestion hypothesis of non circulative virus transmission**. In Aphids as Virus Vectors, ed. KF Harris, K Maramorosch, p. 166–208, New York: Academic, 1977.

HERRERA-ISIDRÓN, L.; OCHOA-SÁNCHE, J.C.; RIVERA-BUSTAMANTE, R.; MARTÍNEZ-SORIANO, J.P. Sequence diversity on four ORFs of citrus tristeza virus correlates with pathogenicity. **Virology Journal**, v.6, n.116, p.1-10, 2009.

HERRON, C.M.; MIRKOV, T.E.; GRAC, J.V.; LEE, R.F. *Citrus tristeza virus* transmission by the *Toxoptera citricida* vector: In vitro acquisition and transmission and infectivity immunoneutralization experiments. **Journal of Virological Methods**, n.134, p. 205–211, 2006.

HILF, M.E.; KARASEV, A.V.; PAPPU, H.R.; GUMPF, D.J.; NIBLETT, C.L.; GARNSEY, S.M. Characterization of *Citrus tristeza virus* subgenomic RNAs in infected tissue.**Virology**, v.208, n.2, p.576-582, 1995.

HOGENHOUT, S.A.; AMMAR, E.; WHITFIELD, A.E.; REDINBAUGH, M.G. Insect vector interactions with persistently transmitted viruses. **Annu. Rev. Phytopathol**., n.46, p.327–59, 2008.

HOGENHOUT, S.A.; Redinbaugh, M.G.; AMMAR, E. Plant and animal rhabdovirus host range: a bug's view. **Trends in Microbiology**, v.11, n.6, p. 264-271, 2003.

IBANEZ, F.; LEVY, J.; TAMBORINDEGUY, C. Identification and expression analyses of vitellogenin in *Bactericera cockerelli* (Šulc). **Journal of Insect Physiology**, n.98, p.205-2013, 2017.

JAGOUEIX, S.; BOVE, J.M.; GARNIER, M. The phloem-limited bacterium of greening disease of citrus is a member of the α subdivision of the Proteobacteria. **International Journal of Systematic and Evolutionary Microbiology**, v.44, n.3, p.379-386, 1994.

JANZEN, D.H. **The natural history of mutualisms**. In: The Biology of Mutualism. D. H. Boucher, ed. Oxford University Press, New York, U.S.A., 1985. p.40-99.

KARASEV, A.V.; BOYKO, V.P.; GOWDA, S.; NIKOLAEVA, O.V.; HILF, M.E.; KOONIN, E.V.; NIBLETT, C.L.; CLINE, K.; GUMPF, D.J; LEE, R.F.; GARNSEY, S.M.; LEWANDOWSKI, D.J.; DAWSON, W.O. Complete sequence of the *Citrus tristeza virus* RNA genome. **Virology**, v.208, p.511-520, 1995.

KENNEDY, J.S.; DAY, M.F.; EASTOP, V. F. Conspectus of aphids as vectors of plant viruses. **Commonwealth Institute of Entomology**, v.95, p.1-114, 1962.

KILLINY, N., PRADO, S.S.; ALMEIDA, R.P. Chitin utilization by the insect-transmitted bacterium *Xylella fastidiosa*. **Applied and Environmental Microbiology**, v.76, n.18, p.6134-6140, 2010.

KITAJIMA, E.W.; SILVA, D. M.; OLIVEIRA, A. R.; MÜLLER, G. W.; COSTA, A.S. Electron microscopical investigations of tristeza. In**: Proceedings of the 3rd Conference of the International Organization of Citrus Virologists – IOCV**, p.1-9. IOCV, Riverside, CA. 1964.

KNOWLTON, G.F.; JANES, M.J. Studies on the biology of *Paratrioza cockerelli* (Sulc). **Annals of the Entomological Society of America**, v.24. n.2, 1931.

KWON, M.O., WAYADANDE, A.C.; FLETCHER, J. Spiroplasma citri movement into the intestines and salivary glands of its leafhopper vector, *Circulifer tenellus*. **Phytopathology**, v. 89*,* n.12, p. 1144-1151, 1999.

LAI, M.M. RNA recombination in animal and plant viruses. **Microbiological reviews**, v.56, n.1, p.61-79, 1992.

LEE, I.M.; BOTTNER, K.D.; MUNYANEZA, J.E.; SECOR, G.A.; GUDMESTAD, N.C. Clover proliferation group (16SrVI) subgroup A (16SrVI-A) phytoplasma is a probable causal agent of potato purple top disease in Washington and Oregon. **Plant disease**, v.88, n.4, p.429-429, 2004.

LEUNG, T.; POULIN, R. Parasitism, commensalism, and mutualism: exploring the any shades of symbioses. **Vie et Milieu**, v.58, n.2, p.107, 2008.

LIEFTING, L.W.; PEREZ-EGUSQUIZA, Z.C.; CLOVER, G.R.G. AND ANDERSON, J.A.D. A new '*Candidatus Liberibacter*'species in *Solanum tuberosum* in New Zealand. **Plant Disease**, v. 92, n.10, p.1474-1474, 2008.

LIEFTING, L.W.; SUTHERLAND, P.W.; WARD, L.I.; PAICE, K.L.; WEIR, B.S; CLOVER, G.R. A new '*Candidatus Liberibacter*'species associated with diseases of solanaceous crops. **Plant Disease**, v.93, n.3, p.208-214, 2009a.

LIEFTING, L.W.; WEIR, B.S.; PENNYCOOK, S.R.; CLOVER, G.R. '*Candidatus* Liberibacter solanacearum', associated with plants in the family Solanaceae. **International Journal of Systematic and Evolutionary Microbiology**, v.59, n.9, p.2274-2276, 2009.

LIN, H.; LOU, B.; GLYNN, J.M.; DODDAPANENI, H.; CIVEROLO, E.L.; CHEN, C.; DUAN, Y.; ZHOU L.; VAHLING, C.M. The complete genome sequence of '*Candidatus* Liberibacter solanacearum', the bacterium associated with potato zebra chip disease. **PLoS One**, v.6, n,4, p.1-13, 2011.

LIST, G.M. The effect of temperature upon egg deposition, egg hatch and nymphal development of *Paratrioza cockerelli* (Sulc). **Journal of Economic Entomology**, v.32, n.1, p. 30-36, 1939.

LIU, D. A; TRUMBLE, J.T. Comparative fitness of invasive and native populations of the potato psyllid (*Bactericera cockerelli*). **Entomologia experimentalis et applicata**, v.123, n.1, p.35-42, 2007.

LOPEZ, C.; NAVAS-CASTILHO, J.; GOWDA, S.; MORENO, P.; FLORES, R. The 23-kDa protein coded by the 39-terminal Gene of *Citrus tristeza virus* is an RNA-binding protein. **Virology**, v.269, p. 462-470, 2000.

LU, R.; FOLIMINOV, A.; SHINTAKU, M.; LI, W.X.; FALKS, B.W.; DAWSON, W.O.; DING, S. Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. **National Academy of Sciences**, v.101, n.44, p. 15742–15747, 2004.

MAWASSI, M.; MIETKIEWSKA, E.; GOFMAN, R.; YANG, G.; BAR-JOSEPH, M. unusual sequence relationships between two isolates of *Citrus tristeza virus*. **Journal of General Virology**, v.77, n.9, p.2359-2364, 1996.

MELZER, M.J.; BORTH, W.B.; SETHER, D.M.; FERREIRA, S.; GONSALVES, D.; HU, J.S. Genetic diversity and evidence for recent modular recombination in Hawaiian *Citrus tristeza virus*. **Virus Genes**, n.40, p.111–118, 2010.

MENEGHINI, M. Sobre la natureza y transmissibilidade de la doencia "Tristeza" de los citros. **O Biológico**, v.12, p.285-287, 1946.

MICHAUD, J.P. A review of the literature on *Toxoptera citricida* (kirkaldy) (homoptera: aphididae). **Florida Entomologist**, v.81, n.1, p.37-61, 1998.

MOREIRA, S. Métodos para identificação e controle da tristeza dos citros. **Boletim técnico da divisão de experimentação e pesquisas Instituto Agronômico**, Campinas: 1954.19p.

MORENO, P.; AMBRÓS, S.; ALBIACH-MARTI, M.R.; GUERRI, J.; PEÑA, L. *Citrus tristeza virus*: a pathogen that changed the course of the citrus industry. **Molecular Plant Pathology**, v.9, n.2, p.251-268, 2008.

MÜLLER, G.W. Pulgão e tristeza dos citros no Brasil. **Laranja**, v.17, n.1, p.292-293, 1996.

MÜLLER, G.W.; TARGON, M.L.P.N.; CARVALHO, S.A.; SOUZA, A.A.; RODRIGUES, J.C.V. **Doenças de citros causadas por vírus e viróides**. In: MATTOS JUNIOR, D.; DE NEGRI, J.D.; PIO, R.M.; POMPEU JUNIOR, J. Citros. Campinas: Instituto Agronômico e Fundag. Centro APTA Citros Sylvio Moreira. p.567-604, 2005.

MUNYANEZA, J.E. Zebra Chip Disease of Potato: Biology, Epidemiology, and Management. **American Journal of Potato Research**, n.89, p.329-350, 2012.

MUNYANEZA, J.E.; CROSSLIN, J.M.; UPTON, J.E. Association of *Bactericera cockerelli* (Homoptera: Psyllidae) with "zebra chip," a new potato disease in southwestern United States and Mexico. **Journal of Economic Entomology**, v.100, n.3, p.656-663, 2007b.

MUNYANEZA, J.E.; FISHER, T.W.; SENGODA, V.G.; GARCZYNSKI, S.F.; NISSINEN, A.; LEMMETTY, A. Association of "*Candidatus* Liberibacter solanacearum" with the

psyllid, *Trioza apicalis* (Hemiptera: Triozidae) in Europe. **Journal of Economic Entomology**, v.103, n.4, p.1060-1070, 2010.

MUNYANEZA, J.E.; GOOLSBY, J.A.; CROSSLIN, J.M.; UPTON, J.E. Further evidence that zebra chip potato disease in the lower Rio Grande Valley of Texas is associated with *Bactericera cockerelli*. **Subtropical Plant Science**, v.59, p.30-37, 2007a.

MUNYANEZA, J.E.; SENGODA, V.G.; CROSSLIN, J.M.; DE LA ROSA-LOZANO G.; SANCHEZ, A. First report of '*Candidatus* Liberibacter psyllaurous' in potato tubers with zebra chip disease in Mexico. **Plant Disease**, v.93, n.5, p.552-552, 2009b.

NACHAPPA, P.; LEVY, J.; TAMBORINDEGUY, C. Transcriptome analyses of *Bactericera cockerelli* adults in response to "*Candidatus* Liberibacter solanacearum" infection. **Molecular genetics and genomics,** v.287, n.10, p.803-817, 2012.

NADARASAH, G.; STAVRINIDES, J. Insects as alternative hosts for phytopathogenic bacteria. **FEMS Microbiology Reviews**, v.35, n.3, p.555-575, 2011.

NAGATA, T.; INOUE-NAGATA, A.; LENT, J.; GOLDBACH, R.; PETERS, D. Factors determining vector competence and specificity for transmission of *Tomato spotted* wilt virus. **Journal of General Virology**, n.83, p.663–671, 2002.

NARADASH, G.; STAVRINIDES, J. Insects as alternative hosts for phytopathogenic bacteria. **FEMS Microbiol Rev**., v.35, p.555-575, 2011.

NAULT, L.R. Arthropod transmission of plant viruses: A new synthesis. **Entomological Society of America**, n.90, v.5, p. 521-541, 1997.

NAVARRE, D.A.; SHAKYA, R.; HOLDEN, J.; CROSSLIN, J.M. LC-MS analysis of phenolic compounds in tubers showing zebra chip symptoms. **American Journal of Potato Research**, v.86, n.2, p.88-95, 2011.

NELSON, W.R., MUNYANEZA, J.E., MCCUE, K.F. AND BOVÉ, J.M. The Pangaean origin of" *Candidatus* Liberibacter" species. **Journal of Plant Pathology**, v.95, n.3, p.455-461, 2013.

NELSON, W.R.; FISHER, T.W.; MUNYANEZA, J.E. Haplotypes of "*Candidatus* Liberibacter solanacearum" suggest long-standing separation. **European Journal of Plant Pathology**, v.130, n.1, p.5-12, 2011.

NG, J.C.K.; FALK, B. Virus-Vector Interactions mediating nonpersistent and semipersistent transmission of plant viruses. **Annu. Rev. Phytopathol**, n.44, p.183–212, 2006.

NG, J.C.K.; PERRY, K. Transmission of plant viruses by aphid vectors. **Molecular Plant Pathology**, n.5, v.5, p.505–511, 2004.

PAN, L..; CHEN, Q.; ZHAO, J.; GUO, T.; WANG, X.; HARITON-SHALEVB, A.; CZOSNEK, H.; LIU, S. Clathrin-mediated endocytosis is involved in Tomato yellow leaf curl virus transport across the midgut barrier of its whitefly vector. **Virology**, v.502, p.152–159, 2017.

PAPPU H.R.; KARASEV, A.V.; ANDERSON, E.J.; PAPPU, S.S.; HILF, M.E.; FEBRES, V.J.; ECKLOFF, R.M.; MCCAFFERY, M.; BOYKO, V.; GOWDA, S.; et al. Nucleotide sequence and organization of eight 3' open reading frames of the citrus tristeza closterovirus genome. **Virology**, v.15, n.1, p.35-46, 1994.

PIEL, J. A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. **Proceedings of the National Academy of Sciences**, v.99, n.22, p.14002-14007, 2002.

PIETERSE, C.M.J.; DICKE, M. Plant interactions with microbes and insects: from molecular mechanisms to ecology. **Trends in Plant Science**, v.12, n.12, p.564-569, 2007.

PRICE, W.C. Flexuous rods in phloem cells of lime plants infected with *Citrus tristeza virus.* **Virology**, v. 29, p. 285-294, 1966.
Psyllid, Vector of the
REHMAN, M.; MELGAR, J.C.; RIVERA C.J.M.; IDRIS, A.M.; BROWN, J.K.. First report of "*Candidatus Liberibacter psyllaurous*" or "*Ca.* Liberibacter solanacearum" associated with severe foliar chlorosis, curling, and necrosis and tuber discoloration of potato plants in Honduras. **Plant Disease**, v.94, n.3, p.376-376, 2010.

ROCHA-PEÑA, M.; LEE, R.F.; CORONA, F.M.O.; YOKOMI, R. *Toxoptera citricida* : Threats to Citrus Production in the Caribbean and Central and North. **Plant disease**, v.79, p.437-445, 1995.

ROOSSINCK, M.J. Plants, viruses and the environment: Ecology and mutualism. **Virology**, v.479, p.271–277, 2015.

SATYANARAYANA, T.; GOWDA, S.; AYLLO, M.; DAWSON, W. Closterovirus bipolar virion: evidence for initiation of assembly by minor coat protein and its restriction to the genomic RNA 5' region. **National Academy of Sciences**, v.101, n.3, p.799-804, 2004.

SATYANARAYANA, T.; GOWDA, S.; AYLLO'N, M.A.; ALBIACH-MARTI, M.R.; RABINDRAN, S.; DAWSON, W. The p23 Protein of *Citrus tristeza virus* controls asymmetrical RNA accumulation. **Journal of Virology**, v.76, n.2, p. 473-483, 2002.

SECOR, G.; RIVERA-VARAS, V. Emerging diseases of cultivated potato and their impact on Latin America. **Suplemento Revista Latino Americana de La Papa**, n.1, p.1-8, 2004.

SECOR, G.A.; LEE, I.M.; BOTTNER, K.D.; RIVERA-VARAS, V.; GUDMESTAD, N.C. First report of a defect of processing potatoes in Texas and Nebraska associated with a new phytoplasma. **Plant Disease**, v.90, n.3, p.377-377, 2006.

SECOR, G.A.; RIVERA, V.V.; ABAD, J.A.; LEE, I.M.; CLOVER, G.R.G.; LIEFTING, L.W.; LI, X.; DE BOER, S.H. Association of '*Candidatus Liberibacter solanacearum*'with zebra chip disease of potato established by graft and psyllid transmission, electron microscopy, and PCR. **Plant Disease**, v.93, n.6, p.574-583, 2009.

SOLOMON, N.U.; JAMES, I.M.; ALPHONSUS, N.O.O; NKIRUKA, R.U. A review of host-parasite relationships. **Annual Research & Review in Biology**, v.5, p.372-384, 2015.

SU, Q.; ZHOU, X; ZHANG, Y. Symbiont-mediated functions in insect hosts. **Communicative & integrative biology**, v 6, p. e23804-1-e23804-7, 2013.

SULC, K. *Trioza cockerelli* n. sp., a novelty from North America, being also of economic importance. **Acta Societatis Entomologica e Bohemiae**, v.6, p.102-108, 1909.

SYLVESTER ,E.S. Circulative and propagative virus transmission by aphids. **Annu. Rev. Entomol.,** n.25, p.257–286, 1990.

TAMBORINDEGUY, C.; HUOT, O.B.; IBANEZ, F.; LEVY, J. The influence of bacteria on multitrophic interactions among plants, psyllids, and pathogen . **Insect Science**, v.1, n.14, p.1-14, 2017.

TANAKA, F.A.O., COLETTA-FILHO, H.D., ALVES, K.C.S., SPINELLI, M.O., MACHADO, M.A.; KITAJIMA, E.W. Detection of the" *Candidatus* Liberibacter americanus" in phloem vessels of experimentally infected cataranthus roseus by scanning electron microscopy. **Fitopatologia Brasileira**, v.32, n.6, p.519-519, 2007.

TATINENI, S.; ROBERTSON, C.; GARNSEY, S.M.; DAWSON, W.O. A plant virus evolved by acquiring multiple nonconserved genes to extend its host range. **National Academy of Sciences**, v.108, n.42, p. 17366–17371, 2011.

