UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
FACULDADE DE CIÊNCIAS AGRONÔMICAS
CAMPUS DE BOTUCATU

CARACTERIZAÇÃO DO *Groundnut ringspot virus* (GRSV) E SEU VETOR (*Frankliniella schultzei*) EM MELANCIA

EVELYNNE URZÊDO LEÃO

Tese apresentada à Faculdade de Ciências Agronômicas da UNESP - Campus de Botucatu, para obtenção do título de Doutor em Agronomia (Proteção de Plantas).

BOTUCATU – SP
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TÍTULO: **"CARACTERIZAÇÃO DO *Groundnut ringspot virus* (GRSV) E SEU VETOR *(Frankliniella schultzei)* EM MELANCIA"**

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À minha mãe, Maria Madalena.

Ao meu pai, Emivaldo.

Ao meu noivo Gentil.

Ao meu irmão, Júnior.

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"There are only two ways to live your life.
One is as though nothing is a miracle.
The other is as though everything is a miracle"

Albert Einstein

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A melancia (*Citrullus lanatus*) é considerada uma das cucurbitáceas mais importantes no mundo e o Brasil é o quarto maior produtor, com área total cultivada de 90.000 ha e produção média de cerca de dois milhões de toneladas. No Brasil, há várias espécies de vírus descritas em melancia pertencentes ao gênero *Potyvirus*, *Cucumovirus*, sendo *Zucchini lethal chlorosis virus* a única espécie até então relatada no gênero *Tospovirus*. Considerando a alta incidência de plantas de melancia exibindo sintomas causados por vírus durante as safras 2012/2013 e a presença de tripes no campo, este trabalho teve como objetivo a identificação e caracterização do vírus e do vetor associado a sua transmissão. No primeiro capítulo intitulado "*Citrullus lanatus* is a new natural host of *Groundnut ringspot virus* in Brazil", foi relatada a ocorrência de *Groundnut ringspot virus* (GRSV) em melancia ocasionando mosaico, deformações e lesões necróticas em folhas e manchas necróticas nos frutos. O GRSV foi coletado durante os anos de 2012 e 2013 nas regiões produtoras dos municípios de Presidente Prudente e Marília. O isolado de GRSV de melancia apresentou um círculo de hospedeiros semelhante aos isolados de GRSV provenientes de solanáceas, com a excessão de não infectar tomateiro cv. Santa Clara e Mariana. Espécimes de tripes coletados no campo foram capazes de transmitir o GRSV para melancia, pimentão e espécies de *Nicotiana*. Este foi o primeiro relato de infecção natural de melancia por GRSV. No segundo capítulo intitulado "A Taqman real-time RT - PCR assay for detection of *Groundnut ringspot virus*" foi possível detectar o GRSV por PCR quantitativo (qPCR) em amostras de melancia e em tripes individuais coletados no campo. A detecção do GRSV por qPCR poderá servir como uma ferramenta útil nos

estudos de identificação e interação do GRSV em diversas culturas. O terceiro capítulo "Efficient detection of *Frankliniella schultzei* (Thripidae: Thysanoptera) by real-time PCR" relata a identificação de *Frankliniella schultzei* pela análise do gene mtCOI. Amostras de tripes foram coletadas de diferentes culturas em diferentes regiões do estado de São Paulo durante os anos de 2013 e 2014 e foram submetidas à identificação molecular por PCR e sequenciamento. Todas as amostras sequenciadas foram identificadas como *F. schultzei*. Oligonucleotídeos específicos para o gene mtCOI de *F. schultzei* foram desenvolvidos e otimizados tanto para detecção por PCR convencional e qPCR. Os oligonucleotideos não detectaram outras espécies de tripes como *F. occidentalis, F. intonsa* e *Thrips tabaci*, mostrando-se altamente específicos.

Palavras chave: *Citrullus lanatus*, *Tospovirus*, tripes, mtCOI, real-time PCR.

CHARACTERIZATION OF *Groundnut ringspot virus* (GRSV) AND ITS VECTOR (*Frankliniella schultzei*) IN WATERMELON

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2. SUMMARY

Watermelon (*Citrullus lanatus*) is considered one of the most important cucurbits in the world and Brazil is the fourth largest producer, with a total cultivated area of 90.000 ha and average production of about two million tons. In Brazil, viruses described in watermelon belong to the genus *Potyvirus* and *Cucumovirus* and *Zucchini lethal chlorosis virus* is the only species of *Tospovirus* genre described on this culture. Considering the high incidence of watermelon plants showing symptoms caused by virus during the 2012/2013 seasons and the presence of thrips in the field, the virus and the vector associated with the disease was identified and characterized. In the first chapter entitled "*Citrullus lanatus* is a new natural host of *Groundnut ringspot virus* in Brazil", the species of tospovirus *Groundnut ringspot virus*(GRSV) was reported causing mosaic, deformation and necrotic lesions on

leaves and fruits of watermelon. The GRSV was collected during the years 2012 and 2013 in producing areas of Presidente Prudente and Marília. GRSV from watermelon, differently from GRSV strains found on solanaceas, didn´t infected tomato plants cv. Santa Clara and Mariana. Thrips specimens collected in the field were able to transmit GRSV to watermelon, peppers and *Nicotiana* species. This was the first report of natural infection of watermelon by GRSV. In the second chapter entitled "A Taqman real-time RT - PCR assay for detection of *Groundnut ringspot virus*" a quantitative PCR test was developed to efficiently detect GRSV from watermelon and from individual thrips. The test might be useful to detect GRSV and for further studies with this virus. The third chapter " Efficient detection of *Frankliniella schultzei* (Thripidae: Thysanoptera) by real-time PCR" reports the efficient detection of *Frankliniella schultzei* by the analysis of the mitochondrial gene cytochrome oxidase I (mtCOI). Thrips samples were collected from different cultures in different regions of the São Paulo state during the years 2013 and 2014 and were subjected to molecular identification by PCR and sequencing. All the sequenced samples were identified as *F. schultzei*. Specific primers for *F. schultzei* mtCOI gene were developed and optimized to detect specifically this species by PCR and qPCR. The primers were highly specific and did not dectect *F. occidentalis*, *Thrips tabaci* and *F. intonsa.*

Keywords: *Citrullus lanatus*, *Tospovirus*, thrips, mtCOI, real-time PCR.

3. INTRODUÇÃO

A melancia (*Citrullus lanatus*) é considerada a principal espécie da família *Cucurbitaceae*, com cerca de 2 milhões de toneladas produzidas no mundo no ano de 2011 em uma área plantada de 90 mil ha. O Brasil é o quarto maior produtor mundial, ficando atrás apenas da China, Irã e Turquia (FAO, 2013).

A ocorrência de doenças é o principal problema no cultivo da melancia em condições tropicais, assim como na maioria das curcubitáceas. A maioria dos vírus de importância econômica para a cultura no Brasil pertencem ao gênero *Potyvirus*, sendo *Papaya ringspot virus* – estirpe W (PRSV-W), *Zucchini yellow mosaic virus* (ZYMV) e *Watermelon mosaic virus* (WMV) as espécies mais frequentes (YUKI et al., 2000). A ocorrência de *Cucumber mosaic virus* (CMV) gênero *Cucumovirus* e *Zucchini lethal chlorosis virus* (ZLCV) gênero *Tospovirus* também são relatadas na cultura (REZENDE et al., 1997; YUKI et al., 2000).

Sintomas de lesões necróticas em folhas e frutos, típicos aos causados por tospovirus, foram observados em plantações comerciais do estado de São Paulo no verão de 2012 e se repetiram nos anos de 2013 e 2014. Presença de tripes principalmente nas flores da melancia foram observados nas áreas apresentando os sintomas descritos acima. Deste modo, foi realizada a identificação do agente causal da doença, concluindo-se que se tratava do tospovirus *Groundnut ringspot virus* (GRSV). Até o momento, no Brasil, a espécie ZLCV era a única do gênero *Tospovirus* descrita em cucurbitáceas e encontrada com mais frequência em abobrinha de moita (BEZERRA et al., 1999; POZZER et al., 1996). Os tospovírus são transmitidos de maneira circulativa

propagativa por insetos da ordem Thysanoptera, conhecidos como tripes (WIJKAMP et al., 1993; HOGENHOUT et al., 2008). São insetos muito pequenos e encontram-se disseminados em todo o mundo. Pelo menos 14 espécies de tripes pertencentes aos gêneros *Frankliniella*, *Thrips*, *Scirtothrips, Ceratothripoides* e *Dictyothrips* já foram descritas como vetores de tospovírus (CIUFFO et al., 2010; KING et al., 2012).

No Brasil existem poucos dados sobre a distribuição geográfica e importância relativa das diferentes espécies de tripes como vetoras de tospovírus. Poucos estudos de reconhecimento e identificação têm sido feitos, sendo a identificação taxonômica de tripes baseada principalmente na morfologia externa analisando-se o número de segmentos da antena e a distribuição e número de cerdas (MEHLE; TRDAN, 2012). Mais recentemente outras metodologias como a análise da sequência de nucleotídeos do gene mitocondrial cytocromo oxidase I (mtCOI) tem se mostrado adequada para a discriminação de espécies na ordem Thysanoptera (BRUNER et al. 2004; ASOKAN et al., 2007; TIMM et al., 2008; ZHANG et al., 2011; KADIRVEL et al., 2013). Neste trabalho, adotando-se a taxonomia tradicional mais o auxílio da análise do gene mtCOI conclui-se que o vetor do GRSV em melancia é a espécie *Frankliniella schultzei*. Para esta espécie, verificada como predominante nas amostragens realizadas no Estado de São Paulo, foi desenvolvido um teste de PCR quantitativo para sua detecção tanto no inseto como em plantas.

Sendo então, esta tese foi dividida em três capítulos na forma de artigos científicos, sendo o primeiro capítulo intitulado "***Citrullus lanatus* is a new natural host of *Groundnut ringspot virus* in Brazil**" redigido conforme as normas da revista *Journal of Phytopathology*, o segundo capítulo intitulado "**A Taqman real-time RT - PCR assay for detection of *Groundnut ringspot virus***" redigido conforme as normas da revista *Tropical Plant Pathology*, e o terceiro intitulado "**Efficient detection of *Frankliniella schultzei* (Thripidae: Thysanoptera) by real-time PCR**" redigido conforme as normas da revista *Journal of Applied Entomology*.

4. REVISÃO BIBLIOGRÁFICA

4.1 A cultura da melancia

A família *Cucurbitaceae* é constituída de cerca de 80 gêneros e mais de 800 espécies. Dentre essas, várias são de grande valor econômico e social na horticultura mundial. No Brasil, as espécies com maior expressão econômica pertencem aos gêneros *Cucurbita* (abóbora, abobrinha e moranga), *Cucumis* (pepino, melão e maxixe), *Citrullus* (melancia), *Sechium* (chuchu) e *Lagenaria* (cabaça caxi) (GIAMPAN, 2007).

A produção mundial de melancia (*Citrullus lanatus*) é de cerca de 2 milhões de toneladas, sendo o Brasil o quarto maior produtor mundial (FAO, 2013). No estado de São Paulo, o cultivo da melancia ocupou uma área de cerca de sete mil hectares, em 2010, com produção de média de 191.884 toneladas de frutos (AGRIANUAL, 2013). Os principais locais de produção no estado são as regiões agrícolas de Presidente Prudente (17%), Itapetininga (10%), Tupã (9%), Marília (8%), Dracena e Jaboticabal (8%) que totalizam 80% da produção estadual (IEA, 2012). Essa produção abastece a grande São Paulo, um dos principais centrosconsumidores, e alguns estados do Sul. No entanto, no período de entressafra a produção vem principalmente dos estados de Goiás, Tocantins, Bahia e Rio Grande do Sul (GRANGEIRO; CECÍLIO – FILHO, 2004).