TATINENI, S.; ROBERTSON, C.J.; GARNSEY, S.M.; MAR-JOSEPH, M.; GOWDA, S.; DAWSON, O.W. Three genes of *Citrus tristeza virus* are dispensable for infection and movement throughout some varieties of citrus trees. **Virology**, v.376, p 297–307, 2008.

TAYLOR, G.P.; COGHLIN, P.C.; FLOATE, K.D.; PERLMAN, S.J. The host range of the male-killing symbiont *Arsenophonus nasoniae* in filth fly parasitioids. **J. Invertebr Pathol**., v.106, n.3, p.371-379, 2011.

TEIXEIRA, D.C.; SAILLARD,C.; EVEILLARD,D.; DANET, J.L.; COSTA, P.I.; AYRES, A.J.; BOVE, J. '*Candidatus Liberibacter americanus'*, associated with citrus huanglongbing (greening disease) in São Paulo State, Brazil. **International Journal of Systematic and Evolutionary Microbiology**, n.55, p. 1858-1862, 2005.

TEIXEIRA, L.; FERREIRA, Á.; ASHBURNER, M. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. **PLoS biology**., v.6, n.12, p. 2754-2763, 2008.

TERESANI, G.R.; BERTOLINI, E.; ALFARO-FERNÁNDEZ, A.; MARTÍNEZ, C., TANAKA, F.A.O.; KITAJIMA, E.W.; ROSELLÓ, M.; SANJUÁN, S.; FERRÁNDIZ, J.C.; LÓPEZ, M.M.; CAMBRA, M. Association of '*Candidatus* Liberibacter solanacearum'with a vegetative disorder of celery in Spain and development of a real-time PCR method for its detection. **Phytopathology**, v.104, n. 8, p.804-811, 2014.

TRAVESET, A.; RICHARDSON, D.M. Mutualistic Interactions and biological invasions. Annu. **Rev. Ecol. Evol. Syst**., v.45, p.89-113, 2014.

TREBICKI, P.; DÁDER, B.; VASSILIADIS, S.; FERERES, A. Insect–plant–pathogen interactions as shaped by future climate: effects on biology, distribution and implications for agriculture. **Insect Science**, n.13, p.1-41, 2017.

UZEST, M.; GARGANI, D.; DRUCKER, M.; HÉBRARD, E.; GARZO, E.; CANDRESSE, T.; FERERES; A.; BLANC, S. A protein key to plant virus transmission at the tip of the insect vector stylet. **Proceedings of the National Academy of Sciences**, v.104, n.46, p.17959-17964, 2007.

VIVES, M.C.; RUBIO, L.; SAMBADE, A.; MRKOV, T.E.; MORENO, P.; GUERRI, J. Evidence of multiple recombination events between two RNA sequence variants within a *Citrus tristeza virus* isolate. **Virology**, v. 331, p.232–237, 2005.

WATSON, M.A.; ROBERTS, F.M. A comparative study of the transmission of *Hyoscyamus*Virus 3, Potato virus Y and Cucumber Virus 1 by the vectors *Myzus persicae* (Sulz.), *M. circumflexus*(Buekton), and *Macrosiphum gei* (Koch). **Proceedings of the Royal Society**, v.127, n.848, p.543-576, 1939.

WATSON, M.A.; ROBERTS, F.M. Evidence against the hypothesis that certain plant viruses are transmitted mechanically by aphides. **Annals of Applied Biology**, v.27, n.2, p.227-233, 1940.

WEINTRAUB, P.G.; BEANLAND, L. Insect vectors of phytoplasmas. **Annu. Rev. Entomol.**, v.51, p.91–111, 2006.

WHITFIELD, A.E.; FALK, B.W.; ROTENBERG, D. Insect vector-mediated transmission of plant viruses. **Virology**, v. 479, p. 278–289, 2015.

WILKINSON, H.H.; SIEGEL, M.R.; BLANKENSHIP, J.D.; MALLORY, A.C.; BUSH, L.P.; SCHARDL, C.L. Contribution of fungal loline alkaloids to protection from aphids in a Grass-Endophyte mutualism. **The American Phytopathological Society**, v.13, n.10, p.1027-1033, 2000.

WU, F., CEN, Y., WALLIS, C.M., TRUMBLE, J.T., PRAGER, S., YOKOMI, R., ZHENG, Z., DENG, X., CHEN, J. AND LIANG, G.The complete mitochondrial genome sequence of *Bactericera cockerelli* and comparison with three other Psylloidea species. **PloS one**, v.11, n.5, p.1-17, 2016.

YANG, X.B.; LIU, T.X. Life history and life tables of *Bactericera cockerelli* (Homoptera: Psyllidae) on eggplant and bell pepper. **Environmental entomology**, v.38, n.6, p.1661-1667, 2009.

YOKOMI, R K. The Brown citrus Aphid and *Citrus tristeza virus*. **Citrograph**, v.6, p.14-18, 1994.

YOKOMI, R.K. The brown citrus aphid, *Toxoptera citricida*. **Options Méditerranéennes**, n.65, p.35-46, 2009.

ZANUTTO, C.A.; SOUZA, I.; GONÇALVES-ZULIANE, A.M.O.; SOARES, L.S.; NUNES, W.M.C.; CORAZZA, M.J. Estabilidade e distribuição do complexo do *Citrus tristeza virus*

em plantas de laranja Pêra avaliado pela análise SSCP. **Tecnol. & Ciên. Agropec**., v.7, p.1-5, 2013.

Chapter 1

Citrus tristeza virus transmission by one single *Toxoptera citricida* vector under Brazilian conditions

RESUMO

Citrus tristeza virus (CTV), membro da família Closteroviridae, gênero *Closterovirus*, é a doença viral mais importante de cítricos em todo o mundo. Atualmente, vários isolados de CTV estão sendo classificados de acordo com a presença e/ou ausência de sintomas, e quão graves esses sintomas são expressos em espécies indicadoras de citros. O CTV pode ser transportado e transmitido por várias espécies de afídeos de forma semi-persistente; no entanto, *Toxoptera citricida* é o vetor mais eficiente. No Brasil, alguns isolados de CTV fracos estão sendo usados para ensaios de premunização que protegem as árvores cítricas contra isolados severos de CTV. Apesar deste conhecimento, as interações moleculares entre o vírus e seu pulgão vetor em condições brasileiras ainda não estão bem estabelecidas. Dessa forma o presente estudo teve como objetivo determinar a capacidade e eficiciência do *T. citricida* para separar o complexo de três diferentes isolados de CTV (PIAC, CS1 e Forte Rolândia). Os isolados foram obtidos de pomares comerciais localizados nas regiões Norte e Noroeste do estado do Paraná- Brasil, em seguida, por meio de testes de transmissão usando um único pulgão, a eficiência de transmissão e o padrão dos sub-isolados de CTV foram determinados. Os resultados mostraram que *T. citricida* transmitiu apenas os sub-isolados de CTV, PIAC e CS1, os quais são considerados como isolados fracos, sob condições brasileiras, mostrando uma eficiência de transmissão de 8% e 4%, respectivamente. Enquanto, Forte Rolândia, um isolado que produz sintomas graves em árvores cítricas localizadas no Norte do estado do Paraná, não foi transmitido por *T. citricida*. A detecção de sub-isolados de CTV, PIAC e CS1, após a transmissão por um único pulgão em plantas cítricas de laranja doce (*Citrus sinensis*), ocorreu em dias diferentes, o primeiro subsolado detectado foi o PIAC aos 150 dias, seguido de CS1 aos 210 dias após inoculação. Além disso, durante este estudo, encontramos diferenças nos padrões de SSCP (Polimorfismo de conformação de filamento único) entre isolados e sub-isolados de CTV, sugerindo que um pulgão pode adquirir e transmitir apenas um subsolado de CTV para plantas cítricas durante sua alimentação via floema.

Palavras-chave: Tristeza dos citros. Closteroviridae. Afídeo.

ABSTRACT

Citrus tristeza virus (CTV), member of the *Closteroviridae* family, genus *Closterovirus,* is the most important viral disease of citrus crops worldwide. Presently, several CTV isolates are being classified according to the presence and/or absence of symptoms, and how severe these symptoms are expressed on citrus indicator species. CTV can be carried and transmitted by several aphid species in a semi-persistent manner; however, *Toxoptera citricida* is the most efficient vector. In Brazil, some weak CTV isolates are being used for preimmunization assays protecting the citrus trees against severe CTV isolates. Despite this knowledge, the molecular interactions between the virus and its aphid vector under Brazilian conditions have not been determined. Thus, the present study aimed to determine the capacity and efficiency of *T. citricida* to separate the complex from three different CTV isolates (PIAC, CS1 and Forte Rolândia). The isolates were obtained from commercial orchards located in the regions North and Northwest of Paraná state, Brazil, then using one-single aphid transmission assays the transmission efficiency and the CTV subisolate pattern was determined. The results showed that *T. citricida* could transmit only the CTV subisolates, PIAC and CS1, considered as mild CTV isolates under Brazilian conditions, showing a transmission efficiency of 8% and 4%, respectively. While, Forte Rolândia, an isolate that produced severe symptoms in citrus trees located in North of Paraná state, was not transmitted by *T. citricida*. The detection of CTV subisolates, PIAC and CS1, after one-single aphid transmission in sweet orange (*Citrus sinensis*) citrus plants occurred at different days, the first subisolate distinguished was PIAC at day 150, followed by CS1 at day 210. Also, during this study, we found differences in the SSCP (Single-strand Conformation Polymorphism) patterns between CTV isolates and CTV subisolates, suggesting that an aphid can acquire and transmit only one CTV subisolate to citrus plants during its phloem feeding.

Keywords: Citrus tristeza. Closteroviridae. Aphid.

INTRODUCTION

Plant diseases caused by viruses are responsible for 47% of the emergent pathologies, causing a major threat to the agriculture worldwide (ANDERSON et al., 2004). Currently, there are over 2.000 species of viruses belonging to about 20 families, affecting different crops cultivated by human (WHITFIELD et al., 2015). In particular, in citrus crops, the citrus tristeza disease caused by the virus *Citrus tristeza virus* (CTV), destroyed millions of trees in the world in the last 70 years (BAR-JOSEPH, 1989; MORENO et al., 2008).

Citrus tristeza virus, member of the Closteroviridae family, genus *Closterovirus,* is the most important viral disease of citrus crops (LEE and KEREMANE, 2013). The virus consists of a helical-shaped flexuous and long particle, measuring about 11 x 2.000 nm in size with single-stranded RNA of positive polarity (KITAJIMA et al., 1964; BAR- JOSEPH et al., 1989; MUNIZ et al., 2012). There are several CTV isolates that were classified according to the presence or absence, and how severe these symptoms occur on citrus indicator species, that distinguished between mild and severe strains (HILF et al., 2005). The typical symptoms induced by CTV in citrus trees are a slow decline, quick decline or death of sweet orange and stem-pitting (DAWSON et al., 2013). In addition to the genetic composition of the virus isolates, the symptomatology can be influenced by citrus cultivar, rootstock and environmental conditions (POWELL et al., 2003; MORENO et al., 2008; GIAMPANI et al., 2017).

The control of citrus tristeza disease, since it was recorded in Brazil in 1937 on several citrus cultivars, had relied on the use of tolerant rootstocks (MÜLLER, 1980a). However, the use of this management has not been effective and/or viable for other citrus species; for example, it did not work in the citrus cultivar Pêra orange (*Citrus sinensis* L.) and acid limes (*Citrus aurantifolia* Swingle) (TEMPORAL et al., 2011). For this reason, new approaches were added to the citrus tristeza disease management in Brazil, these methods rely in cross-protection or pre-immunization, in which CTV-isolates that cause mild or no symptoms have been used to avoid severe symptoms caused by other CTV-isolates (LEE et al., 1987), this method was used in several citrus species, such as Sweet orange (*Citrus sinensis*); Tahiti lime (*Citrus latifolia* Tanaka) and grapefruit (*Citrus paradise* Macf.) (STUCHI et al., 2002; CORAZZA-NUNES et al., 2001; CORAZZA-NUNES et al., 2006). One of these CTV protective isolates called PIAC, found by MÜLLER in 1980, improved the tolerance in Pêra orange citrus. These two strategies (tolerant rootstocks and pre-

immunization) to control citrus tristeza disease had allowed the Brazilian citrus orchards to maintain its production (MÜLLER, 1980b).

Nevertheless, pre-immunization in citrus cultivars had not been completely successful in Brazil, because severe symptoms of citrus tristeza were observed in commercial orchards previous to pre-immunized (MÜLLER, 1999). One of the reasons why this method might fail is related to the characteristic of the virus. Single-stranded RNA viruses are known to undergo rapid genetic changes, and nucleotide substitution is the most commonly observed mechanism of mutation, these genetic changes occurring on CTV might change the transcription and/or translation of important proteins that are triggering the immune system in citrus plants (LAI, 1992).

CTV can be carried and transmitted by several aphid species including *Aphis gossip* (Glover), *Aphis spiraecola* (Patch) and *Toxoptera citricida* (Kirkaldy). Among them, *Toxoptera citricida* is the most efficient vector of CTV isolates (YOKOMI et al., 1994; COSTA et al., 2010). This insect species has two types of reproduction depending on its location and environmental conditions, sexual reproduction can be observed in cold regions, (e.g. Japan), and parthenogenic in tropical and subtropical regions, such as Brazil. *T. citricida* feed on newly expanded buds, leaves and flower buds of its hosts (MICHAUD, 1998).

Toxoptera citricida can acquire and transmit the virus, in a semi-persistent type transmission, once the insect is feeding on CTV-infected plants, it can acquire the virus in 5 to 10 minutes, however the efficiency of transmission increases when insects can feed up to 24 hours (COSTA e GRANT, 1951). After CTV acquisition, the transmission occurs within 30 minutes to the new host or the insect can remain viruliferous for up to 48 hours since the virus does not multiply or circulate in this aphid (YOKOMI, 2009).

Despite, the association *Citrus tristeza virus* - *Toxoptera citricida* is economically important, the mechanisms of transmission of the subisolates, the composition of the virus used for preimmunization and how these are acquired and lately transmitted to citrus plants, are still not well understood under the Brazilian environmental conditions. Therefore, the objectives of this study will be the following, First, determine the ability of *T. citricida* to separate the complex of subisolates in three different CTV isolates (PIAC, CS1, and Forte Rolândia). Second, characterize the isolates and subisolates using SSCP technique. And third, define the efficiency of transmission under the environmental conditions of Maringá, Brazil.

MATERIALS AND METHODS

1. Virus isolates

For this study, we would like to define the following terms. First, isolates were defined as natural sources of CTV obtained from citrus plants found in the field, the second definition, subisolate, was obtained when a single aphid fed on these CTV-infected citrus plants. Three isolates of CTV have used: two CTV mild protective isolates (PIAC and CS1) and the severe isolate Forte Rolândia. The isolate PIAC was gifted by Centro de Citricultura Sylvio Moreira in 2015, the isolate CS1 was collected in 2015 from commercial citrus orchards located in the northwestern of the Paraná state, and the isolate Forte Rolândia (CARRARO et al., 2003; ZANUTTO et al., 2013a) was obtained from commercial citrus orchards placed in northern region of the Paraná state. These isolates were obtained by graft, using CTV-infected sweet orange variety Pêra (*Citrus sinensis* (L.) Osbeck) grafted on clove lemon (*C. limonia* Osbeck) and kept for one year in an insect-free greenhouse at the Núcleo de Pesquisa em Biotecnologia Aplicada (NBA) of the State University of Maringá (UEM), Brazil. To detect and confirm the presence of CTV and its subisolates on these plants, Polymerase chain reaction (PCR) and Single-strand conformation polymorphism (SSCP) assays were performed.

2. Insect source

Brown aphid, *Toxoptera citricida,* colonies were obtained and collected from commercial citrus orchards during summer of 2016, insects were divided and reared in groups in at least 4 uninfected sweet orange seedlings (Figure 1A) under normal Brazilian conditions (approximately 34º C).

2.1.Aphid acquisition and transmission

Before acquisition and transmission assays at least 40 adult insects were collected from the insect source colonies, aphids were transferred to uninfected clove lemon seedlings for a period of 72 hours (Figure 1B), the purpose of this step was to avoid a possible CTV

infection in these aphids, similar as was described in (BRLANSKY et al., 2003). In detail, the CTV acquisition was performed during a period of 48 hours, groups of 40 uninfected (CTV-free) adult aphids were transferred to Pêra orange seedlings previously infected with each CTV-isolate, PIAC, CS1, and Forte Rolândia, per separated (Figure 1C). To perform the transmission assays, a single aphid was transferred to CTV-free Pêra orange citrus plants (25 replicates) during 48 hours (Figure 1D), this transmission method was previously described in Herron et al. (2006). After 48 hours, the aphids were eliminated with an insecticide spray (the organophosphate, Methidathion), then the inoculated plants (N=25) were returned to the greenhouse under normal Brazilian conditions (approximately 34º C). The transmission rate was determined by the number of CTV-positive plants divided by the total number of plants used for the inoculations of each CTV-isolate.

Figure 1. Experimental design of acquisition and transmission assay. (A) *Toxoptera citricida* colonies kept in sweet orange plants under greenhouse conditions; (B) aphids transferred to clove lemon plants for feeding during a period of 72 hours; (C) Acquisition assay, aphids were allowed to fed for 48 hours on sweet orange plants infected with CTV isolates PIAC, CS1, and Forte Rolândia; (D) Transmission assay, one-single aphid was allowed to fed on Orange Pêra plants per CTV-subisolate.

2.2.*Citrus tristeza virus* colonization

The colonization of CTV on Pêra orange citrus plants was achieved detecting the presence of virus by PCR and differentiating the subisolates by Single-Stranded Conformational Polymorphism (SSCP). Sampling was performed in diverse parts of each plant (up, middle and below); also at different times, the sampling was achieved every 30 days reaching a maximum of 270 days, similar to a study previously published (ZANUTTO et al., 2013a).

3. RNA extraction and cDNA synthesis

Total RNA was extracted from the central vein of the leaves selected from different parts of each plant (upper, middle and lower parts), per CTV-isolate and at different time points. The tissues were homogenized mechanically using liquid nitrogen with a pestle and mortar, after homogenization (frozen powder) the RNA was isolated using 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. The integrity of the total RNA was analyzed using 1% agarose gels stained with ethidium bromide.

The reverse transcription of complementary DNA (cDNA) was achieved following the manufacturer's instructions (Invitrogen). Briefly, the cDNA synthesis was performed using 300 ng of total RNA and the following reagents, in a final volume of 25 µL: 5X first-strand buffer (Invitrogen), 2.5 mM of each deoxynucleotides (dNTPs), 10 mM of Dithiothreitol (DTT), 20 Units of RNase inhibitor (Invitrogen), 1 µg of random primers and 100 Units of M-MLV reverse transcriptase (Invitrogen).

4. CTV-detection assay

To detect the presence of CTV in plant tissues, a specific PCR was performed to detect the capsid protein gene p25 (GCP). The PCR was achieved using the following reagents: 2.5 µL of cDNA template, 10X of DNA polymerase buffer (Invitrogen), 2.5 mM of $MgCl_2$, 0.1 mM of each dNTPs, 100 ng of each primer CN-119 (5'-

AGATCTACCATGGACGACGAAACAAAG-3') and CN-120 (5'-GAATTCGCGGCCGCTCAACGTGTGTTAAATTTCC-3') and 1 Unit of Taq DNA polymerase (Invitrogen) in a final volume of 25 µL. The PCR conditions used for this assay were 95°C for 2 min; followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 5 min. The resulting DNA amplicons were analyzed by electrophoresis, on 1% agarose gel containing ethidium bromide and each image was captured using an Ultraviolet gel-documentation system (UVP-Biomalgin).

5. Single Stranded Conformational Polymorphism (SSCP) assay

After the detection of viral infection by PCR, an SSCP analysis was performed to evaluate the electrophoretic patterns of the CTV strains obtained for each isolate, the methodology used was described by Souza et al. (2000) and Corazza-Nunes et al. (2001). Briefly, for SSCP analysis were achieved using 10 µL of DNA amplicon obtained for capsid protein gene p25 (GCP) amplification, each PCR product was mixed with an equal volume of denaturing buffer (95% formamide, 2 mM EDTA and 0.05% bromophenol blue). Then, these samples were denatured at 95°C during 10 minutes in a thermal block and then placed immediately on ice. After samples were loaded in 8% polyacrylamide non-denaturing gels and the electrophoresis was performed using TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.5) at 200 volts for 16 hours at 25°C. After electrophoresis, the gels were stained with a solution of silver nitrate according to the procedure described by Beidler et al. (1982). The images of each gel were documented and the SSCP patterns were analyzed in relation to the number of bands in each lane for each CTV-isolate and subisolate.

RESULTS

1. Total RNA integrity

Plant tissues (CTV-infected or uninfected) were used to isolate total RNA, the integrity of RNA samples was observed on agarose gels stained with ethidium bromide (Figure 2), it was possible to observe that almost all samples showed a good integrity, in which the ribosomal RNA (rRNA) subunits 28S and 18S were detected, except the sample loaded in Lane 9. Figure 2 was a representative image of the RNA extractions and all samples with a bad integrity were discarded.

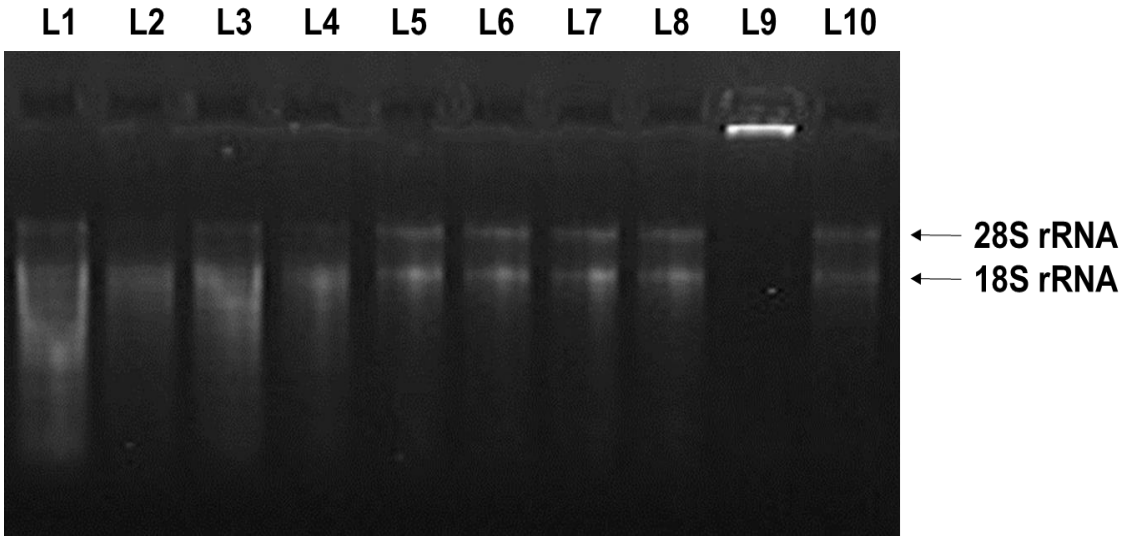

Figure 2. Agarose gel of RNA samples from plant tissues. Samples were observed and separated using electrophoresis observing the rRNA subunits to determine the integrity of total RNA samples isolated from CTV-infected and uninfected plant tissues (leaves from upper, middle and lower parts). This is a representative image of total RNA extractions from plant tissues.

2. Detection of CTV isolates

To performed the analyses of transmission and colonization time, the presence of PIAC, CS1, and Forte Rolândia was determined in those citrus plants used for the acquisition assay, the DNA amplicons obtained by PCR amplification showed that CTV-infected plants collected from the field were positive (Figure 3).

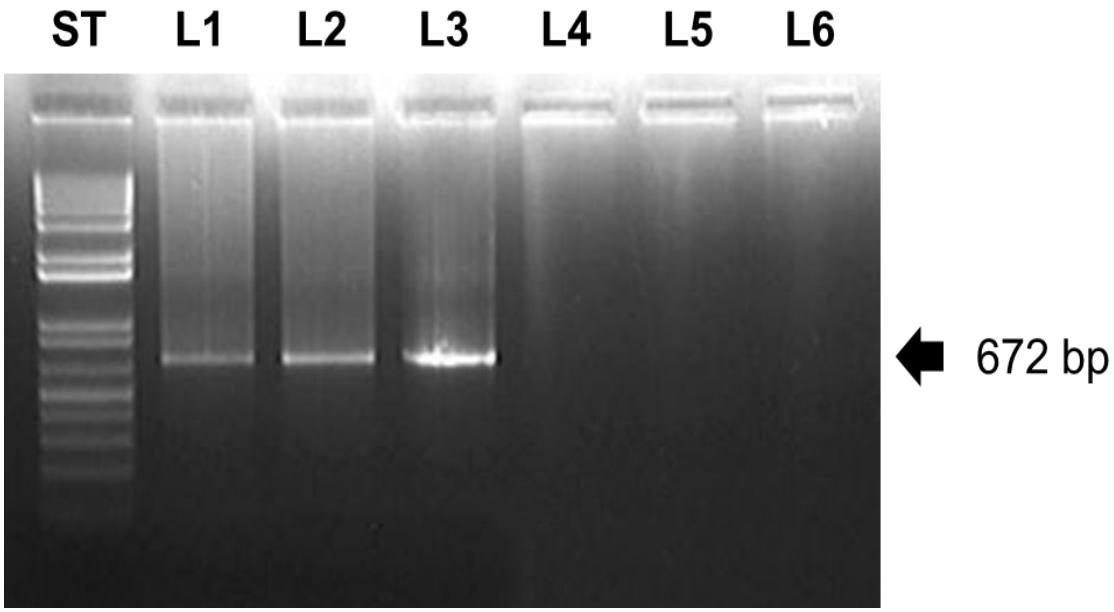

Figure 3. Detection of PIAC, CS1, and Forte Rolândia CTV isolates**.** The CTV-infected plants showed the DNA amplicon of the expected size, 672 bases pair (bp). ST is the DNA ladder and lanes 1 to 3 correspond to CTV isolates and lanes 4 to 6 are the PCR negative controls.

3. Detection of CTV subisolates and colonization

Colonization time of CTV subisolates on citrus plants exposed to one-single aphid fed on infected plants that possessed the PIAC, CS1 and Forte Rolândia (Figure 1D) were analyzed during 270 days for each plant (N=25). The PCR results showed the detection of CTV subisolates occurred at different times (colonization times) among samples (Figure 4). The first subisolate detected on plants was PIAC, occurring at day 150, then the subisolate CS1 was detected at day 210; however, the severe subisolate Forte Rolândia was not detected during this analysis. These results were verified observing the symptoms associated with CTV in citrus plants at day 0 (pre-inoculation) and day 210 (post-inoculation) (see Figure 5).

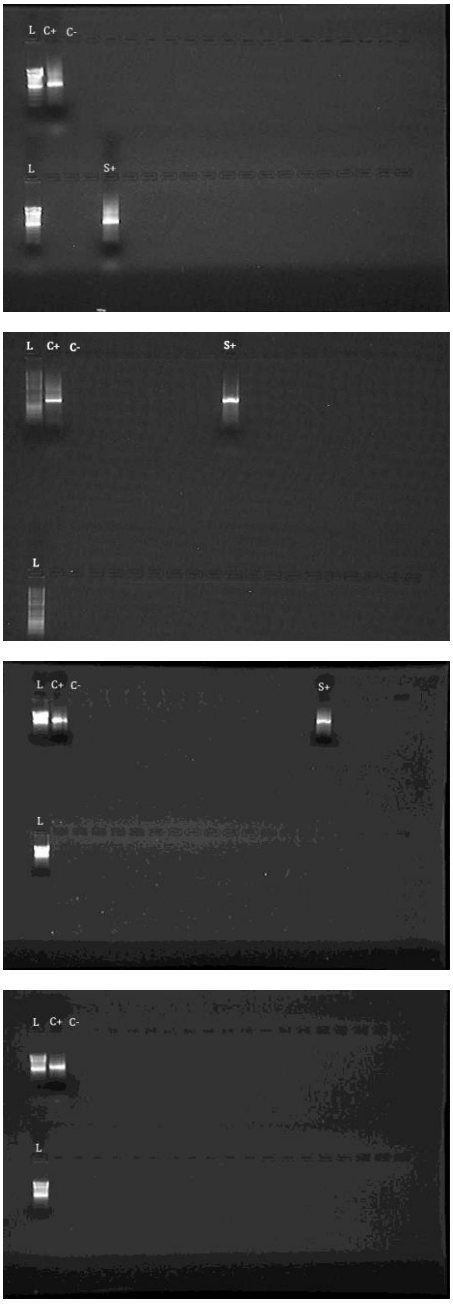

Figure 4. Detection of CTV subisolates (PIAC, CS1, Forte Rolândia) after one-single aphid transmission in 25 individual citrus plants. Representative images of CTV-detection by PCR at different time points. The first PCR-detections were obtained at day 150 by PIAC (1 positive samples) and at day 210 for second sample by PIAC and CS1 (1 positive sample), no PCR amplicons were detected in citrus plants infected with Forte Rolandia subisolate. (L) Ladder, (C+) Positive Control, (C-) Negative Control, (S+) Positive Sample.

Figure 5. Citrus plants cultivar Pêra orange pre- and post-inoculation of CTV subisolates**.** In the pictures, it is possible to observe that plants did not show symptoms associated with citrus tristeza disease.

After CTV detection (Figure 4), the pattern of each CTV-positive sample was analyzed using SSCP (Figure 6). Figure 6A showed the SSCP patterns found CS1, PIAC and Forte Rolândia CTV isolates, the CS1 isolate show 3 upper bands and 2 lower bands, the PIAC isolate showed 1 upper band and 2 lower bands and Forte Rolândia isolate possessed 4 upper bands and 1 lower band. In Figure 6B it was possible to observed how the CTV subisolates patterns occurred after one-single aphid transmission, CS1 subisolate displayed only 3 upper bands, while PIAC subisolate showed 1 upper band and 1 faint lower band (bands are indicated with numbers).

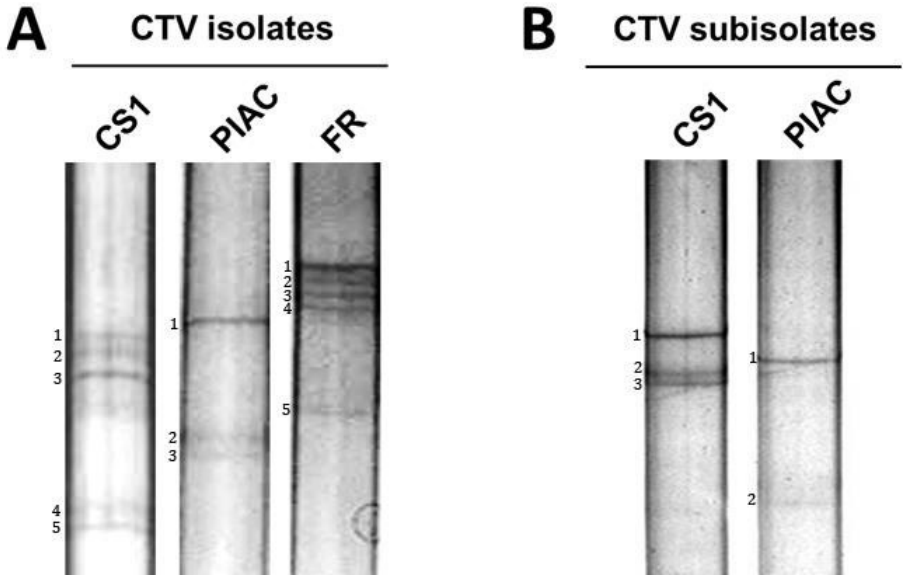

Figure 6. Single-Stranded Conformational Polymorphism patterns of CTV isolates and CTV subisolates. (A) SSCP patterns identified in field citrus plants infected with the CTV isolates: CS1, PIAC and Forte Rolândia (B) SSCP patterns of CTV subisolates identified in citrus plants infected with one-single aphid transmission, only CS1, and PIAC subisolates were identified.

4. Aphid transmission assay

The transmission was performed with one-single aphid, our results showed that one plant was positive after being infected with CS1 CTV subisolate and two plants were positive after being infected with PIAC CTV subisolate, showing a transmission rate of 4% and 8%, respectively (Table 1). There was not transmission using Forte Rolândia subisolate to any of the plants used during this experiment. The transmission efficiency of PIAC subisolate was higher than the CS1 subisolate, as was observed during this assay.

Table 1. Transmission rate using one-single aphid (*Toxopetera citricida*) and CS1, PIAC and Forte Rolândia subisolates, under Brazilian conditions.

CTV subisolate	Positive / inoculated plants
CS1	1/25
PIAC	2/25
Forte Rolândia	0/25

DISCUSSION

The present study was focused on the transmission of several subisolates of CTV, (PIAC, CS1, and Forte Rolândia) by one single aphid (*T. citricida*) under environmental conditions of Maringá, Brazil.

Among the three isolates, PIAC and CS1 were previously identified as protective subisolates. The PIAC was identified as a protective isolate that offered an effective and lasting protection (pre-immunization) for Pêra orange cultivar plants (MÜLLER, 1980b). Also, a recent study from the Núcleo de Pesquisa em Biotecnologia Aplicada (NBA) belonging to the University of Maringá, Paraná-Brazil, found that CS1 might possess the capacity of protection for Pêra orange cultivar plants (ZANUTTO et al., 2013a). The final CTV isolate, Forte Rolândia, was identified to be the one that produced severe symptoms of citrus tristeza disease, causing an economic damage in commercial citrus orchards in the northern regions of Paraná state (CARRARO et al., 2003; ZANUTTO et al., 2013a).

One of the main characteristics of CTV might be also one of the major problems for pre-immunization studies, CTV is a virus of single-stranded RNA (KITAJIMA et al., 1964). Therefore, its genome is prone to a high mutation rate (LAI, 1992). The high mutation rate, in addition to environmental conditions, has generated problems for a prolonged use of the same CTV isolate in the pre-immunization assays (CORAZZA et al., 2012). This problem was observed in when a mild isolate, previously used for pre-immunization in commercial orchards, began to show severe symptoms of citrus tristeza disease (MÜLLER, 1999).

CTV isolates consist of a population of variants that are highly divergent (RUIZ-RUIZ et al., 2007; LEONEL et al., 2015). The composition in a CTV-infected plant may vary by several factors: the high mutation rate observed in CTV (CORAZZA et al., 2012), repeated acquisitions produced by multiple inoculations of CTV-variants found in other citrus trees (AYLLÓN et al., 2006). In this study, we used three CTV isolates (PIAC, CS1, and Forte Rolândia) to infect citrus plants, these isolates were detected by PCR (Figure 3) and its SSCP pattern identified on field citrus plants (Figures 6A). Then, with the purpose of identifying the specific CTV variant transmitted and determine the rate of transmission under the Brazilian environment conditions, aphids were fed on this field plants and then one-single aphid per citrus plant was used for our experiments. The main results found in this study indicated that there are differences in efficiency of transmission by *T. citricida* between mild isolates (PIAC and CS1) compared to the severe isolate (Forte Rolândia). The subisolates of PIAC and CS1 showed an efficiency of transmission of 8% and 4%, respectively. While Forte Rolândia was

not transmitted by *T. citricida* in this assay. These results were similar to a previous study in which the efficiency of transmission to citrus plants varied between 0% to 3.6% using the vector, by *T. citricida*; however, a higher efficiency of transmission using severe CTV isolates were found under greenhouse conditions in Florida, US (LIN et al., 2002).