4.2 Doenças da melancia

A cultura da melancia, assim como a maioria das culturas, pode ser acometida por dezenas de patógenos, que causam os mais diversos sintomas. Além de doenças bióticas, existem as abióticas, que também podem causar sérios danos a cultura, caso não sejam adotadas medidas preventivas (YUKI et al., 2000; SANTOS et al., 2005). Os genótipos de melancia utilizados nos cultivos, com algumas exceções, foram desenvolvidos para condições diferentes daquelas existentes no Brasil e enfrentam sérios problemas com pragas e doenças (SILVEIRA et al., 2005).

4.2.1 Viroses

A ocorrência de viroses em curcubitáceas cultivadas é dinâmica, podendo variar em função da espécie de vírus e suas estirpes, da população e migração dos vetores, das espécies e cultivares vegetais e das condições climáticas (MOURA et al., 2001).

A ocorrência de vírus é o principal problema no cultivo da melancia em condições tropicais, assim como na maioria das curcubitáceas. A maioria dos vírus em melancia no Brasil, de importância econômica, pertencem ao gênero *Potyvirus*, sendo eles: *Papaya ringspot virus* – strain W (PRSV-W), *Zucchini yellow mosaic virus* (ZYMV) e *Watermelon mosaic virus* (WMV) (YUKI et al., 2000). O *Cucumber mosaic virus* (CMV), gênero *Cucumovirus* também ocorre em todas as regiões brasileiras (YUKI et al., 2000). O *Squash mosaic virus* (SqMV) gênero *Comovirus* e *Zucchini lethal chlorosis virus* (ZLCV) do gênero *Tospovirus* são também encontrados infectando melancia (LIMA et al., 2011; BEZERRA et al., 1999).

4.2.1.1 Gênero *Tospovirus*

Os tospovírus são encontrados em todos os continentes e infectam uma grande variedade de cultivares de hortaliças, grãos e plantas ornamentais economicamente importantes como crisântemo, íris, cebola, tomate, pimentão, alface, abobrinha, melancia, batata, amendoim, soja e feijão (PAPPU et al., 2009; OLIVEIRA et al., 2012; ZHOU et al., 2011).

A doença foi descrita pela primeira vez na Austrália em 1915 (BRITTLEBANK, 1919) e apenas em 1930 o agente etiológico foi nomeado de *Tomato spotted wilt virus* (TSWV) (SAMUEL et al., 1930). Milne et al. (1984) notaram a similaridade entre TSWV e os vírus da família *Bunyaviridae* que, até então, era composta apenas por vírus que infectam animais. Trabalhos posteriores validaram a criação e a posição do gênero *Tospovirus*, o único infectando plantas dentro desta família, sendo *Tomato spotted wilt virus* a espécie tipo (FAUQUET et al., 2005). Atualmente, a família *Bunyaviridae* é composta por cinco gêneros: *Orthobunyavirus*, *Hantavirus*, *Nairovirus*, *Phlebovirus* e *Tospovirus*.

Os tospovírus são constituídos de partículas pleiomórficas de 80-120 nm de diâmetro, genoma de RNA fita simples tripartido com polaridade negativa ou ambisenso e são envelopados. Os segmentos genômicos são denominados RNA S (*Small*), RNA M (*Medium*) e RNA L (*Large*). Possuem sequências terminais com uma complementaridade parcial que permite o surgimento de uma conformação pseudocircular (de HAAN et al., 1989). O RNA S codifica uma proteína não estrutural supressora de silenciamento gênico no sentido viral denominada NSs e a nucleoproteína (N) no sentido complementar viral (de HAAN et al., 1990; TAKEDA et al., 2002). O RNA M codifica uma proteína não estrutural de movimento no sentido viral denominada NSm e o precursor das glicoproteínas do envelope (Gn e Gc) no sentido complementar viral (KORMELINK et al., 1992; STORMS et al., 1998). Já o RNA L, diferentemente dos segmentos anteriores que apresentam sequências ambisenso, possui somente polaridade negativa e codifica uma grande proteína (proteína L) com motivos conservados e encontrados em RNA polimerases dependentes de RNA (de HAAN et al., 1991). Assim, o vírion é composto por ribonucleoproteínas (RNP), que são os segmentos de RNA encapsidados por nucleoproteínas (N) e por algumas cópias da RNA polimerase viral dependente de RNA (proteína L) (van POELWIJK et al., 1993). O envelope que circunda estes complexos ribonucleoprotéicos é oriundo de membranas celulares dos hospedeiros e possui projeções na sua superfície compostas pelas glicoproteínas virais Gn e Gc (KIKKERT et al., 1999).

No Brasil, doenças causadas por tospovírus são citadas em várias culturas de interesse econômico. A doença é conhecida como vira-cabeça (COSTA; FORSTER, 1941). Vários estudos já identificaram a presença de outras espécies no Brasil, como *Tomato chlorotic spot virus* (TCSV), *Groundnut ringspot virus* (GRSV), *Iris yellow spot virus* (IYSV), *Chrysanthemum stem necrosis virus* (CSNV) e ZLCV (de ÁVILA et al.,

1990; RESENDE et al., 1996, BEZERRA et al., 1999, POZZER et al., 1999). Recentemente foi identificada a espécie tentativa Bean necrotic mosaic virus em feijoeiro (BeNMV) (OLIVEIRA et al., 2012).

Nota-se que das espécies de *Tospovirus* relatadas no mundo, ZLCV é restrita ao Brasil, e têm sido reportada em cultivo de curcubitáceas nos estados de São Paulo, Tocantins e Distrito Federal (HALLWASS et al., 2012). O número de espécies de tospovírus descritas no Brasil torna a América do Sul a segunda porção continental com maior diversidade, ficando atrás do continente Asiático (PAPPU et al., 2009). Para determinação de novas espécies a caracterização biológica e molecular se faz necessária. O círculo de hospedeiros, sintomatologia, ensaio de transmissão com tripes, testes sorológicos e análise genética da proteína N são comumente utilizados para caracterização, diferenciação e classificação taxonômica (de ÁVILA et al., 1990; FAUQUET et al., 2005; DONG et al., 2008; HASSANI-MEHRABAN et al., 2010).

Até o momento no Brasil a espécie ZLCV era a única do gênero *Tospovirus* infectando cucurbitáceas (BEZERRA et al., 1999; POZZER et al., 1996), algumas vezes associada a grandes danos na produção (GIAMPAN, 2003; NAGATA et al., 1998; REZENDE et al., 1997; STANGARLIN et al., 2000; YUKI et al., 2000). Segundo Yuki et al., (2000) a presença de ZLCV foi verificada em 7,8% das amostras analisadas em curcurbitaceas no Estado de São Paulo. Rodrigues (2011) relatou incidência de aproximadamente 12% para infecção por ZLCV em diferentes regiões produtoras de melancia no estado de Tocantins. Recentemente, foi detectada a presença de um outro tospovirus, o GRSV, em frutos de pepino (SPADOTTI et al., 2014).

Em outras partes do mundo outros tospovirus foram descritos infectando curcubitaceas, como o *Watermelon silver mottle virus* (WSMoV) relatado em Okinawa no Japão em 1982 infectando melancia e posteriormente em Taiwan no ano de 1992 (YEH et al., 1992). Além disso, duas espécies tentativas tem sido propostas: Watermelon bud necrosis virus (WBNV) reportada em melancia na Índia (JAIN et al., 1998) e Melon yellow spot virus (MYSV) em melão no Japão (KATO et al., 2000). *Melon severe mosaic virus* (MeSMV) também foi descrito infectando melão, pepino, abóbora e melancia no México (CIUFFO et al., 2009).

4.2.1.2 Transmissão dos tospovirus

Os tospovírus são transmitidos de maneira circulativa propagativa por insetos da ordem Thysanoptera, vulgarmente conhecidos como tripes (WIJKAMP et al., 1993). São insetos muito pequenos (1.1-1.5 mm de comprimento) e encontram-se disseminados por todo o mundo. Existem cerca de 5.000 espécies identificadas, porém somente algumas são conhecidas como potenciais vetores de tospovírus, sugerindo uma co-evolução entre vírus e vetor referente à especificidade de transmissão (BRUNNER et al., 2002; PAPPU et al., 2009).

Quatorze espécies de tripes pertencentes aos gêneros *Frankliniella* (9 espécies), *Thrips* (2), *Scirtothrips* (1), *Ceratothripoides* (1) and *Dictyothrips* (1) já foram descritas como vetores de tospovírus (CIUFFO et al., 2010; KING et al. 2012). Até a década de 1980, *T. tabaci* era considerada a principal espécie vetora, devido principalmente a sua ampla distribuição geográfica. A partir dessa década *F. occidentalis* tornou-se o principal vetor em decorrência de sua rápida disseminação no hemisfério Norte (GOLDBACH; PETERS, 1994). Outras espécies que merecem destaque devido a sua importância como vetores de tospovírus são *F. schultzei* e *F. fusca* (ROSELLÓ et al., 1996). Segundo Nagata et al., (2004), os principais vetores de GRSV, com relação à eficiência de transmissão, são *F. schultzei* (93,1%) e *F. occidentalis* (17,5%). No Brasil dispõe-se de poucos dados atuais sobre a distribuição geográfica e importância relativa das diferentes espécies de tripes vetoras de tospovírus. Em melancia, por exemplo, há poucos relatos sobre a diversidade de tripes associadas a cultura, sendo a espécie *F. schultzei* frequentemente relatada como praga da cultura (MONTEIRO et al., 1999). Estudos realizados por Costa et al., (2015) observaram a predominância de *F. schultzei* e *Haplothrips gowdeyi* em áreas agrícolas da região Nordeste.

No processo de transmissão o inseto adquire o vírus principalmente no primeiro estádio larval (WIJKAMP et al., 1993), a transmissão só é observada no final do segundo ínstar, mas é realizada com maior eficiência pelos adultos virulíferos (van de WETERING et al., 1996). Os estádios de pré-pupa e pupa não se alimentam e consequentemente não adquirem e não transmitem o vírus (NAGATA; PETERS, 2001). Após a ingestão das partículas virais, os vírions chegam ao intestino médio, primeiro sítio de ligação e entrada nas células do inseto. Lá, replicam-se e por um mecanismo não conhecido atravessam a membrana basal chegando as células musculares viscerais, onde

continuam a se propagar (NAGATA et al., 1999). Posteriormente, atravesssam a membrana basal dessas células e adentram a glandula salivar. Os vírions saem da glândula salivar através da membrana apical e são expelidos com as secreções salivares nos tecidos vegetais durante a alimentação do tripes vetor (WHITFIELD et al., 2005). Não há transmissão vertical do vírus de insetos adultos para sua prole, ou seja, cada geração de tripes só adquire o tospovírus no estágio larval alimentando-se em uma planta doente (WIJKAMP et al., 1996).

4.2.1.2.1 Métodos de identificação de tripes

A identificação de espécies de tripes é geralmente realizada de forma tradicional, utilizando as características morfológicas como o número de segmentos da antena e a distribuição e número de cerdas para distinguir uma espécie da outra (MEHLE; TRDAN, 2012), dependendo de um taxonomista.

Além da identificação taxonômica, a análise do genoma mitocondrial (mtDNA) de insetos tornou-se uma ferramenta poderosa em estudos de genética de população e filogenia. Nos últimos anos, sequências de oligonucleotídeos para amplificação por PCR de genes mitocondriais de diferentes insetos foram publicados (KAMBHAMPATI; SMITH, 1995; DINSDALE et al., 2010; de BARRO et al., 2011). O genoma mitocondrial na maioria dos organismos é herdado maternalmente e é conhecido por apresentar maiores taxas de mutação em comparação com as regiões de codificação no genoma nuclear (BROWN et al., 1982). O gene mitocondrial de insetos é uma molécula de DNA circular de cerca de 18,5 kb de tamanho (HOY, 1994). Em geral, em cada mitocôndria acredita-se conter entre 2 – 10 cópias do genoma. Células individuais contém até várias centenas de mitocôndrias e consequentemente, milhares de cópias de genes mitocondriais (LIGHTOWLERS et al. 1997; SCHEFFLER, 2000). Isto proporciona condições ótimas para análises baseadas em PCR tornando o mtDNA uma ferramenta para diagnóstico molecular.

A análise da sequência de nucleotídeos do gene citocromo oxidase I (mtCOI) tem possibilitado a discriminação de espécies na ordem Thysanoptera (BRUNER et al., 2002; FREY; FREY, 2004; ASOKAN et al., 2007; TIMM et al., 2008; ZHANG et al., 2011; KADIRVEL et al., 2013). Métodos baseados em PCR quantitativo também têm sido desenvolvidas para a identificação de tripes utilizando o gene mtCOI

(KOX et al., 2005). O grau de conservação do gene mtCOI confere baixa variação intra-específica acoplado com variação apreciável entre espécies que permite alta precisão na identificação de espécies de tripes.

Embora vários métodos baseados em PCR estão disponíveis para a identificação de espécies de tripes, a maior parte deles têm sido limitados a uma espécie específica ou algumas espécies (KADIRVEL et al., 2013; BRUNER et al., 2002). A identificação taxonômica e a análise do mtDNA dos tripes são ferramentas importantes na prevenção da entrada de novas espécies pragas e em programas de controle de espécies específicas, especialmente tripes que já relatados com resistência a alguns inseticidas, como *F. occidentalis* e *Thrips tabaci* (JENSEN, 2000; BIELZA, 2008).

4.2.1.3 Manejo do vira cabeça

O manejo de algumas espécies do gênero *Tospovírus* é difícil devido a amplo círculo de hospedeiros e ao ineficiente controle químico do vetor. A resistência à inseticidas em *F. occidentalis* já foi documentada em uma série de categorias de produtos químicos incluindo os organoclorados, organofosforados, carbamatos, piretróides e spinosins (BIELZA, 2008). Para *T. tabaci* também já foi relatada a ineficiência de cinco diferentes inseticidas (TODA; MORISHITA, 2009).

Práticas culturais têm sido recomendadas para manejo do vira-cabeça e incluem combate do tripes vetor mediante o uso de agentes de controle biológico, como o percevejo predador *Orius* sp. (KAWAI, 1995; MEIRACKER, 1999; SILVEIRA, 2003), o ácaro predador *Stratiolaelaps scimitus* já utilizado comercialmente (FREIRE; MORAES, 2007), e também o uso do *Metarhizium anisopliae* como agente biopesticida (NIASSY et al., 2012).

Algumas espécies de *Solanum* possuem resistência natural a tospovirus, porém a maioria das fontes de resistência são específicas a certas espécies (STEVENS et al., 1992) e de natureza poligênica (PATERSON et al., 1989). A primeira fonte de resistência natural foi identificada em *Solanum peruvianum*, o qual apresentou elevado nível de resistência a isolados de TSWV (STEVENS et al., 1992). No Brasil, foram pesquisadas populações derivadas do cultivar "Stevens", originadas de cruzamentos entre *S. peruvianum* e *S. esculentum*, obtidas na África do Sul (STEVENS et al., 1992). Essa resistência introduzida na cultivar "Stevens" é mediada pelo gene *Sw-5*, que confere

resistência ampla às espécies: TSWV, GRSV e TCSV (SPASSOVA et al., 2001). Plantas que expressam o gene *Sw-5* restringem a infecção sistêmica do vírus e as folhas inoculadas produzem uma reação do tipo hipersensibilidade (STEVENS et al., 1992).

Cultivares de tomates comerciais, que expressam o gene de resistência *Sw-5*, têm sido usadas em vários países como África do Sul, Espanha, Itália, Austrália e Estados Unidos (SIVPASARD; GUBBA, 2011). No Brasil, a cultivar de tomate para processamento industrial "Viradoro" possui resistencia à TSWV, TCSV e GRSV (GIORDANO et al., 2000). A cultivar "Viradoro" teve origem a partir de cruzamentos realizados entre os cultivares "IPA-5" (resistente a altas temperaturas eaos fungos *Verticillium*, *Fusarium*, *Stemphylium* e ao nematóide *Meloidogyne*) e "TSW-10" que contém o gene *Sw-5* (GIORDANO et al., 2000). O gene *Sw-5* confere resistência a tospovirus em tomate e em outras espécies da família *Solanaceae*, como o fumo (*Nicotiana tabacum*) e a berinjela (*Solanum melongena*) (LAU et al., 2006). Em *Capsicum chinense* "PI 159236" e "PI 152225 foi relatado outro tipo de gene dominante de resistência denominado *Tsw*, efetivo apenas contra a espécie TSWV" (BOITEUX; de ÁVILA, 1994).

Atualmente, não existe informação disponível sobre genótipos de melancia com resistência a tospovírus no Brasil. No entanto, Pandey e Pandey (2001) avaliando sessenta cultivares de melancia em campo altamente infectado com WBNV observou que as cultivares Durgapur Selection, RHR\VH-2 e uma linhagem de melancia denominada C-393243, mostraram-se livres de infecção por este vírus. Resistência utilizando melancia transgênica contendo parte do gene da nucleoproteína de WSMoV já foi relatada (LIN et al., 2012), mas a resistência transgênica a MYSV, outra espécie do gênero *Tospovirus*, ainda não foi observada (YANG et al., 2014).

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CAPÍTULO I

*Citrull*us *lanatus* is a new natural host of *Groundnut ringspot virus* in Brazil[1]

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Citrullus lanatus is a new natural host of *Groundnut ringspot virus* in Brazil

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ABSTRACT

In 2012 and 2013, watermelon (*Citrullus lanatus*) plants from commercial crops in São Paulo State were found showing mosaic, necrotic lesions, leaf deformation and necrotic spots on the fruits, suggestive of tospovirus infection. Leaf and fruit samples were separately tested by PTA-ELISA and reverse transcription polymerase chain reaction (RT-PCR) against tospovirus was performed. The virus was identified as *Groundnut ringspot virus* (GRSV) and was sap transmissible. The host range was similar to isolates of GRSV found naturally infecting Solanaceae, except that this isolate infected watermelon systemically but did not infect tomato cvs. Santa Clara and Mariana. Thrips collected in the field transmitted GRSV to watermelon, sweet pepper and *Nicotiana* species. To our knowledge, this is the first report on the natural infection of watermelon by GRSV.

Keywords: Survey, watermelon, *Groundut ringspot virus*, tospovirus, RT-PCR

1 INTRODUCTION

Watermelon (*Citrullus lanatus*) is one of the most important cucurbit crops in the world and Brazil is the fourth largest producer, with a total cultivated area of 90.000 ha and average annual production of about 2 million t (FAO 2013). The crop can be affected by several species of virus, for example, the genera *Potyvirus*, *Cucumovirus* and *Tospovirus* in

most Brazilian states. The only tospovirus species reported to infect watermelon in Brazil is *Zucchini lethal chlorosis virus* (ZLCV) (Yuki et al. 2000).

Diseases caused by tospoviruses, commonly known as "vira-cabeça," have been observed affecting several crops of economic interest in Brazil. Seven tospovirus species were reported: *Tomato spotted wilt virus* (TSWV), *Tomato chlorotic spot virus* (TCSV), *Groundnut ringspot virus* (GRSV), *Iris yellow spot virus* (IYSV), *Chrysanthemum stem necrosis virus* (CSNV), ZLCV and Bean necrotic mosaic virus (BeNMV) (Ávila et al. 1990; Resende et al. 1996; Bezerra et al. 1999; Pozzer et al. 1999; Oliveira et al. 2012). Tospoviruses are exclusively transmitted from plant to plant by a few species of thrips (Thysanoptera: Thripidae) in a circulatory propagative manner (Ullman et al. 1997; Jones 2005; Turina et al. 2012).

We report here the molecular and biological characterization of the GRSV isolated from watermelon plants. The phylogenetic relationship of their sequences with those of other GRSV isolates from Brazil and other countries is analyzed.

2 MATERIAL AND METHODS

Watermelon plants exhibiting mosaic and necrotic lesions on the leaves (Fig. 1A), leaf malformation and necrotic spots on fruits (Fig. 1B), associated with the presence of thrips, suggestive of tospovirus infection, were collected in the producing regions of Marília and Presidente Prudente, São Paulo State, during surveys in 2012 and 2013. Leaf and fruit samples were separately tested by plate-trapped antigen (PTA) enzyme-linked immunosorbent assay (ELISA) (Mowat and Dawson 1987) using polyclonal antisera against GRSV, TSWV, TCSV and ZLCV, kindly provided by Dr. Alice K. Inoue-Nagata, Embrapa, CNPH, DF, Brazil.

Some of the same samples from both localities were used for total RNA extraction, using Norgen's total RNA purification kit (Norgen Biotek Corporation, Canada), according to the manufacturer's instructions. Total RNA was used as a template for amplification in the one-step reverse transcription and polymerase chain reaction (RT-PCR) in a final volume of 25 µl, using 12.5 µl of Master Mix buffer (PCR Master Mix, Promega, USA), with the addition of 1 unit of AMV and 1 mM of each generic primer BR 60 (5' CCCGGATCCTGCAGAGCAATTGTGTCA 3') and BR 65 (5' ATCAAGCCTTCTGAAAGTCAT 3') (Eiras et al. 2002). The PCR product was analyzed

in 1% agarose gel stained with Neotaq Brilliant Green Plus DNA Stain (Neobio, Brazil). PCR products were purified using a Qiagen PCR purification kit (Qiagen, Inc. Valencia, CA, USA), according to the manufacturer's protocol. The RT-PCR amplicons were directly sequenced at Macrogen Inc., Seoul, Korea. The resulting nucleotide sequences were edited and then aligned using the program ClustalW2 (EBI;http://www.ebi.ac.uk/Tools/msa/clustalw2/). The nucleotide and deduced amino acid sequences were compared with the corresponding sequences of other tospoviruses deposited in GenBank. Phylogenetic analysis was conducted using MEGA software version 5.0 (Tamura et al. 2011), and a phylogenetic tree was constructed using the neighbor-joining method.

Twelve species of plants from different families (Amaranthaceae, Curcubitaceae and Solanaceae) were used for the host range study. Four to eight plants of each species were sap-inoculated with one isolate from Presidente Prudente (named GRSV-PP) and another from Marília (GRSV-Ma), and symptoms were observed for 40 days in a greenhouse. All inoculated plants were tested for tospovirus infection by PTA-ELISA and RT-PCR.

Thrips collected from flowers of field-infected watermelon plants were identified as *Frankliniella schultzei* by molecular and morphological analysis (data not shown). Groups of 20–25 adult thrips collected in the field were individually transferred onto eight watermelon plants, four *Nicotiana tabacum* cv. Havana and four sweet pepper (*Capsicum annuum*) cv. Magali plants. After 2 days of inoculation access period, the plants were sprayed with imidacloprid. Test plants were kept in a greenhouse for further analysis. In order to test the natural infectivity of the thrips, RT-PCR was used to detect the virus inside the vector (data not shown).