The reduced efficiency of CTV-transmission observed using the protective isolates PIAC and CS1 (8% and 4%, respectively) might be the result of the high temperatures (~34°C) measured in Maringá, Brazil. Our reduced transmission rates might be supported by experiments previously performed in CTV-transmission under controlled temperatures, in which the efficiency of transmission observed in Madame Vinous sweet orange transmitted by melon aphid *Aphis gossypii* varied between 60.8% and 12.2% under constant temperatures of 22±2°C and 31°C, respectively (BAR-JOSEPH and LOEBENSTEIN, 1973). In other studies that involved the transmission of CTV by single and/or multiple aphids of *T. citricida,* the transmission rates vary between 0% to 59% (BROADBENT, 1996; YOKOMI et al., 1994; LIN et al., 2002; HERRON et al., 2006).

In addition, other causes for low transmission rates are several studies that suggested that the capsid protein is involved in the lack/loss of transmissibility of circulating and non-circulating viruses. The transmission process of some non-circulating virus species, such as CTV, the vector is dependent of factors encoded by the virus for effective transmission, called "helper" (PIRONE and BLANC, 1996; HERRON et al., 2006). This association was described for *Cauliflower mosaic virus* (CaMV), which is spread by aphids in a non-circulatory transmission. The CaMV uses a protein “helper” during the transmission process. A protein that had been suggest or identified to be the “helper”, it is a non-structural viral protein (P2) that assists in the adhesion and retention of viral particles in the cuticle of the aphid stylets, promoting an effective transmission (LEH et al., 2001; PALACIOS et al., 2002). In the case of CTV, several gene products were suggested to be “helper” proteins (HERRON et al., 2006) that might assist with the adhesion and retention of CTV particles in the cuticle of the aphid, *T. citricida* (BAR-JOSEPH, 1985; KARASEV et al., 1995; FLORES et al., 2013).

Also, other factors can influence the virus-plant interaction, plants can defend themselves against viruses of single strand RNAs (ssRNAs) using a mechanism in which they can synthesize double-stranded replicative intermediate reducing the virus replication (WATERHOUSE et al., 2001). The defense process occurs when plants synthetize small interference RNA (siRNAs) and microRNAs (miRNAs) that aligned to the viral RNAs

(CHENG et al., 2015). This process can counterfeit by viruses blocking the expression of RNA silencing suppressors (RSSs) (METTE et al., 2001; GROENENBOOM e HOGEWEG, 2012), in the CTV genome, the gene products p20, p23 and p25, have been identified as proteins involved in the suppression of the RSSs (LU et al., 2004).

In this study, we also found differences in the SSCP patterns between CTV isolates and CTV subisolates (see Figure 6A and 6B, respectively), the differences in CTV patterns are because of the use of one single aphid to perform the transmission, one aphid can acquire only one subisolate, process known as segregation (BROADBENT, 1996; TSAI et al., 2000; BRLANSKY, 2003; HERRON et al., 2006; LIN et al., 2002). This was too observed in a field study, in which aphids (*T. citricida*) fed on citrus CTV-infected plants transmitted different subisolates to citrus plants source (NOLASCO et al., 2008).

The differences found in detection times of PIAC and CS1 subisolates occurred after 150 and 210 days, respectively, might be explain by several causes, such as, amount of tissue used for CTV detection, how heterogeneous is the distribution of CTV in the infected plants and the differences in specificity of the primers used for CTV identification (NOLASCO et al., 2008). On the other hand, a hypothesis not yet explored, but with a potential effect for this investigation is the synergism of CTV isolates, and how these isolates and/or subisolates interact with the host plant. The definition of synergism can be understood as the process in which a co-infection with two or more virus isolates and/or subisolates potentiates the infection and multiplication of both within the host (PRUSS et al., 1997). It is already known and proven in several studies (BROADBENT, 1996; TSAI et al., 2000; BRLANSKY, 2003; HERRON et al., 2006; LIN et al., 2002) that CTV is composed by several isolates (CORRAZA-NUNES et al., 2001; COSTA et al., 2010), thus, when only a fragment of this virus is transmitted by a single aphid from those CTV isolates, this new CTV subisolate acquired could have a different SSCP pattern, and might not have a high rate of infection capacity and consequently affect the multiplication within the host plant, as was observed in the present research.

CONCLUSION

In conclusion, this is the first study conducted in Brazil using one-single vector, *T. citricida*, for separation of two different type (mild and severe) of CTV-subisolates. Based on the observations of the data obtained in our results, we concluded that there were differences in the efficiency of transmission between mild and severe isolates using as vector one-single aphid, under the environmental conditions of Maringá, Brazil (~34ºC). The transmission assays showed that the mild CTV isolates, PIAC and CS1, had a low efficiency of transmission. While, the severe CTV-isolate, Forte Rolândia, was unable to be detected or transmitted to citrus plants. Also, the SSCP patterns varied between the mild CTV isolates and their respective subisolates obtained after one-single aphid transmission. In overall, we might suggest future studies on in-vitro acquisitions of the CTV-isolates, PIAC, CS1 and Forte Rolândia, and the detection and characterization of these isolates on aphid stylets before and after transmission, in order to understand the specific mechanisms that are occurring in aphid-virus interaction, with the final goal to reduce the economic impact associated with citrus tristeza disease.

REFERENCES

ANDERSON, P.K.; CUNNINGHAM, A.A.; PATEL, N.G.; MORALES, F.J.; EPSTEIN, P.R.; DASZAK, P. Emerging infectious diseases of plants: pathogen pollution, climate change and agrotechnology drivers. **Trends Ecol. Evol**., n.19, p.535–544, 2004.

AYLLÓN, M.A.; RUBIO, L.; SENTANDREU, V.; MOYA, A.; GUERRI, J.; MORENO, P. Variations in two gene sequences of *Citrus tristeza virus* after host passage. **Virus genes**, v.32, n.2, p.119-128, 2006.

BAR-JOSEPH, M., GUMPF, D.J., DODDS, J.A., ROSNER, A. AND GINZBERG, I. A simple purification method for *Citrus tristeza virus* and estimation of its genome size. **Phytopathology**, v.75, n.2, p.195-198, 1985.

BAR-JOSEPH, M.; MARCUS, R.; LEE, R.F. The continuous challenge of *Citrus tristeza virus* control. **Annual Review of Phytopathology**, v.27, p. 291-316, 1989.

BAR-JOSEPH, M; LOEBENSTEIN, G. Effects of strain, source plant, and temperature on the transmissibility of *Citrus tristeza virus* by the melon aphid. **Phytopathology**, v.63, n.6, p.716-720, 1973.

BEIDLER, J.L.; HILLIARD, P.R.; RILL, R.L. Ultrasensitive staining of nucleic acids with silver. **Analytical biochemistry**, v.126, n.2, p.374-380, 1982.

BRLANSKY, R.H.; DAMSTEEGT, V.D.; HOWD, D.S.; ROY, A. Molecular analyses of *Citrus tristeza virus* subisolates separated by aphid transmission. **Plant Disease**, v.87, n.4, p.397-401, 2003.

BROADBENT, P.; BRLANSKY, R.H.; INDSTO, J. Biological characterization of Australian isolates of *Citrus tristeza virus* and separation of subisolates by single aphid transmissions. **Plant Disease**, v.80, n.3, p.329-333, 1996.

CARRARO, B.P.; DE CARVALHO NUNES, W.M.; CORAZZA-NUNES, M.J.; MACHADO, M.A.; STACH-MACHADO, D.R. Avaliação de complexos do *Citrus tristeza virus* da região Norte do Estado do Paraná por meio de testes imunológicos e SSCP do gene da capa protéica. **Acta Sci. Agron**., v.25, n.2, p.269-273, 2003.

CHENG, C.Z.; YANG, J.W.; YAN, H.B.; BEI, X.J.; ZHANG, Y.Y.; LU, Z.M.; ZHONG, G.Y. Expressing p20 hairpin RNA of *Citrus tristeza virus* confers Citrus aurantium with tolerance/resistance against stem pitting and seedling yellow CTV strains. **Journal of Integrative Agriculture**, v.14, n.9, p.1767-1777, 2015.

CORAZZA, M.J., ZANUTTO, C.A., ZANINELI-RÉ, M.L., MÜLLER, G.W. AND NUNES, W.M.D.C. Comparison of *Citrus tristeza virus* (CTV) isolates by RFLP analysis of the coat protein nucleotide sequences and by the severity of the symptoms. **Tropical Plant Pathology**, v.37, n.3, p.179-184, 2012.

CORAZZA-NUNES, M.J.; MACHADO, M.A.; MÜLLER, G.W.; STACH-MACHADO, D.R.; SOUZA, A.A.; NUNES, W.M.C. Evaluation of *Citrus tristeza virus* (CTV) complexes in preimmunized Marsh seedless grapefruit. **Summa Phytopathologica**, v.27, p.11-16, 2001.

CORAZZA-NUNES, M.J.; MACHADO, M.A.; STACH-MACHADO, D.R.; NUNES, W.M.C.; CARVALHO, S.A.D.; MÜLLER, G.W. Characterization of *Citrus tristeza virus* isolates from grapefruit (*Citrus paradisi* Macf.) accessions of Citrus Active Germplasm Bank. **Summa Phytopathologica**, v.32, n.4, p.322-327, 2006.

COSTA, A.T.; CARVALHO-NUNES, W.M.; ZANUTTO, C.A.; MÜLLER, G.W. Stability of *Citrus tristeza virus* protective isolates in field conditions. **Pesq. agropec. bras**., v.45, n.7, p.693-700, 2010.

COSTA, A.S.; GRANT, T.J. Studies on transmission of the tristeza virus by the vector, *Aphis citricidus*. **Phytopathology**, v.41, n.2, 1951.

DAWSON, W.O.; GARNSEY, S.M.; TATINENI, S.; FOLIMINOVA, S.Y.; HARPER, S.J.; GOWDA, S. *Citrus tristeza virus*-host interactions. **Frontier in Microbiology**, v.4, n.88, p.1-10, 2013.

FLORES, R.; RUIZ-RUIZ, S.; SOLER, N.; SANCHEZ-NAVARRO, J.; FAGOAGA, C.; LÓPEZ, C.; NAVARRO, L.; MORENO, P.; PEÑA, L. *Citrus tristeza virus* p23: a unique protein mediating key virus–host interactions. **Frontiers in Microbiology**, v.4, p.1-9, 2013.

GIAMPANI, J.S.; SANTOS, K.S.; SILVA, C.C.; DE OLIVEIRA MOLINA, R.; RODRIGUES, L. K.; EIRAS, M.; JÚNIOR, R. P. L. Severity and genetic diversity of Citrus tristeza virus complexes infecting 'Pêra Bianchi'sweet orange in Paraná, Brazil. **Tropical Plant Pathology**, v. 43, n. 1, p. 59-68, 2017.

GROENENBOOM, M.A.; HOGEWEG, P. Modelling the dynamics of viral suppressors of RNA silencing. **Journal of The Royal Society Interface,** v.9, p.436-447, 2012.

HERRON, C.M.; MIRKOV, T.E.; DA GRAÇA, J.V.; LEE, R.F. *Citrus tristeza virus* transmission by the *Toxoptera citricida* vector: In vitro acquisition and transmission and infectivity immunoneutralization experiments. **Journal of virological methods**, v.134, n.1, p.205-211, 2006.

HILF, M.E.; MAVRODIEVA, V.A.; GARNSEY, S.M. Genetic marker analysis of a global collection of isolates of *Citrus tristeza virus*: characterization and distribution of CTV genotypes and association with symptoms. **Phytopathology**, v.95, n.8, p.909-917, 2005.

KARASEV, A.V.; BOYKO, V.P.; GOWDA, S.; NIKOLAEVA, O.V.; HILF, M.E.; KOONIN, E.V.; NIBLETT, C.L.; CLINE, K.; GUMPF, D.J; LEE, R.F.; GARNSEY, S.M.; LEWANDOWSKI, D.J.; DAWSON, W.O. Complete sequence of the *Citrust tristeza virus* RNA genome**. Virology**, v.208, p.511-520, 1995.

KITAJIMA, E.W.; SILVA, D. M.; OLIVEIRA, A. R.; MÜLLER, G. W.; COSTA, A.S. Electron microscopical investigations of tristeza. In: **Proceedings of the 3rd Conference of the International Organization of Citrus Virologists - IOCV.** p.1-9. IOCV, Riverside, CA. 1964.

LAI, M.M. RNA recombination in animal and plant viruses. **Microbiological reviews**, v.56, n.1, p.61-79, 1992.

LEE, R.F. AND KEREMANE, M.L. Mild strain cross protection of tristeza: a review of research to protect against decline on sour orange in Florida. **Frontiers in microbiology**, v.4, n.259, p.1-11, 2013.

LEE, R.K.; BRLANSKY, H.; GARNSE, S.M.; YOKOM, R.K. Traits of *Citrus tristeza virus* important for mild strain cross protection of citrus: the Forida approach. **Phytophylactica**, v.19, n.2, p.215-218, 1987.

LEH, V.; JACQUOT, E.; GELDREICH, A.; HAAS, M., BLANC, S.; KELLER, M.; YOT, P. Interaction between the open reading frame III product and the coat protein is required for transmission of cauliflower mosaic virus by aphids. **Journal of virology**, v.75, n.1, p.100-106, 2001.

LEONEL, W.M.S.; CORAZZA, M.J.; ZANUTTO, C.A.; MÜLLER, G.W.; CARVALHO, S.A.; NUNES, W.M. Stability of *Citrus tristeza virus* protective isolate 'Pêra IAC' according to SSCP analysis of old and new lines of three sweet orange varieties. **Summa Phytopathol**. v.41, n.1, p.8-12, 2015.

LIN, Y.; BRLANSKY, R.H.; POWELL, C.A. Inefficient transmission of *Citrus tristeza virus* from grapefruit by single brown citrus aphids. **HortScience**, v.37, n.6, p.936-939, 2002.

LU, R.; FOLIMONOV, A.; SHINTAKU, M.; LI, W.X.; FALK, B.W.; DAWSON, W.O.; DING, S.W. Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. **Proceedings of the National Academy of Sciences of the United States of America,** v.101, n.44, p.15742-15747, 2004.

METTE, M.F.; MATZKE, A.J.M.; MATZKE, M.A. Resistance of RNA-mediated TGS to HC-Pro, a viral suppressor of PTGS, suggests alternative pathways for dsRNA processing. **Current Biology**, v.11, n.14, p.1119-1123, 2001.

MICHAUD, J.P. A review of the literature on *Toxoptera citricida* (kirkaldy) (homoptera: aphididae). **Florida Entomologist**, v.81, n.1, p.37-61, 1998.

MORENO, P.; AMBRÓS, S.; ALBIACH-MARTI, M.R.; GUERRI. J.; PEÑA, A.L. *Citrus tristeza virus*: a pathogen that changed the course of the citrus industry. **Molecular Plant Pathology**, v.9, n.2, p.251-268, 2008.

MÜLLER, G.W. Tristeza control by cross protection. **Plant Disease**, v.64, n.6, 1980.

MÜLLER, G.W. Use of mild strains of *Citrus tristeza virus* (CTV) to reestablish commercial production of 'Pera'sweet orange in Sao Paulo, Brazil. **Proc. Fla. State Hort. Soc.,** v. 93, p. 62-64, 1980b.

MÜLLER, G.W.; TARGON, M.L.N.; MACHADO, M.A. Trinta anos de uso do clone pré-imunizado "Pêra" IAC na citricultura paulista. **Laranja**, v.20, n.2, p. 399-408, 1999.

MUNIZ, F.R.; DE SOUZA, A.J.; STIPP, L.C.L.; SCHINOR, E.; FREITAS, W.; HARAKAVA, R.; STACH-MACHADO, D.R.; REZENDE, J.A.M.; MOURÃO FILHO; F.A.A.; MENDES, B.M.J. Genetic transformation of *Citrus sinensis* with *Citrus tristeza virus* (CTV) derived sequences and reaction of transgenic lines to CTV infection. **Biologia Plantarum**, v.56, n.1, p.162-166, 2012.

NOLASCO, G.; FONSECA, F.; SILVA, G. Occurrence of genetic bottlenecks during *Citrus tristeza virus* acquisition by *Toxoptera citricida* under field conditions. **Arch Virol**., v.153, p. 259–271, 2008.

PALACIOS, I.; DRUCKER, M.; BLANC, S.; LEITE, S.; MORENO, A.; FERERES, A. Cauliflower mosaic virus is preferentially acquired from the phloem by its aphid vectors. **Journal of General Virology**., v. 83, n.1, p.3163-3171, 2002.

PIRONE, T.P.; BLANC, S. Helper-dependent vector transmission of plant viruses. **Annual review of phytopathology**, v.34, n.1, p.227-247, 1996.

POWELL, C.A.; PELOSI, R.R.; RUNDELL, P.A.; COHEN, M. Breakdown of cross-protection of grapefruit from decline-inducing isolates of *Citrus tristeza virus* following introduction of the brown citrus aphid. **Plant disease**, v.87, n.9, p.1116-1118, 2003.

PRUSS, G.; GE, X.; SHI, X.M.; CARRINGTON, J.C.; VANCE, V.B. Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. **The Plant Cell**, v.9, n.6, p.859-868, 1997.

RUIZ-RUIZ, S.; MORENO, P.; GUERRI, J.; AMBRÓS, S. A real-time RT-PCR assay for detection and absolute quantitation of *Citrus tristeza virus* in different plant tissues. **Journal of Virological Methods**, v.145, n.2, p.96-105, 2007.