3 RESULTS AND DISCUSSION

The symptomatic watermelon samples collected from the field reacted only with the polyclonal antisera against GRSV in PTA-ELISA. The RT-PCR assay with generic tospovirus primers amplified an expected 453 bp fragment, representing part of the nucleocapside (N) gene. Nucleotide sequences for 16 isolates, 14 collected in the region of Marília and four in the region of Presidente Prudente, were obtained. The nucleotide (426 nt) sequences, one isolate from each location, were deposited in GenBank (GRSV-PP and

GRSV-Ma under accession numbers KJ183076 and KJ183077, respectively). A comparison with the sequences available at GenBank showed 98 to 99% nucleotide sequence identity with the GRSV isolate from Argentina (Dewey et al. 1995; accession number GRU49702).

The nucleotide and deduced amino acid sequence for the GRSV-Ma isolate compared with other GRSV isolates deposited in GenBank revealed that they share 96 to 98% of nucleotide identity and 97% deduced amino acid identity, respectively. For GRSV-PP the nucleotide sequence identity varied from 96 to 97%, and the deduced amino acid sequence showed 98% identity. It is worth mentioning that the 90% amino acid identity threshold is used for species delineation in the genus Tospovirus (King et al. 2012). When nucleotide sequences for GRSV-PP and GRSV-Ma isolates were compared with the corresponding nucleotide sequences for TCSV, TSWV, ZLCV, CNSV and IYSV, the identities varied from 82 to 56%, and 82 to 56%, respectively. Phylogenetic analysis of the partial nucleotide sequences of the N protein showed that watermelon isolates of GRSV clustered with other Brazilian isolates of GRSV, as well as with isolates from Argentina (Fig. 2). The reaction of 11 species to mechanical inoculation with GRSV-PP and GRSV-Ma isolates is shown in Table 1. In thrips transmission tests, *F. schultzei* were able to transmit GRSV to watermelon cv. Crimson Sweet, *N. tabacum* cv. Havana and sweet pepper cv. Magali. GRSV was detected in *F. schultzei* by RT-PCR. The amplified fragment of 410 bp showed 97% identity with GRSV (accession number U49702).

The results indicate that GRSV was identified as the tospovirus species causing disease in watermelon crops in Brazil. The host range of the watermelon GRSV isolates was very similar to the GRSV described in other hosts, except that the former induced systemic necrosis in watermelon, but did not infect tomato (*Solanum lycopersicum*) cvs. Santa Clara, Mariana. Tomato plants inoculated with viruliferous thrips, collected from field-infected watermelon plants, remained symptomless and virus free.

Despite the differences in host range, the watermelon GRSV isolates were transmitted by *F. schultzei* in transmission tests. GRSV is efficiently transmitted by *F. schultzei* and *F. occidentalis* under field conditions (Nagata et al. 2004) and by *F. gemina* under experimental conditions (de Borbón et al. 2006).

It is known that GRSV was first isolated from tomato in South America (de Ávila et al. 1993; Dewey et al. 1995). GRSV is known to affect the production of peanuts (*Arachis hypogaea*) (de Breuil et al. 2007), as well as sweet pepper and tomato in South

America (Pappu et al. 2009).Webster et al. (2010) recently reported GRSV in tomato in North America. A high incidence of peanut plants infected with GRSV was recently reported in São Paulo State, Brazil (García et al. 2014).

GRSV may not have been detected in the other watermelon-producing regions in Brazil yet, or it may occur only sporadically and a larger number of samples need to be tested to verify its occurrence. A natural infection of watermelon with GRSV has not been reported; however, a cucumber fruit was detected recently infected with GRSV in Brazil (Spadotti et al. 2014). So far, it is known that the tospovirus infection in watermelon plants has been observed only in Japan and Taiwan with *Watermelon silver mottle virus* (WSMoV) (Yeh et al. 1992), in India and Mexico with the putative species respectively, Watermelon bud necrosis virus (WBNV) (JAIN et al. 1998) and Melon severe mosaic virus (MeSMV) (Ciuffo et al. 2009), and in Brazil with ZLCV (Yuki et al. 2000).

To the best of our knowledge, this is the first report on the natural infection of watermelon by GRSV, suggesting the need for regular assessment of the virus status of cucurbit crops. Thus, GRSV can be considered an emerging virus in watermelon in Brazil.

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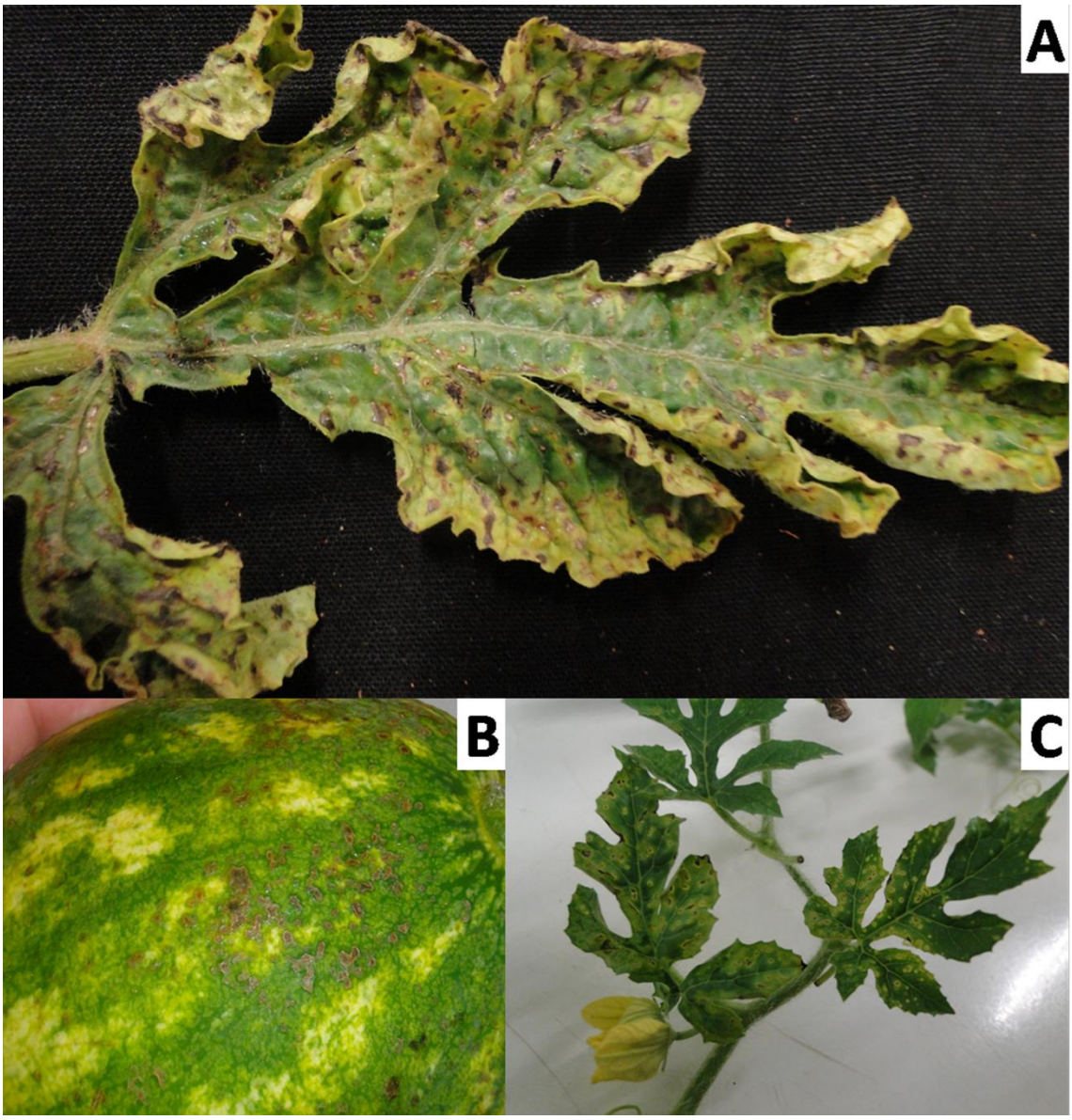

Figure 1. Necrotic lesions and mosaic on watermelon leaves in the field (A), necrotic spots on watermelon fruits (B), and a necrotic spot on watermelon cv. Fairfax leaves mechanically inoculated (C).

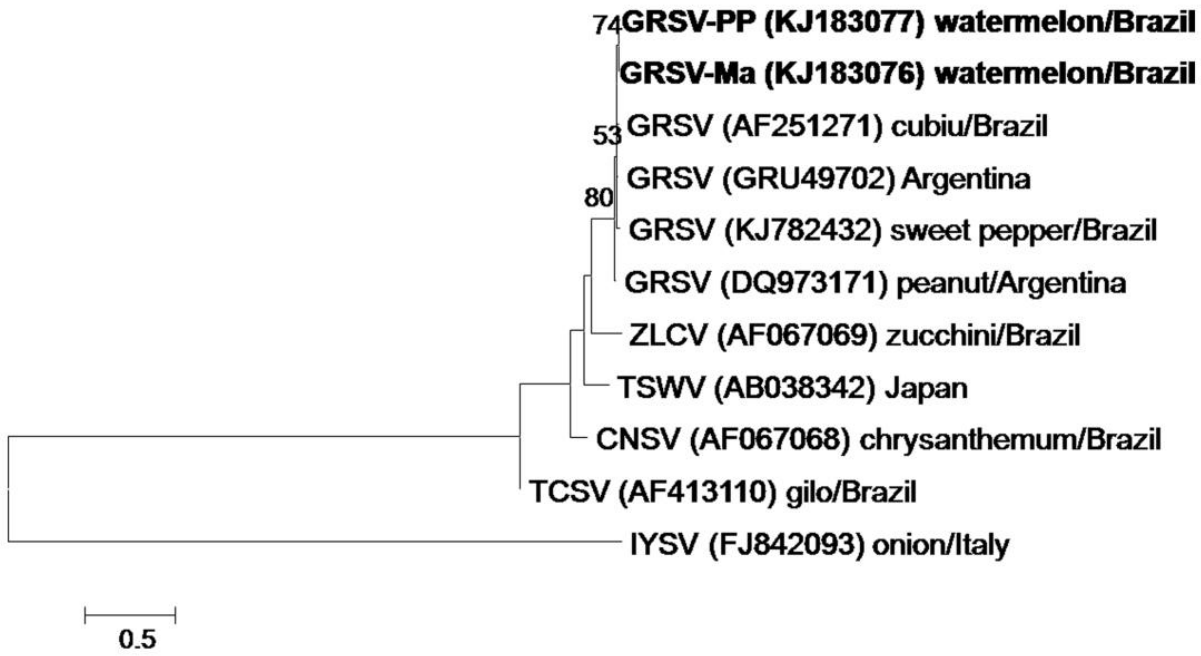

Figure 2. Phylogenetic tree constructed with the partial nucleotide sequences of the N protein of *Groundnut ringspot virus* derived from watermelon plants (GRSV-Ma and GRSV-PP) and from sequences from other reported tospoviruses (accession number, host and country of origin). Numbers at the nodes indicate the frequency of the cluster after bootstrap analysis (2000 replicates). Only bootstrap values above 50% are shown.

Table 1
Host range of *Groundnut ringspot virus* isolates and symptoms caused on various hosts

Family	Species	GRSV-PP[a]				GRSV-Ma[a]			
		No. infected plants/total inoculated	Symptoms[b]		ELISA/ RT-PCR[c]	No. infected plants/total inoculated	Symptoms[b]		ELISA/ RT-PCR[c]
			Local	Systemic			Local	Systemic	
Solanaceae	*Solanum lycopersicum* cv Santa Clara	0/8	ni	ni	-	0/6	ni	ni	-
	S. lycopersicum cv Mariana	nt	nt	nt	nt	0/8	ni	ni	-
	Capsicum annuum cv Magali	7/7	cr	cr, m, cl	+	4/6	cr	m,cr,cl	+
	Datura stramonium	nt	nt	nt	nt	2/2	ns	m,n, ld	+
	Nicotiana benthamiana	nt	nt	nt	nt	2/3	cr	m, n,cr	+
	N. glutinosa	0/10	ni	ni	-	3/3	cr	m,n,cr	+
	N. tabacum cv Havana	2/2	cr	cr, m	+	13/18	cr	m,n, mo,cr	+
	N. tabacum cv TNN	2/6	cr	cr,m,n	+	2/4	cr	n,cr	+
Amaranthaceae	*Chenopodium quinoa*	1/6	cll	ni	+	4/4	cll	ni	+
	Gomphrena globosa	1/4	nll,rh	m, ld	+	4/4	nll, rh	m,ld	+
Cucurbitaceae	*Cucumis sativus* cv Caipira	0/14	ni	ni	-	0/8	ni	ni	-
	C. sativus cv Aodai	0/14	ni	ni	-	0/10	ni	ni	-
	Citrullus lanatus cv Crimson Sweet	10/15	nll	n	+	9/14	ns	m, n	+
	C. lanatus cv Fairfax	nt	nt	nt	nt	5/15	ns	m, n	+
	C. lanatus hybrid Olímpia	nt	nt	nt	nt	12/23	ns	m, n	+
	C. lanatus hybrid Top Gun	nt	nt	nt	nt	4/4	ns	m, n	+
	C. lanatus hybrid Manchester	nt	nt	nt	nt	2/4	ns	ns	+
	Cucurbita pepo cv Caserta	nt	nt	nt	nt	0/4	ni	ni	-
	Cucumis melo cv. Eldorado	nt	nt	nt	nt	0/6	ni	ni	-

[a] GRSV isolated from watermelon leaves from Presidente Prudente (GRSV-PP) and Marília (GRSV-Ma); [b]cr, concentric rings; m, mosaic; ns, necrotic spot; n, necrosis; mo, mottle; cll, chlorotic local lesions; nll, necrotic local lesions; ld, leaf distortion; cl, chlorosis on leaves; rh, reddish halo; ni, no infected; nt, not tested;[c](+) positive and (-) negative results.

CAPÍTULO II

A Taqman real-time RT - PCR assay for detection of *Groundnut ringspot virus*[2]

[2] Redigido conforme as normas da revista *Tropical Plant Pathology* (Short communication)

A Taqman real-time RT - PCR assay for detection of *Groundnut ringspot virus*

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ABSTRACT

Groundnut ringspot virus (GRSV) is a tospovirus species transmitted in a circulative propagative manner by several thrips species. Traditionally, GRSV is detected by ELISA and RT-PCR, so this paper describes a rapid diagnostic system for the reliable detection of GRSV in plants and individual thrips by quantitative RT-PCR (qRT-PCR). Watermelon GRSV infected leaves and fruits, and thrips individuals (*Frankliniella schultzei*) collected from flowers of infected watermelons were used in the qRT-PCR test. The GRSV TaqMan assay we designed detected efficiently GRSV in all samples. The method had high specificity and could distinguish GRSV from the other tospoviruses species as *Tomato spotted wilt virus* (TSWV), *Tomato chlorotic spot virus* (TCSV) and *Zucchini lethal chlorosis virus* (ZLCV). The test will help collecting epidemiological data for GRSV in plants and thrips.

Key words: *Citrullus lanatus*; *Tospovirus;* GRSV*; Frankliniella schultzei*; RT-PCR.

Groundnut ringspot virus (GRSV), a member of the genus *Tospovirus*, causes spotted wilt disease, one of the most serious diseases for *Solanaceae* in Brazil, central America and South Africa (Pappu et al., 2009). Tomato (*Solanum lycopersicum* L.) and sweet pepper (*Capsicum annum* L.) are the most common crops affected by GRSV worldwide. In general, the symptoms are bronzing, mosaic, mosaic with ringspots on

leaves, yellowing and stem necrosis. GRSV is an important threat to horticulture since, in addition to an overall reduction in crop yield, the marketing quality of the harvested product is seriously affected by pronounced symptoms on fruits (Webster et al., 2010).

Recently GRSV was detected on watermelon [*Citrullus lanatus* (Thunb.) Matsum & Nakai] and cucumber (*Cucumis sativus* L.) (Leão et al., 2014; Spadotti et al., 2014) in Brazil. GRSV is transmitted in a circulative-propagative manner by several species of thrips including *Frankliniella occidentalis* (Pergande) (Wijkamp et al., 1995; Nagata et al., 2004), *F. schultzei* (Trybom) (Wijkamp et al., 1995; de Borbón et al., 2006; Nagata et al., 2004), *F. intonsa* (Trybom) (Wijkamp et al., 1995) and *F. gemina* (Bagnall) (de Borbón et al., 1999). The virus multiplies in the insect, but it is not transmitted to the eggs (Wijkamp et al., 1995).

Traditionally tospovirus species are detected by enzyme-linked immunosorbent assays (ELISA) (Resende et al., 1991; Webster et al., 2010), DOT-BLOT hybridization (Eiras et al., 2001) and conventional reverse transcription (RT) polymerase chain reaction (PCR) with generic (Eiras et al., 2001) or specific primers (de Breuil et al., 2007). Other methods, such as host range and electron microscopy, have also been utilized (Boari et al., 2002; Camelo-García et al., 2014).

Quantitative RT-PCR (qRT-PCR) technology has already proved to be an efficient tool for the detection of other plant tospoviruses (Roberts et al., 2000; Boonham et al., 2002; Chen et al., 2013; Boben et al., 2007). In this study, we describe a routine diagnostic system for the reliable detection of GRSV in plants and inside individual thrips by qRT-PCR (TaqMan). Furthermore, the method is quantitative and could be used as a tool for examining tospovirus interaction within thrips.

Watermelon leaves and fruits showing tospovirus-like symptoms were collected in two regions of São Paulo state (Marília and Presidente Prudente). The virus species infecting these plants was identified as GRSV by PTA-ELISA and RT-PCR sequencing using generic primers (BR60 and BR65), located in the tospovirus nucleoprotein (N) gene (Eiras et al., 2001). The specimens of *F. schultzei* were collected from flowers of GRSV-infected watermelon plants and stored in 70% ethanol until analysis. GRSV isolate from *Datura stramonium* L. was used as positive control.

The primers and probe for qRT-PCR assay were designed within the conserved regions of the N protein gene (Figure 1) of the GRSV isolates deposited in GenBank (access number AF251271). The design of primers and probes were carried out using Primer Express™ software (PE-Biosystems).

Total RNA from watermelon leaves and fruits was extracted using Norgen's total RNA purification kit (Norgen Biotek Corporation, Canada), according to the manufacturer's instructions. For thrips, the RNA was extracted using Trizol reagent, following the manufacturer's protocol, with some modifications. Briefly, individual thrips were placed in eppendorf tubes (1.5 ml) and homogenized with a micropestle in 250 µl of extraction buffer (Trizol reagent) and incubated at room temperature for 5 min. Then, 50 µl of chloroform isoamyl alcohol (24:1) were added after 3 min at room temperature, and centrifugation at 12.000 g for 10 min at 4°C, the supernatant was moved to another eppendorf tube, with 1 µl of glycogen (10ng/ml) and mixed with 125 µl of cold isopropanol, incubated for 10 min at room temperature. After a final centrifugation at 12 000 g for 10 min at 4°C, the pellets were washed with 100 µl of 70% ethanol, dried in a vacuum chamber and re-suspended in 8 µl of water Milli-Q following incubation for 15 min at 60 °C.

First-strand cDNA was synthesized using 1 µl of total RNA from plants and 8 µl for thrips with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions. qRT-PCR reactions were performed in a 96 well plates (Bio-Rad, California, USA) and contained 1 µl cDNA, 0.3 µl of each primers (final primer concentration of 10 mM each), 0.2 µl of TaqMan probe (10 mM) and 5 µl of iTaq™ Universal Probes Supermix (Bio-Rad), in a final volume of 10 µl. The qRT-PCR amplifications were undertaken in a CFX Connect™ Real-Time PCR detection system (Bio-Rad) with a CFX Manage Software (Bio-Rad). The amplification reactions were run at least in triplicate. The real-time PCR reactions were performed under the following conditions: initial at 95°C for 3 min followed by 40 cycles consisting of denaturation at 95°C for 10 sec, and annealing/sinthesis at 60°C for 30 sec. Protocol of optimization was done using differential dilution of cDNA. An internal control was used. All plant samples were tested with primer-probe combination designed in a highly conserved region of the plant mtCOX1 gene (GenBank X83206) (Weller et al., 2000).

The qRT-PCR assay was subjected initially to an optimization in a 5-fold serial cDNA dilution of a positive sample to determine the end-point limit of detection and the linearity of the assay. The slope (-3.554) and correlation coefficient (91.1%) of the dilution curve showed that this assay could be used to detect target RNA in infected watermelon tissue and individual viruliferous thrips (Figure 2). The final concentration of primers and probe (10 mM) was suitable for detection of virus in plant and thrips.

GRSV TaqMan assay successfully detected GRSV isolates from watermelon leaves and fruits (Figure 3A). Ct-value observed for infected watermelon samples was less than 20 cycles, showing the specificity of the primers and the sensibility of the probe. The internal control used was capable to amplify only the mtCOX1 gene from the plant. The internal controls are naturally present in the biological sample, they are used as indicator of perfect nucleic acid extraction, quality of samples, and quality of PCR. In addition, the assay could detect GRSV in individual viruliferous thrips (Figure 3B). Although, the extracted RNA from individual viruliferous thrips provides the lowest Ct-value (30.0), healthy thrips resulted in no detection of cDNA in the assay conditions. Low Ct-value (28.0) was also observed in TSWV infected thrips (*F. occidentalis*) (Boonham et al. (2002) and in *Impatiens necrotic spot virus* (INSV)infected thrips (Ct-value - 30.0) (Chen et al., 2013).

We also performed the same assay including positive controls of other tospovirus species, such as *Tomato spotted wilt virus* (TSWV), *Tomato chlorotic spot virus* (TCSV) and *Zucchini lethal chlorosis virus* (ZLCV). The results obtained showed that the primers and probe used in this study were able to detect only GRSV (data not shown).

Molecular-based detection tools are used for routine detection of tospoviruses in infected plants (Eiras et al., 2001; de Breuil et al., 2007). Quantitative RT-PCR tests was reported to detect TSWV and INSV in plants and thrips vector (Roberts et al., 2000; Boonham et al., 2002; Chen et al., 2013), *Chrysanthemum stem necrosis virus*, CSNV (Boben et al. 2007) and *Iris yellow spot virus*, IYSV (Tiberini et al., 2012).

The qRT-PCR assay to detect GRSV in plant host and on individual thrips, developed in our study, was demonstrated to be highly sensitive, specific and accurate to detect GRSV in low concentrations (RNA dilution 1:15625). Detection of GRSV in thrips has only been reported using ELISA (Wijkkamp et al., 1995), but according to authors false positive results were frequent due to the small size of thrips, low concentration of virus in thrips and interference of insect tissue with the immune reaction.

Thrips monitoring in greenhouse is usually done with the utilization of sticky traps, as an important component for integrate pest management system for tospovirus control. This procedure, however, does not provide any indication for the proportion of viruliferous insects at any time. Testing the trapped thrips using this method may be useful as an early warning for the presence of viruliferous thrips. A change in the thrips

management system may then be able to prevent the spread of virus in the very early stages of the infection process. Boonham et al. (2002) showed that it is possible to detect TSWV by qRT-PCR in thrips trapped on sticky traps for 24 h and seven days after their collection.

In conclusion, qRT-PCR assay was used successfully to detect GRSV in plants and thrips. In addition, the optimization of primers for GRSV detection in qRT-PCR assays might serve as a useful tool in the identification studies, epidemiology and interaction of GRSV with different crops.