SOUZA, A.A., MÜLLER, G.W., TARGON, M.L.P.N. AND MACHADO, M.A. Evaluation of changes which occurred in a mild protective *Citrus tristeza virus* isolate in pera sweet orange trees by using RFLP and SSCP analyses of the coat protein gene. In **Proc. 14th Conf. IOCV Univ. of Calif**., Riverside, CA. p. 136-140, 2000.

STUCHI, E.S., DONADIO, L.C.; SEMPIONATO, O. Produtividade e tamanho das plantas do clone cnpmf-01, premunizado contra a tristeza dos citros, da limeira-ácida 'tahiti' em Bebedouro (SP). **Laranja**, Cordeirópolis. v.23, n.1, p.221-230, 2002.

TEMPORAL, W.M.; CORAZZA, M.J.; ZANUTTO, C.A.; NUNES, W.D.C.; MÜLLER, G.W. SSCP analysis of *Citrus Tristeza Vvirus* protectives isolates in Pêra sweet orange clones under northern Paraná state, Brazil conditions. **Citrus Research & Technology**, v.32, n.1, p.9-16, 2011.

TSAI, J.H.; LIU, Y.H.; WANG, J.J.; LEE, R.F. Recovery of orange stem pitting strains of *Citrus tristeza virus* (CTV) following single aphid transmissions with *Toxoptera citricida* from a Florida decline isolate of CTV. **Proceedings of the Florida State Horticultural Society**, v.113, p.75-78, 2000.

WATERHOUSE, P.M.; WANG, M.B.; LOUGH, T. Gene silencing as an adaptive defence against viruses. **Nature**, v.41, p.834-842, 2001.

WHITFIELD, A.E.; FALK, B.W.; ROTENBERG, D. Insect vector-mediated transmission of plant viruses. **Virology**, v. 479, p.278–289, 2015.

YOKOMI, R K. The Brown citrus Aphid and *Citrus tristeza virus*. **Citrograph**, v.6, p.14-18, 1994.

YOKOMI, R.K. The brown citrus aphid, *Toxoptera citricida*. **Options Méditerranéennes**, n.65, p.35-46, 2009.

ZANUTTO, C.A.; CORAZZA, M.J.; NUNES, W.M.D.C.; MÜLLER, G.W. Evaluation of the protective capacity of new mild *Citrus tristeza virus* (CTV) isolates selected for a preimmunization program. **Scientia Agricola,** v.70, n.2, 2013a.

ZANUTTO, C.A.; SOUZA, I.; GONÇALVES-ZULIANE, A.M.O.; SOARES, L.S.; NUNES, W.M.C.; CORAZZA, M.J. Estabilidade e distribuição do complexo do *Citrus tristeza virus* em plantas de laranja Pêra avaliado pela análise SSCP. **Tecnol. & Ciên. Agropec**., v.7,p.1-5, 2013b.

Chapter 2

Effects of '*Candidatus* Liberibacter solanacearum' (haplotype B) on *Bactericera cockerelli* (Šulc) fitness and vitellogenesis[1]

[1] The chapter 2 of this dissertation refers to the article accepted for publication in the Insect Science Journal (**https://doi.org/10.1111/1744-7917.12599**) of authorship by: Angélica Albuquerque Tomilhero Frias, Freddy Ibanez, Azucena Mendoza, William Mario de Carvalho Nunes and Cecilia Tamborindeguy.

RESUMO

Candidatus Liberibacter solanacearum (Lso) são bactérias gram negativas restritas ao floema e não cultivadas. Atualmente, cinco haplótipos foram identificados em todo o mundo, mas apenas os haplótipos A e B estão associados à *Bactericera cockerelli* (Šulc.) nas Américas. Estudos anteriores mostraram que infeção por Lso reduziu capacidade reprodutiva de *B. cockerelli*. Para entender a interação entre Lso haplótipo B e *B. cockerelli*, a aptidão e a expressão de um gene envolvido diretamente na reprodução do inseto foram analisadas. Diferenças estatísticas no número de ovos ovipositados, número de ninfas e adultos produzidos foram encontrados entre cruzamentos de insetos que abrigavam ou não o patógeno bacteriano. A progênie dos adultos F_1 de mães infectadas com LsoB mostrou uma feminização da população de *B. cockerelli*. Uma redução significativa do tipo *BcVg1* foi observada em cruzamentos realizados com fêmeas infectadas com LsoB em comparação com o controle de Lso-livre de bactéria. Em diferentes grupos de fêmeas de mesma idade, uma redução significativa na expressão do tipo *BcVg1* foi observada em fêmeas infectadas com LsoB de 7 dias de idade (virgens e acasaladas) em comparação com fêmeas sem Lso de 7 dias de idade (virgens e acasaladas). A redução da transcrição do tipo *BcVg1* foi associada com baixo número de oócitos em desenvolvimento observados nos sistemas reprodutivos femininos. No geral, este estudo representa o primeiro passo para entender a interação do LsoB com *B. cockerelli*, destacando a função de *BcVg1* na diminuição do número de ovos e no desenvolvimento de oócitos.

Palavras-chave: Infecção bacteriana. Reprodução. Expressão Gênica.

ABSTRACT

Candidatus Liberibacter solanacearum (Lso) are phloem-restricted and unculturable gram-negative bacteria. Presently five haplotypes have been identified worldwide, but only haplotypes A and B are associated with *Bactericera cockerelli* (Šulc.) in the Americas. Previous studies showed that Lso-infection reduced *B. cockerelli* the reproductive output. To understand the interaction of Lso haplotype B and *B. cockerelli*, the fitness and the expression of a gene involved directly in the insect reproduction were analyzed. Statistical differences in the number of eggs oviposited, number of nymphs and adults produced were found among crosses of insects harboring or not the bacterial pathogen. The F_1 progeny adults from mothers infected with LsoB showed a feminization of *B. cockerelli* population. A significant reduction of the *BcVg1-like* expression was observed in crosses performed with LsoB-infected females compared to control Lso-free. In different female cohorts, a significant reduction of *BcVg1-like* was measured in 7-day-old LsoB-infected females (virgin and mated) compared with 7-day-old Lso-free females (virgin and mated). The reduction of *BcVg1-like* transcript was associated with a low number of developing oocytes observed in female's reproductive systems. Overall, this study represents the first step to understand the interaction of LsoB with *B. cockerelli*, highlighting the function of *BcVg1-like* in egg production and oocyte development.

Keywords: Bacterial infection. Reproduction. Gene expression.

INTRODUCTION

Many plant pathogenic bacteria depend on insect vectors to spread between hosts (WEINTRAUB and BEANLAND, 2006, ORLOVSKIS et al., 2015, PERILLA-HENAO and CASTEEL, 2016, TAMBORINDEGUY et al., 2017). The association between the microorganism and the insect can result in numerous symbiotic interactions that can affect the insect fitness; these associations have been well described in the literature as mutualism, parasitism and commensalism (LEUNG AND POULIN, 2008, SU et al., 2013, SOLOMON et al., 2015).

In last 30 years, emergent diseases caused by plant pathogenic bacteria associated with insects had caused significant economic losses worldwide (NADARASAH and STAVRINIDES, 2011). One of these associations involves psyllid insect species with the plant pathogenic bacteria '*Candidatus* Liberibacter spp.'. Psyllids are phloem-feeding hemipterans that transmit these plant pathogenic bacteria threatening several crops production (AUBERT, 1992, BOVÉ, 2006, MANJUNATH et al., 2008).

Zebra chip has recently become an important economic disease in potato crops, documented in several countries of the Americas and also in New Zealand (LIEFTING et al., 2008, TEULON et al., 2009, SECOR et al., 2009, CROSSLIN et al., 2010, MUNYANEZA et al., 2010, MUNYANEZA, 2012). The causative agent of zebra chip is '*Candidatus* Liberibacter solanacearum' (Lso) a phloem-restricted and unculturable gram-negative bacteria transmitted by *Bactericera cockerelli* (Šulc.) (Hemiptera: Triozidae), also known as potato psyllid (MUNYANEZA et al., 2007, HANSEN et al., 2008, LIEFTING et al., 2008, SECOR et al., 2009). Worldwide, five different Lso haplotypes have been described: LsoA, LsoB, LsoC, LsoD and LsoE (NELSON et al., 2011, NELSON et al., 2013, TERESANI et al., 2014). But, only Lso haplotypes A and B are associated with *B. cockerelli* and solanaceous crops (Nelson et al., 2011) in the Americas and New Zealand, whereas the haplotypes C, D and E are associated with *Trioza apicalis* and *Bactericera trigonica* in the old world (MUNYANEZA et al., 2010, TERESANI et al., 2014).

Despite the economic importance of Lso, few studies have been performed in the insect fitness of *B. cockerelli* infected with Lso. Previous studies showed that Lso-infected psyllid females had a reduced number of eggs oviposited on tomato plants compared to uninfected females (NACHAPPA et al., 2012, NACHAPPA et al., 2014, YAO et al., 2016). Similarly, other studies performed in *Drosophila melanogaster*, *Anopheles gambiae*, *Euoniticellus intermedius* and *Teleogryllus oceanicus*, showed that exposure to pathogenic

bacteria resulted in a reduced number of eggs (AHMED and HURD, 2006, REANEY and KNELL, 2010, NYSTRAND and DOWLING, 2014, MCNAMARA et al., 2014). However, the molecular mechanisms that might affect the egg production in Lso-infected psyllids are still undetermined.

The reproductive success of oviparous species depends on egg production, insects are not an exception. However, egg production is energetically demanding and can be influenced by external or internal factors. During oocyte development, yolk proteins (including vitellogenins), lipids, maternal RNAs, ribosomes, and organelles provide nutrients and/or patterning information for the future zygote (CHEN et al., 1997, ARUKWE and GOKSOYR, 2003). In most insect species, juvenile hormone (JH) initiates the vitellogenic process and egg development (TUFAIL and TAKEDA, 2008), in particular in members of the Hemiptera order, such as *B. cockerelli, Nilaparvata lugens*, *Lethocerus deyrollei* and *Riptortus clavatus* (SHINODA et al., 1996, NAGABA et al., 2010, NAGABA et al., 2011, IBANEZ et al., 2017).

The synthesis of vitellogenins (Vgs), essential for egg production, occurs in most insects in the fat body. After synthesis, Vgs are secreted into the hemolymph and they accumulate in the developing oocytes by a receptor-mediated pathway, as vitellin proteins (RAIKHEL, 1992). In *B. cockerelli*, *BcVg1-like* have been described and it is highly expressed in females after mating and following the exogenous application of JH III (IBANEZ et al., 2017).

In this study, the effects of LsoB in *B. cockerelli* was evaluated analyzing life table parameters of reciprocal crosses performed with LsoB and/or Lso-free females and males, also the relative expression of *BcVg1-like*, a gene involved directly in the reproduction of *B. cockerelli* females was investigated. This study has as a final goal, the beginning of unveiling the cause(s) of the reduced reproductive output observed in LsoB-infected *B. cockerelli* females previously reported (NACHAPPA et al., 2012, NACHAPPA et al., 2014, YAO et al., 2016).

MATERIALS AND METHODS

The present study was conducted in the laboratory of Dr. Cecilia Tamborindeguy at Texas A&M University, Texas, USA from April to September 2017.

1. Plant Material

Tomato (*Solanum lycopersicum* cv. Moneymaker) were cultivated in a room temperature (23 ± 3 °C) in plastic pots filled with Metro-Mix 900 growing mix (Sungro, Horticulture Distribution, Inc., Bellevue, WA, USA) and grown with a photoperiod of 16:8 h (Light: Dark). The plants were watered once every 2 days.

2. Insect source

The Lso-free *B. cockerelli* colony used for this study was obtained in 2013 from Dr. Don Henne, AgriLife Research Weslaco, TX. The colony was maintained on tomato plants in 14'' x 14'' x 24" insect cages (BioQuip, Rancho Dominguez, CA, USA) at room temperature and a photoperiod of 16:8 h (Light: Dark). Insects from the Lso-free colony were transferred to tomato plants infected with LsoB in order to create laboratory psyllid colonies harboring LsoB haplotype. Insects and plants were tested regularly using SSR1 primers previously published (LIN et al., 2012), previous to all analyses as was reported (Yao et al., 2016) to verify the presence and the haplotype of Lso.

With the objective to obtain all insects of the same age, nymphs (4^{th}-5^{th} instar) from each colony (Lso-free and LsoB) were transferred to 4-week-old uninfected tomato plants. On the day adults emerged, they were sexed under a dissection microscope (Leica EZ4W, I. Miller Precision Optical Instruments, Inc.), then females and males of each colony were separated and kept as same age cohorts in different tomato plants until required.

2.1.Insect crosses

To perform the individual crosses, one couple of 3-day-old insects (a male and a female) was placed in a 1.7mL Eppendorf tube for copulation during 4-5 hours or until they

mated (Figure 1A). Different crosses were performed using females and males, including Lso-free x Lso-free; Lso-free x LsoB; LsoB x Lso-free; LsoB x LsoB, each group consisted of at least 7 couples. After mating, males were discarded and females were transferred to 4-week-old tomato plants and allowed to oviposit for four days (Figure 1B). In the fourth day after mating, eggs were counted and started to analyze life-history parameter and females (mothers) were collected and flash frozen in liquid nitrogen and stored at -80ºC for further gene expression analysis.

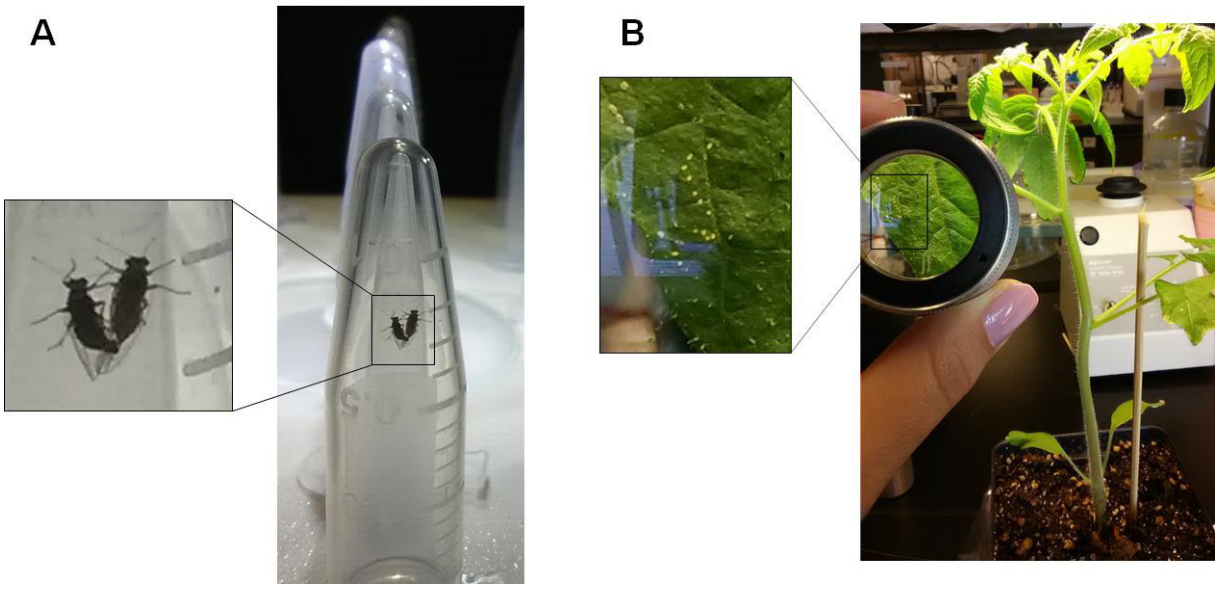

Figure 1. Experimental desing of crosses and egg counting using *B. cockerelli* couples (females and males). (A) Microtube containing a couple of *B. cockerelli* during copulation; (B) Counting the number of eggs oviposited by mated females of *B. cockerelli* on tomato leaves.

2.2.Life-history analyses

To understand the effects of LsoB in *B. cockerelli* insects, we analyzed several the life-history traits using three biological replicates consisting of 7 mated females. Among these analyses were oviposition, hatching percentage, average number of nymphs per female, sex ratio, the total number of adults; and LsoB sexual transmission in individuals from F_1 progeny.

2.3.Insect adult cohorts

Cohorts of 1-, 3- and 7-days-old virgin females were obtained as previously described. To obtain 7-day-old mated females, groups of ten 1-day-old females from the Lso-free and LsoB colonies were kept on 4-week-old uninfected tomato plants with ten 1-day-old males from the Lso-free colony, until they reached 7-days old. At day 7, the males were discarded and females were collected for follow-up analyses such as dissection of reproductive systems and *BcVg1-like* gene and protein expression. Each 10-female group was considered a biological replicate.

3. DNA extraction and Lso-detection

DNA extraction was performed on single psyllids (mothers and progeny F_1) from each cross following a procedure previously described (NACHAPPA et al., 2011). The presence of Lso on insects were determined by PCR reaction using the Lso primers (see Table 1). Each PCR reaction consisted in 50 ng of genomic DNA, 5 µL of Green Master Mix (Promega,), 3.5 µL of water, 10 µM each Lso primer, the PCR conditions were the following: 94°C for 5 min (denaturation), 40 cycles (amplification) of 95°C for 30 sec; 60°C for 30 sec and 72°C for 80 sec; and a final step (extension) of 72°C for 7 min. The amplicons were analyzed by electrophoresis, on 1% agarose gel containing ethidium bromide and the image of each gel was captured using a gel-documentation system (Fotodyne Incorporated, Hartland, WI).

4. RNA extraction

Total RNA was extracted from pools of seven 7-day-old mated females (mothers from the crosses) and from pools of 10 insects per biological replicate (1-, 3-, and 7-day-old virgin and 7-day-old mated females) and homogenized during 1 minute on ice with a plastic pestle using 500 µL of TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Genomic DNA contamination was removed by DNase treatment using Turbo DNase (Ambion, Invitrogen, CA). Quantity and purity of the total RNA samples were determined using an Infinite® 200 PRO NanoQuant (Tecan, Männedorf, Switzerland)

and RNA integrity was visualized by electrophoresis in agarose gels at 1.2% stained with ethidium bromide.