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  1 ATGTCTAAGG TCAAGCTCAC AAAAGAAAAC ATTGTCTCTC TTTTAACTCA ATCTGCAGAT
 61 GTTGAGTTTG AAGAAGACCA AAACCAGGTC GCATTCAACT TTAAAACTTT CTGTCAGGAA
121 AATCTTGACC TGATTAAGAA AATGAGTATC ACTTCATGTT TGACTTTCTT GAAGAATCGC
181 CAAAGCATCA TGAAAGTCGT GAACCAAAGT GATTTTACTT TTGGTAAGGT CACGATAAAG
241 AAAAATTCTG AGAGAGTTGG AGCCAAAGAC ATGACTTTCA GGAGGCTTGA TAGCATGATA
301 AGAGTGAAGC TCATAGAAGA GACTGCAAAC AATGAGAATC TTGCTATCAT CAAGGCAAAA
361 ATTGCCTCCC ACCCTCTGGT TCAAGCCTAC GGGCTGCCTC TGGCGGATGC AAAATCTGTG
421 AGACTTGCTA TAATGCTTGG AGGTAGTATC CCTCTCATTG CTTCTGTTGA CAGCTTCGAG
            Forward                          Probe
481 ATGATCAGTG TTGTTCTTGC CATATATCAA GATGCAAAGT ACAAGGAGCT AGGGATTGAA
       Reverse
541 CCGACTAAGT ACAACACTAA GGAAGCTCTG GGGAAGGTTT GCACAGTGCT TAAAAGCAAA
601 GGATTTACAA TGGATGATGC ACAGATAAAC AAAGGAAAAG AATATGCCAA GATACTTAGT
661 TCTTGCAATC CCAATGCTAA GGGAAGCATT GCTATGGACT ATTACAGTGA CAATCTTGAC
721 AAGTTCTATG AAAGTGTTGG AGTCAAGAAA GAAGCCAAGA TTGCTGGTGT TGCATGA
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Figure 1. Partial sequence of the nucleoprotein (N) of *Groundnut ringspot virus* (GRSV). The GRSV-F and GRSV-R primer sequences and probe are highlighted.

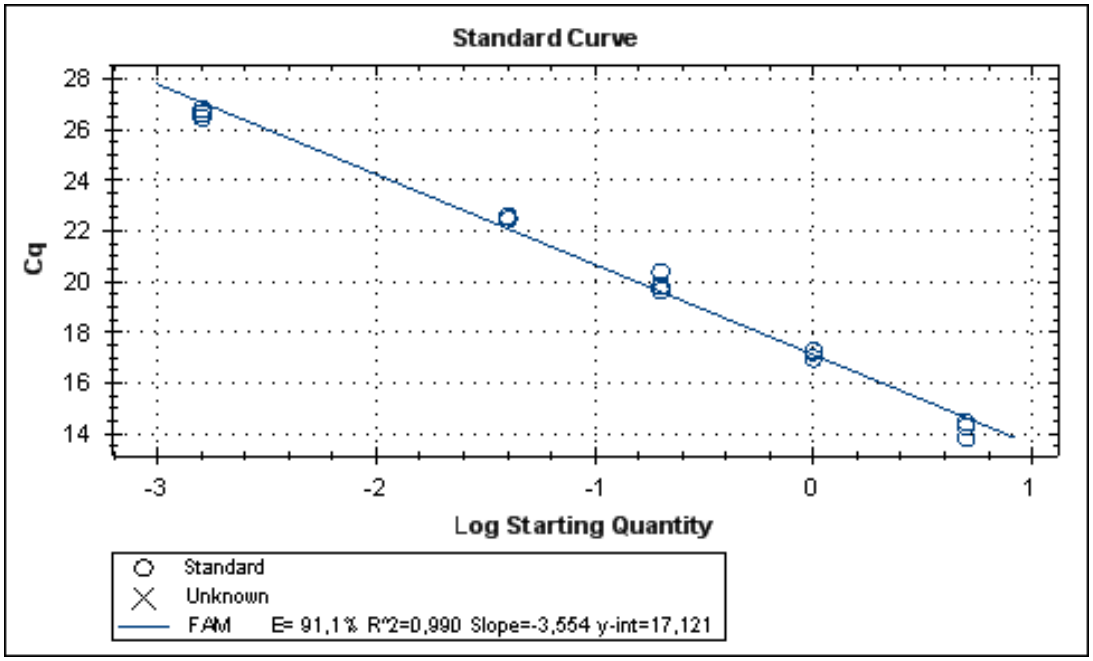

Figure 2. Standard curve of *Groundnut ringspot virus* extracted from positive samples (*Datura stramonium*).

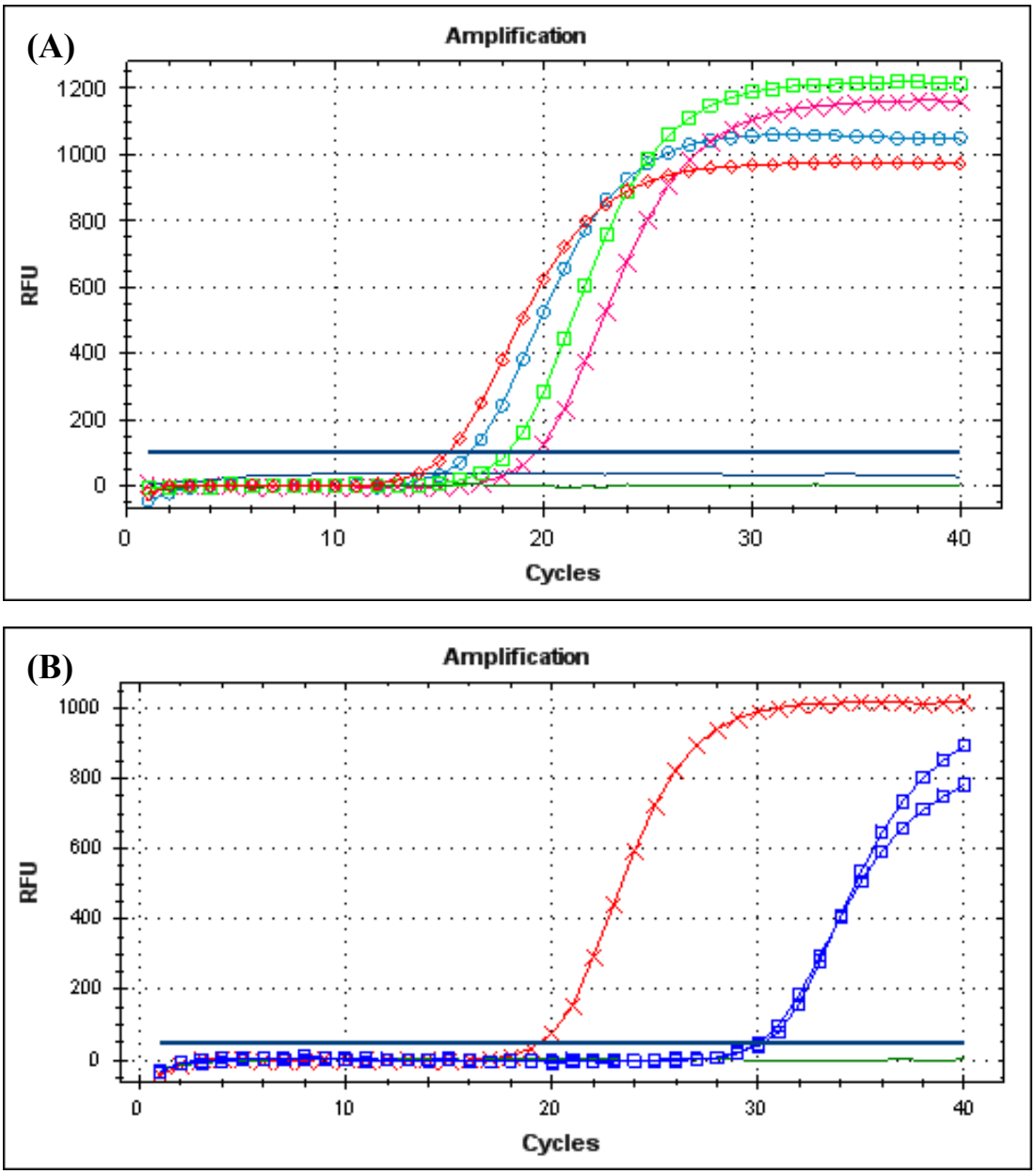

Figure 3. Detection of *Groundnut ringspot virus* (GRSV) in total plant RNA (A) and in individual thrips (B) by qRT-PCR. Amplification plot: (A) Red – GRSV positive control (*Datura stramonium*); blue – watermelon GRSV-Presidente Prudente; green – watermelon GRSV-Marília; pink – watermelon fruit; blue dark – healthy plant; dark green – water. (B) Red –positive plant extract; blue – viruliferous thrips; green – GRSV negative thrips.

CAPÍTULO III

Efficient detection of *Frankliniella schultzei* (Thripidae: Thysanoptera) by real-time PCR[3]

[3] Redigido conforme as normas da revista *Journal of Applied Entomology*

Efficient detection of *Frankliniella schultzei* (Thripidae: Thysanoptera) by real-time PCR

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ABSTRACT

The importance of thrips in the last decades has increased considerably worldwide. The taxonomic identification of thrips species is based mainly on external morphology, sometimes difficult for a non taxonomic expertise. DNA based methods for species identification are technically simple and applicable to large-scale screening. In this work, we propose a rapid and efficient molecular method for the identification of *Frankliniella schultzei*, an important pest, vector of tospoviruses on various vegetable crops worlwide. Species-specific primers for the *F. schultzei* mitochondrial cytochrome oxidase I gene (mtCOI) gene were developed and optimized for detection by conventional PCR and qPCR. The primers were tested on thrips adults and young stages collected from watermelon, pumpkin, tomato, sweet pepper, cucumber, lettuce and also from two weeds (*Emilia sonchifolia* and *Tridax procumbens*) from different regions of São Paulo state, Brazil. All samples collected were identified as *F. schultzei*, indicanting the prevalence of this species in this region. *F. occidentalis*, *Thrips tabaci* and *F. intonsa* were not detected by these primers, indicanting the high specificity of this test in both conventional PCR and real – time PCR

Keywords: thrips**,** identification, molecular diagnostics, qPCR

1 INTRODUCTION

Thrips are small insects widespread throughout the world causing direct damages (feeding and oviposition wounds) and indirect problems associated with the transmission of tospoviruses. Fourteen species of thrips belonging to the genera *Frankliniella* (9 species), *Thrips* (2), *Scirtothrips* (1), *Ceratothripoides* (1) and *Dictyothrips* (1) have been described as tospoviruses vectors in the world (King et al. 2012; Ciuffo et al. 2010). In Brazil there are five species reported: *Frankliniella schultzei*, *F. occidentalis, F. zucchini, Thrips tabaci* and *T. palmi,* associated with differents hosts (Monteiro et al., 2001).

The common blossom thrips or tomato thrips, *F. schultzei*¸ is native to South America (Mound, 2002) and is one of the major pests of some ornamentals and vegetable crops in tropical regions (Sakurai, 2004). In Cuba and Brazil it is one of the key pests of tomato (*Solanum lycopersicum*) (Haji et al. 1998; Jones 2005; Monteiro et al. 1999) and in Brazil this vector is associated with the transmission of tospoviruses to different crops such as tomato and sweet peper (*Capsicum annuum*) (Monteiro et al. 2001), watermelon (*Citrullus lanatus*) (Leão et al. 2014), cucumber (*Cucumis sativus*) (Spadotti et al. 2014) and peanuts (*Arachis hypogaea*) (Camelo-Garcia et al. 2014) .

One of the major constraints in studying the relationship between thrips and tospovirus is the dificulty to identify the thrips species. The taxonomic criteria is based mainly on external morphology but different species are similar in appearance, differing only in minute details (Mehle and Trdan 2012). On the other hand, nucleotide sequence analysis of the mitochondrial cytochrome oxidase I gene (mtCOI) has been shown to be well suited for species discrimination in the order Thysanoptera (Bruner et al. 2004; Frey and Frey, 2004; Kox et al. 2005; Asokan et al. 2007; Timm et al. 2008; Zhang et al. 2011; Kadirvel et al. 2013). Real-time quantitative PCR for the mtCOI also have been developed for *Thrips palmi* detection (Kox et al. 2005).

Given the importance of *F. schultzei* in Brazil, we developed primers, based on the mtCOI gene, for the rapid and accurate identification of this species by PCR and qPCR. The primers were highly specific for *F. schultzei* species.

2 MATERIALS AND METHODS

Thrips adults and larvae were collected from watermelon, pumpkin, tomato, sweet pepper, cucumber, lettuce and also from two weeds (*Emilia sonchifolia* and *Tridax procumbens*) in different cities of São Paulo state during the years 2012 – 2014 (table 1). The specimens were collected by using a hand-held aspirator, preserved immediately in 70% ethanol and stored at -20ºC until analysis. The Brazilian *F. occidentalis* specimes were provided by Dr. Grazielle Furtado Moreira, UNESP – FCAV, São Paulo, Brazil. Other species like *F. occidentalis, F. intonsa* and *Thrips tabaci* were kindly provided by Dr. Lara Bosco, University of Turin, Turin, Italy. Morphological identification (fig. 1) was realized prior the molecular studies under a stereomicroscope at 100X magnification using taxonomic keys (Mound et al. 1976; Palmer et al. 1989).

For the first PCR assay with primer pair mtD-7.2F and mtD-9.2R designed to amplify part of the mtCOI gene (Brunner et al. 2002), total DNA was extracted from each individual thrips following a modified Chelex method (Boonham et al. 2002). Briefly, thrips adults were crushed and homogenized in a 50 µl of Chelex 5% solution in a 0.5 ml eppendorf tube and then incubated at 94°C for 5 min. After centrifugation at 14.000 g for 5 min, the supernatant was collected and used as template for the PCR amplification. Each PCR reaction contained 3 µl of DNA, 12.5 µl Taq DNA Polymerase Master Mix Ampliqon III (Ampliqon ApS, Copenhagen, Denmark), 12.5 µl of 1.5 Mm $MgCl_2$ and 0.25µl of each primer (10 mM) in a final volume of 25 µl and was performed in termocycler with the following parameters: 3 min at 94° C, 35 cycles of 30 s at 95°C, 30 s at 55°C, and 45 s at 72°C, followed by a final extension for 5 min at 72°C. After amplification, 6 µl of the PCR products were subjected to electrophoresis on a 1 % agarose gel stained with Neotaq Brilliant Green Plus DNA Stain (Neobio, Brazil). PCR products were purified (QIAquick Gel Extraction Kit Qiagen) and sequenced (Macrogen, South Korea) using the primer mtD-7.2F. Sequence chromatograms were manually checked and edited in BioEdit software (version 7.1). DNA sequences were compared with the corresponding sequences of other tospoviruses deposited in GenBank and aligned using Clustal W in MEGA (version 5.0). A phylogenetic tree was constructed with the neighbour-joining method with 2000 bootstrap replications using MEGA software (Tamura et al. 2011).

For the PCRs with species specific primers and real – time PCR, individual thrips were taken in a 0.5 ml PCR eppendorf tube containing 10 µl of a Milli-Q water and ground thoroughly using sterile plastic micro pestle. The homogenate was incubated in boiling water for 5 min, stored at 20°C for 10 min and centrifuged at 8000 g for 5 min. Three micro liters of the supernatant were used as template for qPCR and 5 µl were used for specific primer PCR analysis.

One set of primers targeted on the region of mtCOI gene specifically for *F. schultzei* were designed and called F_S.rev and F_S.for (table 2). The specificity of the *F. schultzei*-specific primers (F_S.rev and F_S.for) was evaluated performing PCR assays on four thrips species, although *F. occidentalis* collected from two countries (table 1). All these thrips species are economically important pests in Brazil transmitting tospovirus, except *F. intonsa*, not recorded in the country (Monteiro and Lima 2011). PCR assay was performed for each thrips sample with a total volume of 25 µl as follows: 5 µl of DNA, 2.5 µl of 10x Buffer, 1.0 µl of $MgCl_2$ (50 mM), 2.5 µl of dNTP's (2 mM), 0.5 µl of each primer (10 µM), and 0.125 µl of Polytaq (Polymed, Florence, Italy). The reaction was carried out in a termocycler using the follow parameters: 3 min at 94° C, 35 cycles of 30 s at 95°C, 30 s at 55°C, and 40 s at 72°C, followed by a final extension for 5 min at 72°C. After amplification, the PCR products were subjected to electrophoresis.

For the qPCR assay, reactions were performed in a 96 well plates (Bio-Rad, California, USA) containing 3 µl DNA, 0.3 µl of primers F_S.rev and F_S.for (final primer concentration of 10 µM each), and 5 µl of iTaq™ Universal SYBR® Green Supermix (Bio-Rad), in a final volume of 10 µl. The SYBR Green PCR amplifications were undertaken in a CFX Connect™ Real-Time PCR detection system (Bio-Rad) with a CFX Manage Software (Bio-Rad). The amplifications reactions were run at least in triplicate. The thermal profile for SYBR PCR was 95 °C 3 min followed by 40 cycles of 95 °C for 10 s and 60 °C 30 sec.

3 RESULTS

Thrips specimens collected from watermelon, pumpkin, tomato, sweet pepper, lettuce, cucumber and in *Tridax procumbens* and *Emilia sonchifolia* were identified as *F. schultzei*. The PCR amplification with primer pair mtD-7.2F and mtD-9.2R designed to amplify part of the mtCOI gene (Brunner et al. 2002), produced only a single band of

450 bp. BLAST results showed significant identity (95–100%) between the sequences of the mtCOI gene of *F. schutzei* with the corresponding nucleotide sequences of *F. schutzei* deposited in GenBank (accession number KF560548).

For the *F. schultzei*-specific COI primers (F_S.rev and F_S.for) a single amplicon (145 bp) was amplified for the previously identified *F. schultzei* specimens. A 10-fold dilution series of DNA isolated from *F. schultzei* was tested in a qPCR using concentrations of 50, 150, 300 and 900 nM for the F_S.rev and F_S.for primers. Cycle threshold (Ct) values were optimal using 300 nM of primers and the limit of dilution from DNA detection was 1:100 (data not shown).

All *F. schultzei* specimens were positive by qPCR with Ct values ranging from 16.05 - 19.65 (fig. 3A; table 1). The test was sensitive to detect adults and young stages of the vector and no positive signal was observed for other species tested. The corresponding dissociation curves of the samples are showed in fig. 3B. The melting temperature (Tm) of the positive samples ranged from 73.50 to 77.50 ºC .

4 DISCUSSION

The small size and cryptic behaviour of thrips make their monitoring and the identification processes difficult. In such a huge and biodiverse country as Brazil, where the Thysanoptera diversity is high, the correct thrips identification can be even more challenging. According to Murai and Toda (2001), in some cases morphological characteristics are also influenced by the environment temperature.

The qPCR assay as described here is rapid, specific and sensitive, easy and quick to perform for identification of young and adults of *F. schultzei*. qPCR assays have been developed for the identification of *T. palmi* (Kox et al. 2005; Walsh et al. 2005) and *F. occidentalis* (Huang et al. 2010).

Although the equipment is expensive, the use of qPCR is rapid and accurate and can be applied in large scale process of identifying species that are found in plants and / or plant material derived from trade. It can also be used with insects caught on sticky traps (Mehle and Trdan 2012). Due to *F. schultzei* prevalence in many countries (Mound, 1996), this method can be essential to the control of plant material from these countries to prevent further spread of this vector in areas where there are not yet or even the entrance of new tospovirus in Brazil transmitted by this species as *Capsicum*

chlorosis virus (CaCV) and *Groundnut bud necrosis virus* (GBNV) (Persley et al. 2006; Lakshmi et al. 1995).

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Table 1 Details of thrips used in this study and results of DNA based methods for molecular identification

Species	Locality	Host plant	Cultivation	Year of collection	Molecular identification (PCR)[b]		
					Direct sequencing	Specific primer	Real -time (ct - value)
Frankliniella schutzei	Presidente Prudente,SP, Brazil	Watermelon	Open field	2012/2013	+	+	16.05
	Marília, SP,Brazil		Open field	2012/2013	+	+	18.13
	Botucatu, SP, Brazil (adult)		Greenhouse[a]	2013/2014	+	nt	18.17
	Botucatu, SP,Brazil (larvae)		Greenhouse[a]	2013/2014	nt	+	16.05
	São Manuel, SP,Brazil		Greenhouse[a]	2013/2014	+	+	17.98
	Borborema, SP, Brazil		Open field	2014	+	+	15.90
	Presidente Prudente, SP, Brazil	Pumpkin	Open field	2013	+	+	14.58
	Botucatu, SP,Brazil	Tomato	Greenhouse	2013/2014	+	+	nt
	São Manuel, SP, Brazil		Greenhouse	2013/2014	+	+	19.65
	Campinas, SP, Brazil	Sweet Pepper	Greenhouse	2014	+	nt	nt
	Campinas, SP, Brazil	Lettuce	Greenhouse	2014	+	+	18.51
	Campinas, SP, Brazil	Cucumber	Greenhouse	2014	+	nt	16.03
	Vitoriana SP, Brazil	*Emilia sonchifolia*	Greenhouse	2012/2013	+	+	17.59
	São Manuel, SP,Brazil	*Tridax procumbens*	Open field	2014	+	nt	nt
F. occidentalis	Andradas, MG, Brazil	Chrysanthemum	Greenhouse	2014	nt	-	31.64
F. occidentalis	Turin, Italy	Sweet Pepper	Greenhouse	2011	nt	-	23.98
F. intonsa	Turin, Italy	Strawberry	Greenhouse	2011	nt	-	23.91
Thrips tabaci	Turin, Italy	Sweet Pepper	Greenhouse	2011	nt	-	29.08

[a]experimental condition. [b](+) positive for *F. schultzei*; (-) negative for *F. schultzei*; nt – not tested;

Table 2 Primers used in this study.

name	sequence 5' – to – 3'	orientation	specificity
mtD-7.2F	ATTAGGAGCHCCHGAYATAGCATT	forward	generic
mtD-9.2R	CAGGCAAGATTAAAATATAAACTTCTG	reverse	generic
F.S_for	ATACCTGCTAAATGAAGGG	forward	*F. schultzei* - specific
F.S_rev	TTCCACCTTCAATAACTTTAC	reverse	*F. schultzei* - specific