5. cDNA synthesis and gene expression analyses

Complementary DNA (cDNA) was synthesized using 300 ng of total RNA from females (life stages), anchored-Oligo (dT) primers and Verso cDNA Synthesis kit (Thermo, Waltham, MA) following the manufacturer's instructions. After synthesis, cDNA samples were stored at -20º C until RT-qPCR analyses.

For gene expression analyses in mothers (crosses), quantitative reverse transcription PCR (RT-qPCR) reactions were performed using SensiFAST SYBR Hi-ROX One-step Kit (Bioline, Taunton, MA) according to manufacturer's instructions. Each reaction contained 50 ng of total RNA, 250 nM of each primer (Table 1) and 1x of SensiFAST SYBR Hi-ROX One-step Mix; the volume was adjusted with nuclease-free water to 10 µL. The real-time PCR program was 45°C for 10 min (reverse transcription) followed 95°C for 2 min (activation) then 40 cycles at 95°C for 5 sec and 60°C for 30 sec. Real-time PCR assays were performed using an Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). The gene expression in different female cohorts (life stages) was performed using PowerUp SYBR Green Master Mix according to manufacturer's instructions. Each reaction contained 5 ng of cDNA, 250 nM of each primer (Table 1) and 1x of SYBR Green Master Mix; the volume was adjusted with nuclease-free water to 10 µL. The real-time PCR program was 95°C for 2 min followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. Real-time PCR assays were performed using an Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems).

Three biological replicates were used and each RT-qPCR reaction was performed in duplicate with a negative control in each run. The threshold cycle (Ct) values and the primer specificity was monitored with melting curve analysis using QuantStudio™ software V1.3 (Applied Biosystems). The relative expression of each transcript was estimated with the delta-delta CT method (SCHMITTGEN and LIVAK, 2008), using *elongation factor-1α* (GenBank KT185020) and a *ribosomal protein subunit 18* (GenBank KT279693) as reference genes (IBANEZ and TAMBORINDEGUY, 2016).

Table 1. Primers used for *Bactericera cockerelli* Lso-detection and RT-qPCR relative gene expression analyses.

Target gene	Name	Primers
LSO	Lso-F	5'-CGAGCGCTTATTTTTAATAGGAGC-3'
	Lso-R	5'-GCCTCGCGACTTCGCAACCCAT-3'
rRNA 28S	rRNA 28S-F	5'-CGCAGACTGGTTCGGGATAC-3'
	rRNA 28S-R	5'-GCGAGGACTCAGTTTCGTGTC-3'
BcVg1-like	BcVg1-like qPCR Fw1	5'-GACCTGTTGGGACTTTTGGA-3'
	BcVg1-like qPCR Rv1	5'-GCTTGTTTGGCGTTCTTCTC-3'

6. Number of developing oocytes

After insects were collected on the seventh day, females were used to determine the number of developing oocytes and males were discarded. Briefly, 7-day-old mated females were anesthetized on ice for 15 minutes, and their reproductive tissue was dissected using cold 1x Phosphate-buffered saline (1x PBS) in a dissection slide as previously described in Ibanez et al. (2014). The dissected samples were transferred to a new 1.7 mL microcentrifuge tube previously filled with 200 µL of 3.8% formaldehyde/1x PBS buffer (fixation buffer) for 1 hour. The fixation buffer was removed by washing the reproductive tissues twice for 15 minutes using 1x PBS. Then, the samples were mounted using 50 µL of Vectashield mounting medium with DAPI (4,6-Diamidino-2-phenylindole-Vector laboratories Inc., Burlingame, CA). The images of at least twenty female reproductive systems per group (♀Lso-free x ♂Lso-free and ♀LsoB x ♂Lso-free) were obtained using an Axioimager A1 microscope (Carl Zeiss microimaging, Thornwood, NY, USA) and visualized with AxioVision SE64 Rel. 4.9.1 software (Carl Zeiss).

7. Statistical analyses

The effect of LsoB-infection in *B. cockerelli* life history analyses, such as, number of eggs and nymphs, nymph survival, number of adults (F_1 progeny), number of developing oocytes and relative expression analyses were analyzed using one-way ANOVA with Tukey's

post hoc test using RStudio (RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/.). The sex ratio statistical analysis was performed using paired t-test using RStudio.

RESULTS

1. Oviposition

The number of eggs oviposited on leaves of 4-week tomato plants by groups of seven mated females from different crosses (Lso-free x Lso-free; Lso-free x LsoB; LsoB x Lso-free; LsoB x LsoB) were counted after an oviposition period of four days (Figure 2). A significant reduction in the number of eggs oviposited by crosses performed with LsoB-infected insects (females or males) compared to the control Lso-free x Lso-free ($P < 0.05$) was observed. No statistical differences were determined among the crosses performed with LsoB-infected insects ($P > 0.05$).

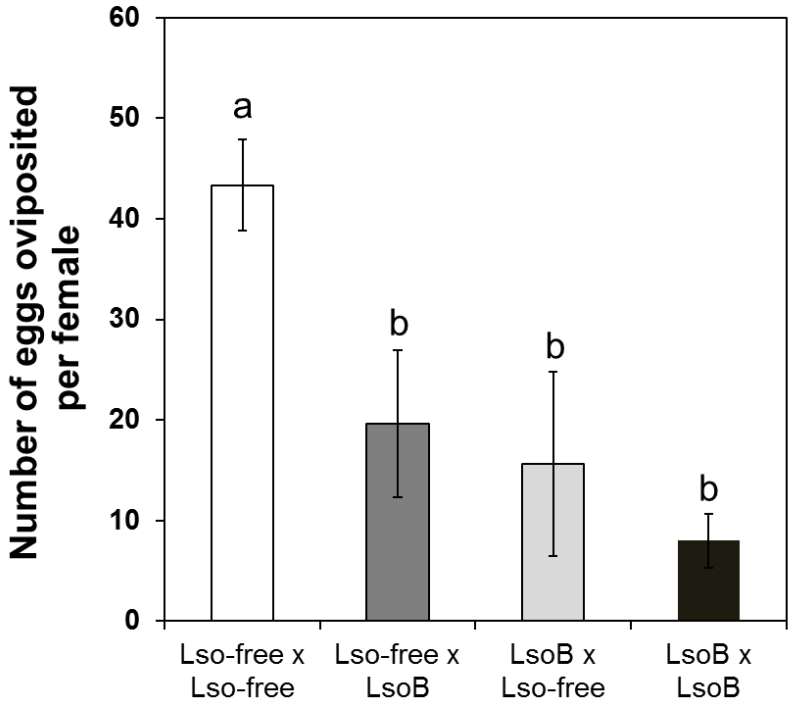

Figure 2. Average number of eggs oviposited per mated *B. cockerelli* female from crosses performed with Lso-free and LsoB-infected insects on tomato leaves in a 4-day period**.** A significant reduction in egg oviposition was observed in the crosses when at least one adult (female or male) was infected with haplotype LsoB. Data represent means ± SD of three independent experiments. Different letters indicate statistical differences between treatments using one-way ANOVA with tukey's post hoc test ($P < 0.05$).

2. Egg hatching percentage

The percentage of egg hatching was analyzed for each group of samples, no statistical differences were found among crosses performed with Lso-free and LsoB-infected insects ($P > 0.05$), see Figure 3.

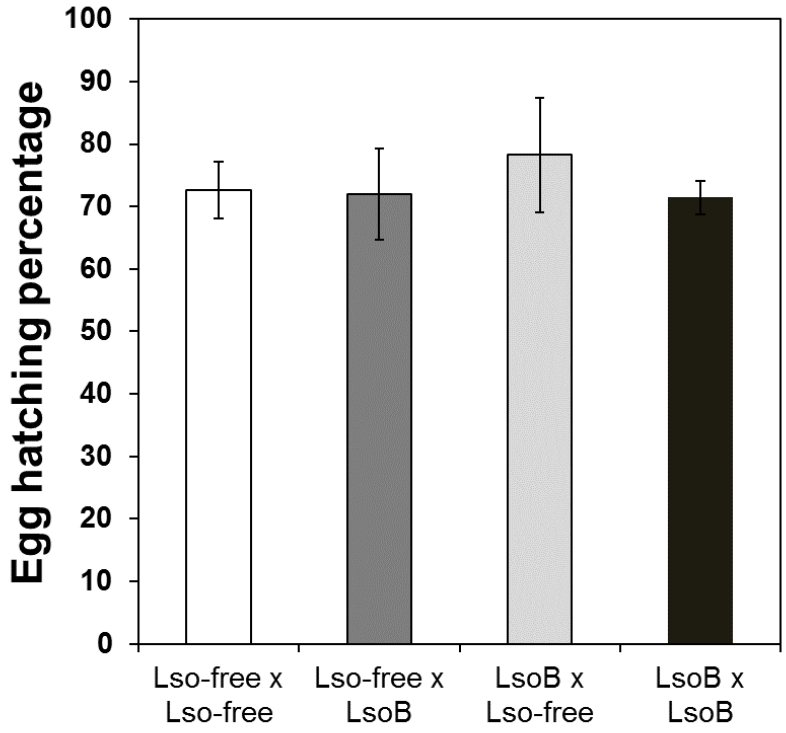

Figure 3. Influence of LsoB infection in egg hatching percentage in *B. cockerelli*. No significant differences in the percentage of egg hatching among the crosses performed with Lso-free and LsoB-infected insects were observed ($P>0.05$). Data represent means ± SD of three independent experiments.

3. Number of nymphs, adults and nymph survival

The number of nymphs on tomato leaves from different crosses (Lso-free x Lso-free; Lso-free x LsoB; LsoB x Lso-free; LsoB x LsoB) were counted and monitored daily during a period of three weeks (Figure 4A). Significant differences were measured between the number of nymphs per female produced by the Lso-free x Lso-free (31 nymphs) compared with those crosses achieved with LsoB-infected insects ($P < 0.05$). In detail, there were fewer

nymphs in the Lso-free x LsoB (8 nymphs), LsoB x Lso-free (14 nymphs) and LsoB x LsoB (7 nymphs) crosses. No statistical differences were identified among crosses performed with LsoB-infected insects ($P > 0.05$). Fewer adults were also produced in the Lso-free x LsoB, LsoB x Lso-free and LsoB x LsoB crosses compared to the Lso-free x Lso-free cross (Figure 4B). However, no differences in nymphal survival were observed among crosses (Figure 4C).

Each F_1 progeny cross was tested for Lso detection, no presence of Lso was identified in Lso-free x Lso-free and Lso-free x LsoB. While all F_1 progeny from crosses LsoB x Lso-free and LsoB x LsoB tested positive for Lso (Figure 5).

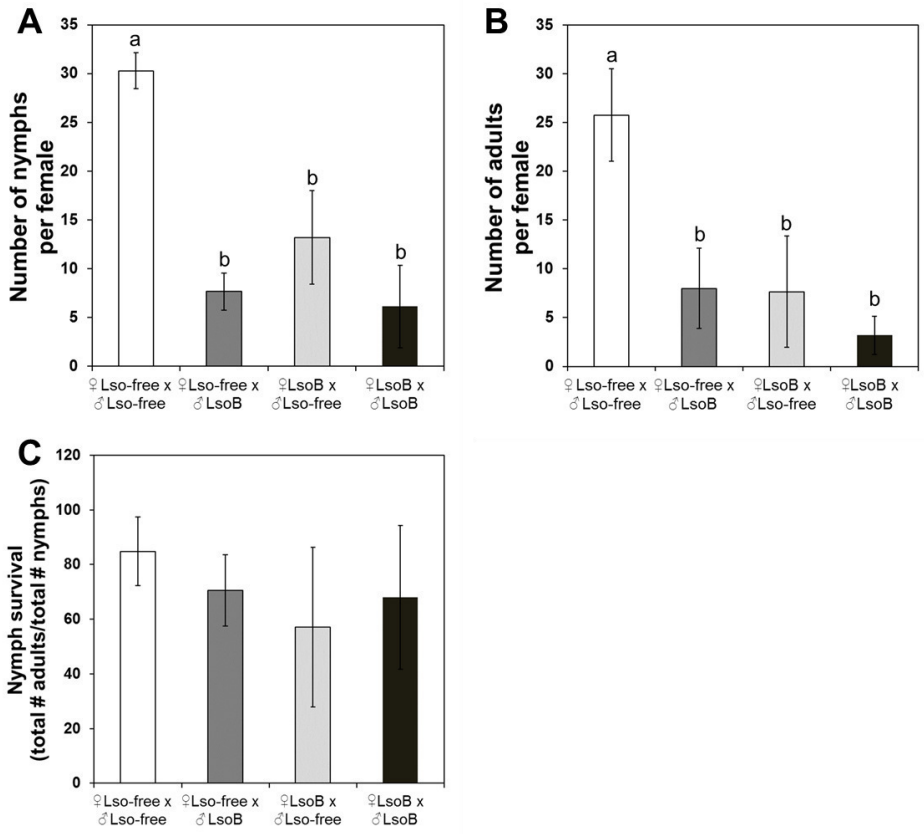

Figure 4. Influence of LsoB infection in number of nymphs, adults and nymphal survival in *B. cockerelli*. (A) A reduction in the number of nymphs was observed in the crosses performed with LsoB-infected insects compared to the cross Lso-free x Lso-free. (B) Fewer adults were obtained in the crosses Lso-free x LsoB and LsoB x Lso-free compared to the Lso-free x Lso-free. (C) No statistical differences in the percentage of nymphal survival was found among crosses. Data represent means ± SD of three independent experiments. Different letters indicate statistical differences between treatments using one-way ANOVA with tukey's post hoc test.

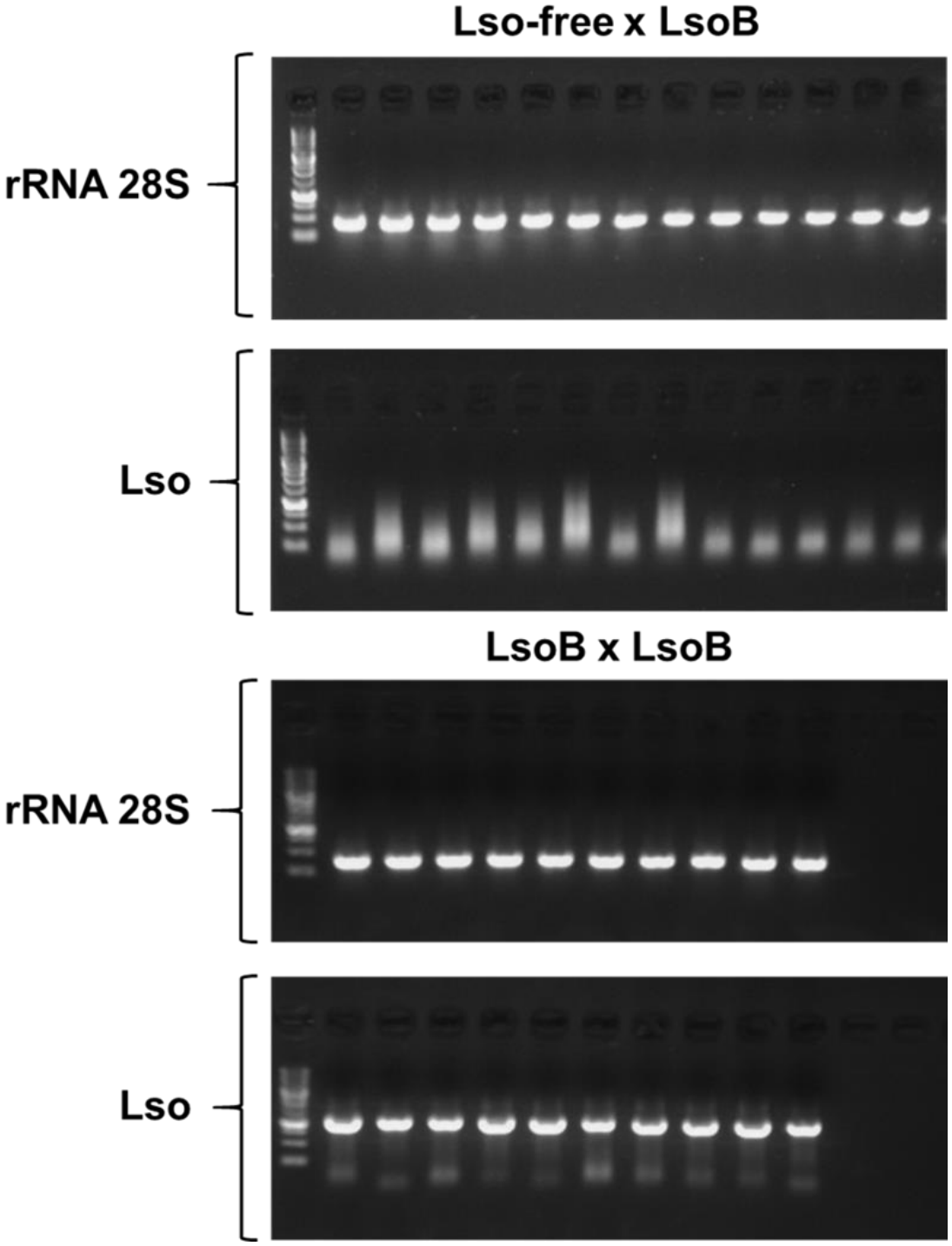

Figure 5. Lso detection in F_1 progenies from Lso-free x LsoB and LsoB x LsoB. We did not detect the presence of Lso in F_1 progeny from the cross Lso-free x LsoB. While, F_1 progeny from cross LsoB x LsoB all samples were positives. Our control of PCR amplification was performed using the rRNA subunit 28S primers.