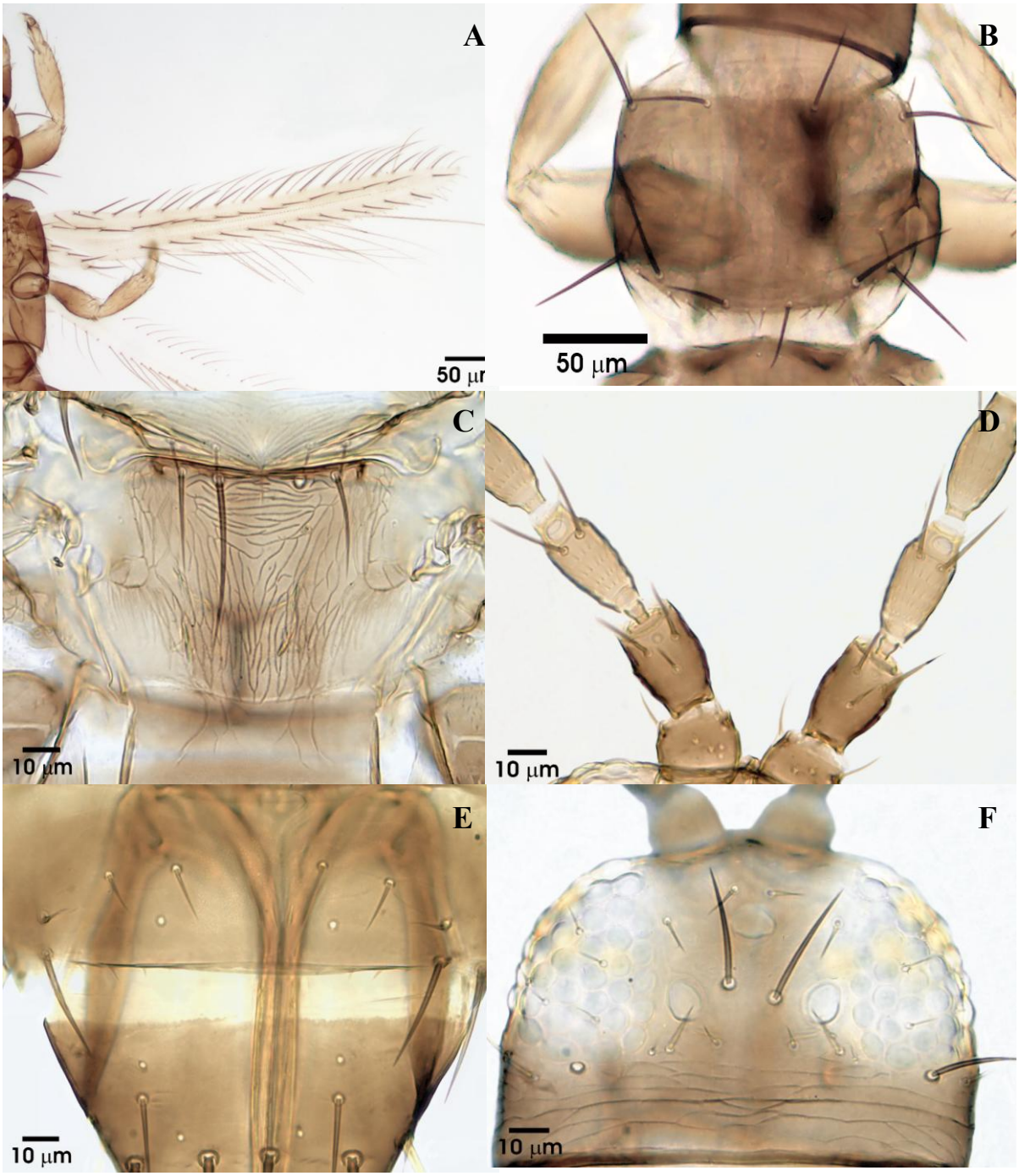

Fig. 1 (A) The fore wing with two complete rows of setae; (B) Prothorax with five pairs of main setae; (C) Absence of companiforme sensilo in the metanotum; (D) Pedicel of the antenna is simple; (E) Abdomen showing a incomplete developed comb; (F) Head showing interocellar setae.

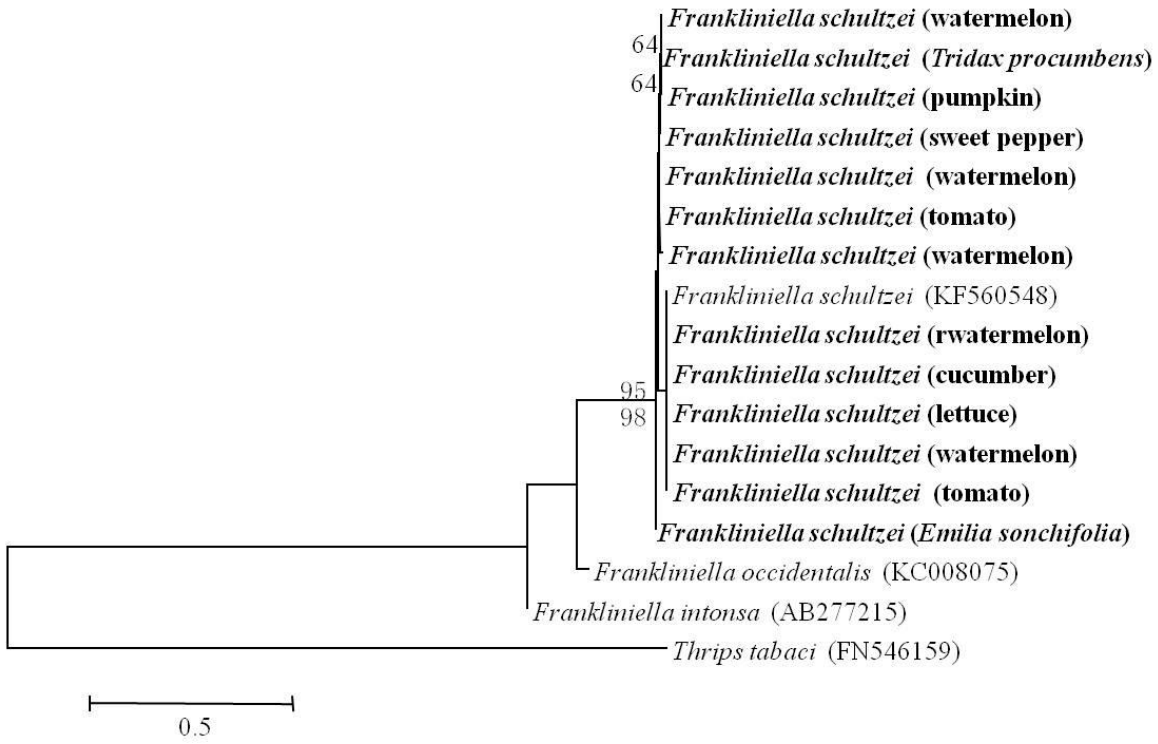

Fig. 2 Phylogenetic relationships of *Frankliniella schultzei* mtCOI gene sequences (450bp) samples collected from different crops and weeds in São Paulo state. The tree was constructed by neighbor-joining method with the MEGA 5.0 software. Numbers at the nodes indicate the frequency of the cluster after bootstrap analysis (2000 replicates). Only bootstrap values above 50% are shown. KF5605488 - *F. schultzei* from USA; KC008075 – *F. occidentalis* from New Zealand; AB2777215 – *F. intonsa* from China; FN546159 – *Thrips tabaci* from USA.

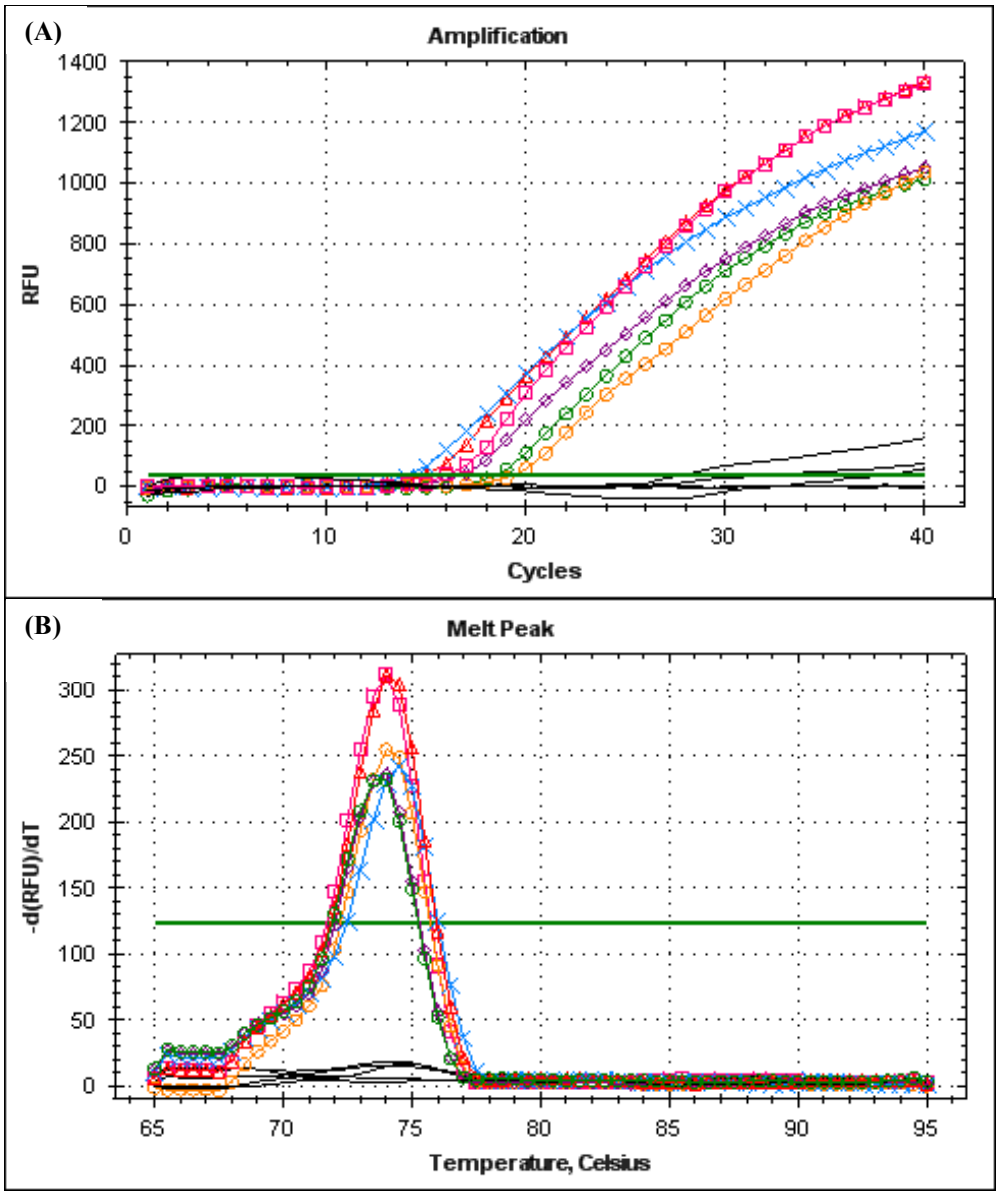

Fig. 3 (A) Specific amplification curve of *F. schultzei*, collected at young stage (larvae - red line) and adult in differents crops (watermelon – pink, purple and green lines; pumpkin – blue; cucumber – orange line). Other four thrips species including *F. occidentalis* (Brazil), *F. occidentalis* (Italy), *F. intonsa* and *Thrips tabaci* did not show any positive signal (black lines). The mean Ct values for each samples are given in a Table. (B) The corresponding dissociation curves of *Frankliniella schultzei.* The melting temperature (Tm) of each amplicon is shown alongside its dissociation curve.

CONCLUSÕES GERAIS

- *Groundnut ringspot virus* infecta melancia e é transmitido pela espécie de tripes *Frankliniella schultzei*;
- qRT-PCR pode ser utilizado para detecção de GRSV em plantas e em tripes individuais;
- Espécies de tripes são eficientemente identificadas pela análise do gene citocromo-oxidase I (mtCOI);
- Primers específicos para o gene mtCOI de *Frankliniella schultzei* podem ser utilizado em ensaios de PCR e real-time PCR para a identificação da espécie;
- *Frankliniella schultzei* é a espécie predominante de tripes nas regiões produtoras de melancia do estado de São Paulo.