4. Sex ratio percentages

Changes in the percentage of *B. cockerelli* females and males were determined in the F_1 progeny produced by different crosses (Lso-free x Lso-free; Lso-free x LsoB; LsoB x Lso-free; LsoB x LsoB) of uninfected and LsoB-infected adults (Figure 6). Crosses performed with uninfected females and males (Lso-free x Lso-free, Lso-free x LsoB and LsoB x Lso-free) did not show statistical differences in sex ratio in F_1 progeny ($P > 0.05$). While the crosses performed with LsoB-infected insects (LsoB x LsoB) had a significantly higher percentage of females (57.2%) than males in F_1 progeny ($P < 0.05$).

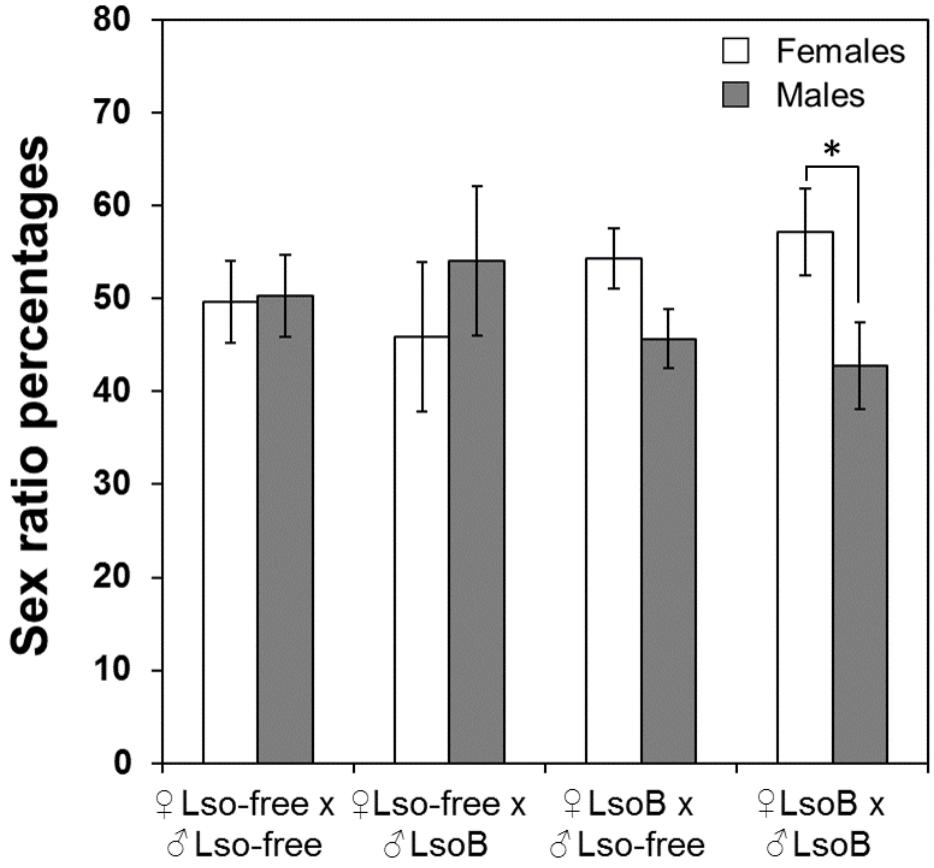

Figure 6. Adult sex ratio in *B. cockerelli* crosses. There were no statistical differences in sex ratio of the progeny from the Lso-free x Lso-free, Lso-free x LsoB and LsoB x Lso-free crosses. However, a female bias was identified in the progeny from LsoB x LsoB cross. Data represent means ± SD of three independent experiments. Asterisks (*) show statistical differences between females and males for each cross in *B. cockerelli* using paired t-test.

5. Expression of *BcVg1-like* in 7-day old mated females (mothers-crosses) and life stages

The relative expression of *BcVg1-like* transcript was determined in the mothers from the different crosses and also among adult females of different ages in specific crosses (Lso-free x Lso-free and LsoB x Lso-free) (Figure 7). The expression of *BcVg1-like* transcript in the 7-day old mothers from the different crosses showed that the control (Lso-free x Lso-free) was statistically higher compared to the expression in the females from the LsoB x Lso-free and LsoB x LsoB crosses. While no statistical differences were found in the *BcVg1-like* expression level between the control and the cross Lso-free x LsoB, a reduction in the relative expression (0.69) was observed in the cross using the LsoB-infected male (Figure 7A).

The *BcVg1-like* expression compared among adult females of different ages from the Lso-free and LsoB-infected insects. Overall, the relative expression analyses showed that *BcVg1-like* was expressed in all samples and its expression increased as females aged and following mating (Figure 7B). In Lso-free females, *BcVg1-like* its expression increased from 1-day-old virgin to 7-day-old virgin females. Also, that its expression was higher in 7-day-old mated females compared to virgin females. However, in LsoB-infected females, no differences in *BcVg1-like* expression were found between 3- and 7-day old virgin females, or between 7-day-old mated and 3-day-old virgin females even on average the expression in 7-day-old mated females was three times higher than in 3- and 7-day old virgin females. *BcVg1-like* expression was significantly higher in 7-day-old virgin and mated Lso-free females compared to 7-day-old virgin and mated Lso-infected females, respectively.

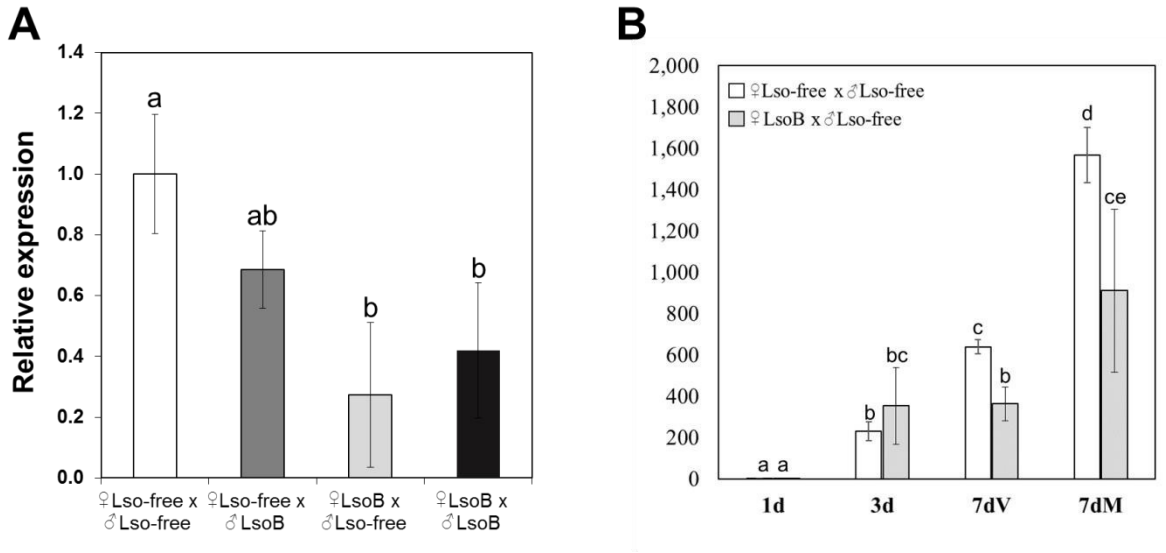

Figure 7. Expression analyses of *BcVg1-like* transcript in mothers from the crosses and among adult females of different ages**.** Relative *BcVg1-like* transcript expression was determined by RT-qPCR and normalized to the expression value of *RpS18* and *Ef-1a* transcripts. (A) The expression of *BcVg1-like* was reduced in crosses performed with LsoB-infected females compared to control Lso-free; however, no statistical differences were observed in the cross Lso-free x LsoB. (B) Overall, *BcVg1-like* relative expression was reduced in 7-day old LsoB-infected females (virgin and mated) compared with 7-day old Lso-free females (virgin and mated). Each bar represents the means ± standard deviation (SD) of three independent experiments. Different letters indicate statistical differences among life stages using one-way ANOVA with t-test post hoc test ($P < 0.05$).

6. Developing mature oocytes

To evaluated a possible behavioral change in oviposition by LsoB-infected females, such as egg retention, we dissected and counted the number of developing oocytes present on female ovaries in 7-day-old Lso-free and LsoB mated females (Figures 8 and 9). A significant reduction in the number of developing oocytes was observed in the reproductive organs of the LsoB-infected females compared with Lso-free females, excluding a possible behavioral change of egg retention (Figure 9).

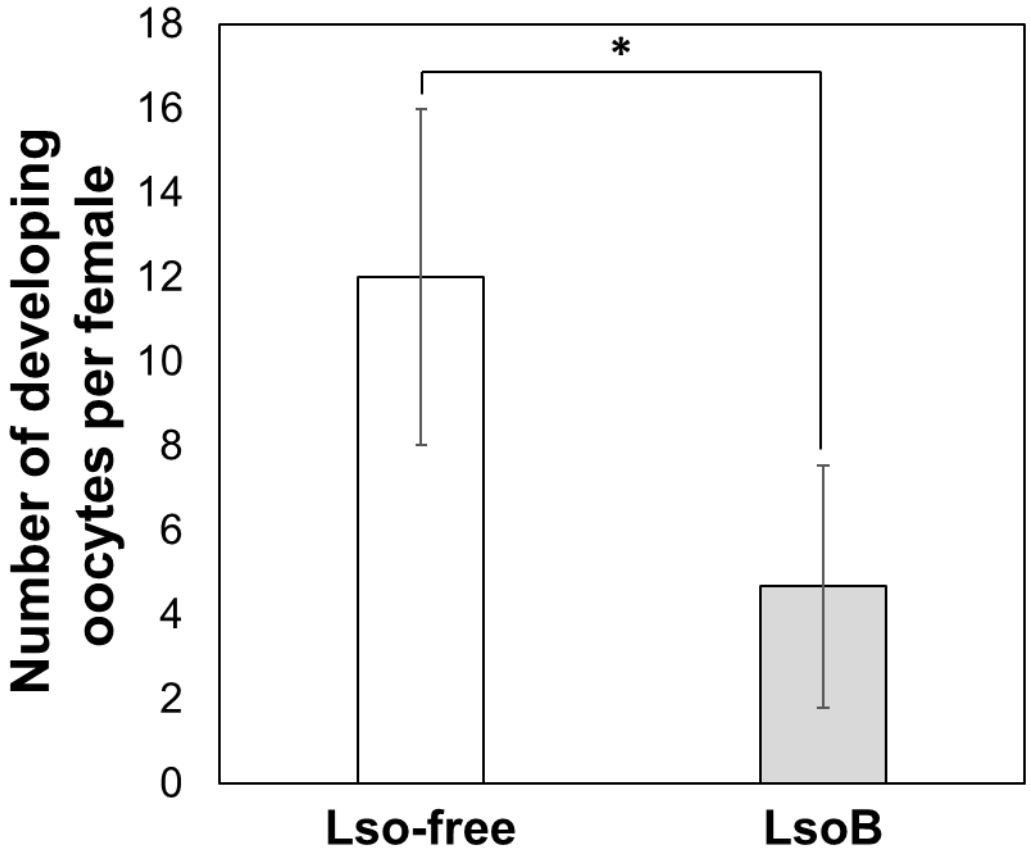

Figure 8. Average number of developing oocytes present in 7-day-old Lso-free and LsoB mated females. We found a significant reduction in the number of developing oocytes observed in LsoB-infected mated females on dissected reproductive systems. Data represent means ± 20 reproductive female tissue samples. Asterisks (*) show statistical differences between treatments using paired t-test ($P < 0.05$).

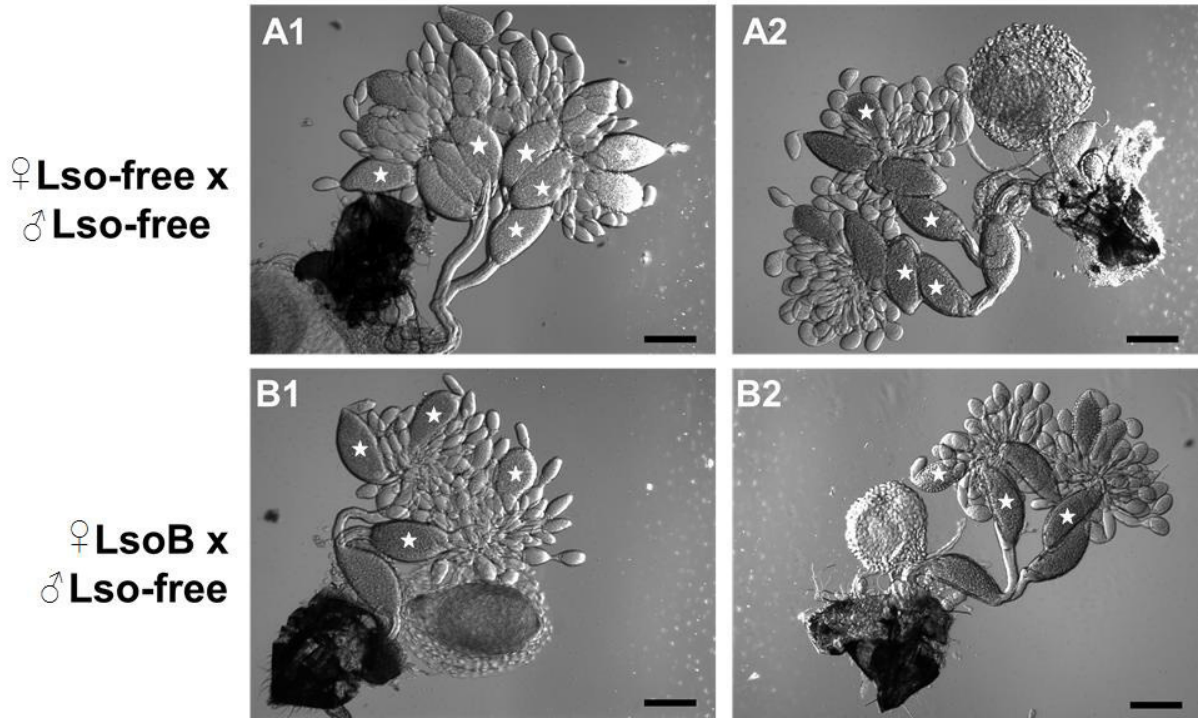

Figure 9. Representative images of female reproductive system from 7-day-old Lso-free and LsoB-infected mated females**.** The dissected ovaries showed reduction in the number of developing oocytes in LsoB-infected females. A1 and A2 are representative images of Lso-free mated females (Lso-free x Lso-free), B1 and B2 correspond to LsoB-infected mated females (LsoB x Lso-free). Scale bar is equal to 200 µm. Examples of developing oocytes are highlighted in white stars.

DISCUSSION

The effect of *Liberibacter* infection in psyllid fitness *B. cockerelli* is not well understood. While, the fitness analyses performed in *Diaphorina citri* revealed that infection by '*Candidatus* Liberibacter asiaticus' (CLas) resulted in an increase in the number of eggs, a reduction in time of nymph development and an increased in female longevity of the insects harboring CLas (PELZ-STELINSKI and KILLINY, 2016, REN et al., 2016), the interaction of *B. cockerelli* with Lso resulted in a reduction of oviposition (NACHAPPA et al., 2012, NACHAPPA et al., 2014). Also, previously, it was showed that the effects of Lso infection in *B. cockerelli* can differ depending on the Lso haplotype. The infection with each Lso haplotype resulted in the decrease of *B. cockerelli* fecundity, but only the infection with LsoB reduced the nymphal survival (YAO et al., 2016). On potato and silver leaf nightshade, Lso-infected *B. cockerelli* nymphs developed faster than uninfected psyllids, but their mortality was higher (THINAKARAN et al., 2015). Also in tomato, Lso-infection resulted in lower nymphal survival (NACHAPPA et al., 2012, NACHAPPA et al., 2014, YAO et al., 2016). However, the effect of LsoB on psyllids reciprocal crosses (♀Lso-free x ♂LsoB and ♀LsoB x ♂Lso-free) and its possible cytoplasmic incompatibility have not been investigated.

Presently, the results of this study in oviposition, number of nymphs and total number of adults showed a significant reduction in all crosses performed with a LsoB adults, these results were similar to previous reports using *B. cockerelli* infected with both Lso haplotypes (NACHAPPA et al., 2012), differences in nymph survival were identified in a previous work (YAO et al., 2016), these might be caused by an extended time of oviposition (2 weeks), compared to the time used in this study (4 days).

According to the frequency-dependent selection hypothesis, the sex ratio reaches an equilibrium and a balanced sex proportion (AYALA and CAMPBELL, 1974). A sex ratio distortion was observed in the progeny F_1 from crosses using ♀LsoB x ♂LsoB (Figure 6); this performance indicated that infection in *B. cockerelli* females by LsoB was associated with a significant female bias. Several studies had shown the relationship of insects with bacteria that induced feminization. For example, in whiteflies (*Bemisia tabaci*), *Rickettsia* was found to be associated with significant female bias in two whitefly genetic lines (CASS et al., 2016). Also, other members of the order *Rickettsiales*, *Wolbachia*, a diverse group of intracellular bacteria with different symbiotic relationships induced diverse phenotypes in their hosts (insect species) including female-bias, parthenogenetic induction and cytoplasmic incompatibility (WERREN et al., 2008). Female bias induced by LsoB on *B. cockerelli* F_1

progeny might have an adaptive strategy in *B. cockerelli* populations increasing the insect fitness; however, this interaction needs to be investigated in more detail.

In a previous study, it was observed that the expression of *BcVg1-like* was correlated with the number of eggs oviposited, suggesting its important role in psyllid reproduction (IBANEZ et al., 2017). However, Vgs have been also associated with other biological functions. For instance, Vgs are involved in immune defense in non-mammalian vertebrates and invertebrates (ZHANG et al., 2011). *Hexagrammos otakii* Vg binds lipopolysaccharides from Gram-negative bacteria, lipoteichoic acids from Gram-positive bacteria, peptidoglycans from both Gram-positive and Gram-negative bacteria, β-1,3-glucans from eukaryotic fungi and laminarin from brown algae (LI et al., 2008). Whereas, the *Bombyx mori* Vg protein can bind the Gram-negative bacterium *Escherichia coli* and the Gram-positive bacterium *Bacillus subtilis*, resulting in a strong antibacterial activity (SINGH et al., 2013). In *Anopheles gambiae,* Vg was able to interfere with the anti-Plasmodium response (RONO et al., 2010). These findings suggest that Vg might be involved in the defense of hosts against microbes. However, this is a hypothesis that needs to be examined in *B. cockerelli*.

Our results showed that mated LsoB-infected females had a significant reduction in the level of *BcVg1-like* transcript (Figure 7). The negative effects of pathogen-infection in insect's reproduction have been shown in *A. gambiae*, *D. melanogaster*, *E. intermedius* and *T. oceanicus* (AHMED and HURD, 2006, NYSTRAND and DOWLING, 2014, MCNAMARA et al., 2014, REANEY and KNELL, 2010). For instance, in *A. gambiae*, the transcription of Vg, the Vg protein concentration in the hemolymph and Vitellin accumulation in oocytes, were reduced following the infection with *Plasmodium yoelii nigeriensis*, when ookinetes invaded the female's mosquito midguts (AHMED et al., 2001). Also, when *A. gambiae* was challenged with lipopolysaccharides, an immune elicitor, a significant decrease in the accumulation of vitellin was observed in the ovaries, also a reduction of eggs oviposited was characteristic in these mosquitoes species (AHMED et al., 2002). In *D. melanogaster* a reduced fecundity was observed during an acute phase of infection of a naturally occurring Gram-negative pathogen*, Providencia rettgeri* (MCKEAn et al., 2008).

In this study, it was clear that Lso haplotype B affects several aspects of *B. cockerelli* fitness (oviposition, nymphs, adults and sex ratio); also, the *BcVg1-like* expression was reduced on those females infected with LsoB. The reasons of why LsoB had these effects in *B. cockerelli* are still not understood and might be the results in changes of in the expression of pathogenesis-related genes, as was proposed in (YAO et al., 2016). The reduction in gene

expression might be implicated in pathogenicity and an activation of the insect immune system could reduce the reproductive output, as was the outcome of this study. This follows the idea of the resource allocation model in which a trade-off arises because of competition for one or more limiting resources (SCHWENKe et al., 2016). Also, it is possible that LsoB may disrupt multiple stages of vitellogenesis, and/or initiate an ovarian pathology that feeds back negatively the fat body tissue. However, LsoB might have an adaptive strategy in which *B. cockerelli* benefits from the infection under specific environmental circumstances and not related to its diminished reproduction. Therefore, this interaction might need to be investigated in detail.

CONCLUSION

In conclusion, this is the first study performed in *Bactericera cockerelli* and its interaction with *Candidatus* Liberibacter solanacearum haplotype B, in which reciprocal crosses were investigated. It was determined that the presence of LsoB reduced the reproduction output of *B. cockerelli*, in all crosses in which females and/or males LsoB-infected were used, the final result was a significant decrease in the life-history parameters (oviposition, number of nymphs, nymphal survival, number of adults and sex ratio) measured in this study. Also, a reduction of the relative expression of *BcVg1-like* mRNA was observed in the crosses, in which one of the couples was LsoB-infected, this low level of *BcVg1-like* expression was associated with a low number of developing oocytes observed in female's reproductive systems. Our result showed that the F_1 progeny adults from mothers infected with LsoB showed a female bias in *B. cockerelli* population.

REFERENCES

AHMED, A.; BAGGOTT, S.; MAINGON, R.; HURD, H. The costs of mounting an immune response are reflected in the reproductive fitness of the mosquito *Anopheles gambiae*. **Oikos**, v.97, p. 371-377, 2002.

AHMED, A.; MAINGON, R.; ROMANS, P.; HURD, H. Effects of malaria infection on vitellogenesis in *Anopheles gambiae* during two gonotrophic cycles. **Insect molecular biolog,**. v.10, p.347-356, 2001.

AHMED, A.M.; HURD, H. Immune stimulation and malaria infection impose reproductive costs in Anopheles gambiae via follicular apoptosis. **Microbes and Infection**, v. 8, p. 308-315, 2006.

ARUKWE, A.; GOKSOYR, A. Eggshell and egg yolk proteins in fish: hepatic proteins for the next generation: oogenetic, population, and evolutionary implications of endocrine disruption. **Comp Hepatol**., v. 2, p.1-21, 2003.

AUBERT, B. Citrus greening disease, a serious limiting factor for citriculture in Asia and Africa. **Proceedings of the International Society of Citriculture**, v.2, p.817-820, 1992.

AYALA, F.J.; CAMPBELL, C.A. Frequency-dependent selection. **Annual review of Ecology and systematics**, v. 5, p.115-138, 1974.

BOVÉ, J. M. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. **Journal of plant pathology,** v.88. n.1, p. 7-37, 2006.

CASS, B. N.; HIMLER, A. G.; BONDY, E. C.; BERGEN, J. E.; FUNG, S. K.; KELLY, S. E.; HUNTER, M. S. Conditional fitness benefits of the *Rickettsia* bacterial symbiont in an insect pest. **Oecologia**, v.180, p.169-179, 2016.

CHEN, J.S.; SAPPINGTON, T. W.; RAIKHEL, A. S. Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. **Journal of Molecular Evolution**, v. 44, p. 440-45, 1997.

CROSSLIN, J. M.; MUNYANEZA, J. E.; BROWN, J. K.; LIEFTING, L. W. Potato zebra chip disease: a phytopathological tale. **Plant Health Progress**, v.17, p.1-9, 2010.

HANSEN, A., TRUMBLE, J., STOUTHAMER, R. & PAINE, T. 2008. A new huanglongbing species,"*Candidatus* Liberibacter psyllaurous," found to infect tomato and potato, is vectored by the psyllid *Bactericera cockerelli* (Sulc). **Applied and environmental microbiology**, v.74, p.5862-5865, 2008.

IBANEZ, F.; HANCOCK, J.; TAMBORINDEGUY, C. Identification and expression analysis of aquaporins in the potato psyllid, *Bactericera cockerelli*. **PloS one**, v. 9, p.111745- 111745, 2014.

IBANEZ, F.; LEVY, J.; TAMBORINDEGUY, C. Identification and expression analyses of vitellogenin in *Bactericera cockerelli* (Šulc). **Journal of insect physiology**, v.98, p. 205-213, 2017.

IBANEZ, F.; TAMBORINDEGUY, C. Selection of reference genes for expression analysis in the potato psyllid, *Bactericera cockerelli*. **Insect molecular biology**, v.25, p. 227-238, 2016.

LEUNG, T.; POULIN, R. Parasitism, commensalism, and mutualism: exploring the many shades of symbioses. **Vie et Milieu**, v.58, p.107, 2008.

LI, Z., ZHANG, S.; LIU, Q. Vitellogenin functions as a multivalent pattern recognition receptor with an opsonic activity. **PLoS One**, v. 3, n.4, p. 1940-1947, 2008.

LIEFTING, L.; PEREZ-EGUSQUIZA, Z.; CLOVER, G.; ANDERSON, J. A new '*Candidatus* Liberibacter'species in *Solanum tuberosum* in New Zealand. **Plant Dis**., v. 92, p. 1474-1474, 2008.

LIN, H.; ISLAM, M.S.; BAI, Y.; WEN, A.; LAN, S.; GUDMESTAD, N.C.; CIVEROLO, E.L. Genetic diversity of '*Cadidatu*s Liberibacter solanacearum'strains in the United States and Mexico revealed by simple sequence repeat markers. **European Journal of Plant Pathology**, v. 132, p. 297-308, 2012.

MANJUNATH, K.; HALBERT, S.; RAMADUGU, C.; WEBB, S.; LEE, R. Detection of '*Candidatus* Liberibacter asiaticus' in *Diaphorina citri* and its importance in the management of citrus huanglongbing in Florida. **Phytopathology**, v.98, p. 387-396, 2008.

MCKEAN, K. A.; YOURTH, C. P.; LAZZARO, B. P.; CLARK, A. G. The evolutionary costs of immunological maintenance and deployment. **BMC evolutionary biology,**. v.8, p.1-19, 2008.

MCNAMARA, K.; LIESHOUT, E.; SIMMONS, L. Females suffer a reduction in the viability of stored sperm following an immune challenge. **Journal of evolutionary biology**, v.27, p. 133-140, 2014.

MUNYANEZA, J.; CROSSLIN, J.; UPTON, J. 2007. Association of *Bactericera cockerelli* (Homoptera: Psyllidae) with "zebra chip," a new potato disease in southwestern United States and Mexico. **Journal of Economic Entomology**, v. 100, p. 656-663, 2007.

MUNYANEZA, J. E. Zebra chip disease of potato: biology, epidemiology, and management. Am J Potato Res. v. 89, 329-350, 2012.

MUNYANEZA, J. E.; FISHER, T. W.; SENGODA, V. G.; GARCZYNSKI, S. F.; NISSINEN, A.; LEMMETTY, A. Association of "*Candidatus* Liberibacter solanacearum" with the psyllid, *Trioza apicalis* (Hemiptera: Triozidae) in Europe. **J Econ Entomol**., v.103, p.1060-1070, 2010.

NACHAPPA, P.; LEVY, J.; PIERSON, E.; TAMBORINDEGUY, C. Diversity of endosymbionts in the potato psyllid, *Bactericera cockerell*i (Hemiptera: Triozidae), vector of zebra chip disease of potato. **Current microbiology**, v.62, p.1510-1520, 2011.

NACHAPPA, P.; LEVY, J.; PIERSON, E.; TAMBORINDEGUY, C. Correlation between "*Candidatus* Liberibacter solanacearum" infection levels and fecundity in its psyllid vector. **Journal of invertebrate pathology**, v.115, p.55-61, 2014.

NACHAPPA, P., SHAPIRO, A. A.; TAMBORINDEGUY, C. Effect of '*Candidatus* Liberibacter solanacearum'on fitness of its insect vector, *Bactericera cockerelli* (Hemiptera: Triozidae), on tomato. **Phytopathology**, v.102, p.41-46, 2012.

NADARASAH, G.; STAVRINIDES, J. Insects as alternative hosts for phytopathogenic bacteria. **FEMS microbiology reviews,** v.35, p.555-575, 2011.

NAGABA, Y.; TUFAIL, M.; INUI, H.; TAKEDA, M. Hormonal regulation and effects of four environmental polutants on vitellogenin gene transcription in the giant water bug, *Lethocerus deyrollei* (Heteroptera: Belostomatidae). **J Insect Conserv**., v.15, n.3, p.421-431, 2010.

NELSON, W. R.; FISHER, T. W.; MUNYANEZA, J. E. 2011. Haplotypes of "Candidatus Liberibacter solanacearum" suggest long-standing separation. **European Journal of Plant Pathology**, v.130, p. 5-12, 2011.

NELSON, W. R.; SENGODA, V. G.; ALFARO-FERNANDEZ, A. O.; FONT, M. I., CROSSLIN, J. M.; MUNYANEZA, J. E. A new haplotype of "*Candidatus* Liberibacter solanacearum" identified in the Mediterranean region. **European Journal of Plant Pathology**, v. 135, p. 633-639, 2013.

NYSTRAND, M.; DOWLING, D. Dose- dependent effects of an immune challenge at both ultimate and proximate levels in *Drosophila melanogaster*. **Journal of evolutionary biology,** v.27, p.876-888, 2014.

ORLOVSKIS, Z.; CANALE, M. C.; THOLE, V.; PECHER, P.; LOPES, J. R.; HOGENHOUT, S.A. Insect-borne plant pathogenic bacteria: getting a ride goes beyond physical contact. **Current Opinion in Insect Science**, v. 9, p.16-23, 2015.

PELZ-STELINSKI, K. S.; KILLINY, N. 2016. Better together: association With *'Candidatus* Liberibacter asiaticus' increases the reproductive fitness of Its insect vector, *Diaphorina citri* (Hemiptera: Liviidae). **Annals of the Entomological Society of America**, v.109, p. 371-376, 2016.

PERILLA-HENAO, L. M.; CASTEEL, C. L. 2016. Vector-borne bacterial plant pathogens: interactions with hemipteran insects and plants. **Frontiers in plant science**, v.7, p. 1-15, 2016.

RAIKHEL, A.S. Vitellogenesis in mosquitoes. **Advances in Disease Vector Research**. v.9, p.1-39, 1992.

REANEY, L.T.; KNELL, R.J. Immune activation but not male quality affects female current reproductive investment in a dung beetle. **Behavioral Ecology**, v.21, p.1367-1372, 2010.

REN, S.L.; LI, Y.H.; ZHOU, Y.T.; XU, W.M.; CUTHBERTSON, A.G.; GUO, Y.J. ;QIU, B.L. Effects of Candidatus Liberibacter asiaticus on the fitness of the vector *Diaphorina cit*ri. **Journal of applied microbiology**, v.121, p. 1718-1726, 2016.

RONO, M. K.; WHITTEN, M. M.; OULAD-ABDELGHANI, M.; LEVASHINA, E. A. ; MAROIS, E. The major yolk protein vitellogenin interferes with the anti-plasmodium response in the malaria mosquito *Anopheles gambiae*. **PLoS Biol**., v. 8, p.1-12, 2010.

SCHMITTGEN, T. D.; LIVAK, K. J. Analyzing real-time PCR data by the comparative CT method. **Nature protocols**, v.3, p. 1101-1108, 2008.

SCHWENKE, R. A.; LAZZARO, B. P.; WOLFNER, M. F. 2016. Reproduction–immunity trade-offs in insects. **Annual review of entomology**, v.61, p. 239-256, 2016.

SECOR, G.; RIVERA, V.; ABAD, J.; LEE, I.-M.; CLOVER, G.; LIEFTING, L.; LI, X.; DE BOER, S. Association of '*Candidatus* Liberibacter solanacearum'with zebra chip disease of potato established by graft and psyllid transmission, electron microscopy, and PCR. **Plant Dis**., v.93, p. 574-583, 2009.

SHINODA, T.; MIURA, K.; TAYLOR, D.; CHINZEI, Y. Vitellogenins and vitellins in the bean bug, *Riptortus clavatus* (Hemiptera: Alydidae): purification, immunological identification, and induction by juvenile hormone. **Archives of insect biochemistry and physiology**, v.31, p. 395-412, 1996.

SINGH, N. K.; PAKKIANATHAN, B. C.; KUMAR, M.; PRASAD, T.; KANNAN, M.; KÖNIG, S.; KRISHNAN, M. Vitellogenin from the silkworm, *Bombyx mori*: an effective anti-bacterial agent. **PloS one**, v.8, p.1-8, 2013.

SOLOMON, N.U.; JAMES, I.M.; ALPHONSUS, N.O.O.; NKIRUKA, R.U. A review of host-parasite relationships. **Annual Research & Review in Biology,** v.5, p.372- 384, 2015.

SU, Q.; ZHOU, X.; ZHANG, Y. Symbiont-mediated functions in insect hosts. **Communicative & integrative biology**, v.6, p.23804.1- e23804.7, 2013.

TAMBORINDEGUY, C.; HUOT, O. B.; IBANEZ, F.; LEVY, J. The influence of bacteria on multi- trophic interactions among plants, psyllids, and pathogen. **Insect Science**, p.1-14, 2017.

TERESANI, G. R.; BERTOLINI, E.; ALFARO-FERNÁNDEZ, A.; MARTÍNEZ, C.; TANAKA, F.A.O.; KITAJIMA, E.W.; ROSELLÓ, M.; SANJUÁN, S.; FERRÁNDIZ, J.C.; LÓPEZ, M.M. Association of '*Candidatus* Liberibacter solanacearum'with a vegetative disorder of celery in Spain and development of a real-time PCR method for its detection. **Phytopathology**, v.104, p.804-811, 2014.

TEULON, D.; WORKMAN, P.; THOMAS, K.; NIELSEN, M. *Bactericera cockerelli*: incursion, dispersal and current distribution on vegetable crops in New Zealand. **New Zealand Plant Protection**, v.62, p.136-144, 2009.

THINAKARAN, J.; YANG, X.B.; MUNYANEZA, J.E.; RUSH, C.M.; HENNE, D. C. Comparative biology and life tables of "*Candidatus* Liberibacter solanacearum"-infected and -free *Bactericera cockerelli* (Hemiptera: Triozidae) on potato and silverleaf nightshade. **Annals of the Entomological Society of America**, v.108, p. 459-467, 2015.

TUFAIL, M.; TAKEDA, M. 2008. Molecular characteristics of insect vitellogenins. **Journal of Insect Physiology,** v.54, p.1447-1458, 2008.

WEINTRAUB, P.G.; BEANLAND, L. Insect vectors of phytoplasmas. **Annu. Rev. Entomol.,** v.51, p.91-111, 2006.

WERREN, J. H.; BALDO, L.; CLARK, M. E. *Wolbachia*: master manipulators of invertebrate biology. **Nature reviews Microbiology**, v.6, 741-751, 2008.

YAO, J.; SAENKHAM, P.; LEVY, J.; IBANEZ, F.; NOROY, C.; MENDOZA, A.; HUOT, O.; MEYER, D.F.; TAMBORINDEGUY, C. Interactions "*Candidatus* Liberibacter solanacearum"—*Bactericera cockerelli*: Haplotype Effect on Vector Fitness and Gene Expression Analyses. **Frontiers in Cellular and Infection Microbiology**, v.6, p.1-13, 2016.

ZHANG, S., WANG, S., LI, H. & LI, L. 2011. Vitellogenin, a multivalent sensor and an antimicrobial effector. **The international journal of biochemistry & cell biology**, v.43, p.303-305, 2011.