RENAN BATISTA QUEIROZ

INTERACTIONS BETWEEN THE CITRUS PATHOGEN '*CANDIDATUS* PHYTOPLASMA AURANTIFOLIA' AND HEMIPTERAN VECTORS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS – BRASIL
2014

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T
Q3i
2014

Queiroz, Renan Batista, 1985-
Interactions between the citrus pathogen '*Candidatus* Phytoplasma aurantifolia' and hemipteran vectors / Renan Batista Queiroz. – Viçosa, MG, 2014.
vii, 72f. : il. (algumas color.) ; 29 cm.

Orientador: Simon Luke Elliot.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Limão - Controle de pragas. 2. *Citrus aurantifolia*. 3. *Diaphorina citri*. 4. Fitoplasma. 5. Bactérias fitopatogênicas. I. Universidade Federal de Viçosa. Departamento de Biologia Animal. Programa de Pós-graduação em Entomologia. II. Título.

CDD 22. ed. 634.337

RENAN BATISTA QUEIROZ

INTERACTIONS BETWEEN THE CITRUS PATHOGEN '*CANDIDATUS* PHYTOPLASMA AURANTIFOLIA' AND HEMIPTERAN VECTORS

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Entomologia, para obtenção do título de *Doctor Scientiae*.

APROVADA: 27 de maio de 2014

_____
Claudine Márcia Carvalho
Coorientadora

_____
Raul Narciso Carvalho Guedes

_____
Emerson Medeiros Del Ponte

_____
Tathiana Guerra Sobrinho

_____
Simon Luke Elliot
Orientador

AGRADECIMENTOS

À Universidade Federal de Viçosa e ao Programa de Pós-Graduação em Entomologia pelas oportunidades e pela qualidade acadêmica e científica.

Ao Instituto Tecnológico Vale por todos os recursos disponibilizados para condução do projeto e pela bolsa de Doutorado Sanduíche (Omã) e ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) pela bolsa de Doutorado (Brasil).

Ao professor Simon Luke Elliot pela orientação, pelas oportunidades apresentadas e por me proporcionar constante aprendizado.

À professora Claudine Márcia Carvalho por toda ajuda, orientação e também pela cessão do Laboratório de Virologia para as análises de biologia molecular dos trabalhos realizados no Brasil.

Ao doutor Fábio Nascimento da Silva por ter me auxiliado durante toda a execução do projeto e pela participação na Banca de Qualificação.

Aos professores Ângelo Pallini, Eliseu Pereira e Raul Guedes por terem participado da Banca de Qualificação.

Aos colegas do Laboratório de Interações Inseto-Microrganismo pela contribuição, discussões científicas, amizade e pelas risadas garantidas nos momentos de descontração.

Aos docentes da UFV, em especial aos professores da Entomologia, pelos ensinamentos repassados desde 2004.

Ao doutor Abdullah Al-Saadi pela orientação durante o Doutorado Sanduíche.

Ao amigo Mr. Issa Hashil Said Al-Mahmooli pela ajuda na coleta de dados em campo e durante os trabalhos no Laboratório de "Plant Pathology" em Omã.

A todos os colegas do Laboratório de "Plant Pathology", em especial ao Mahmood, Hamed, Waleed, Ahmed, Aisha, Shayka e Elham, pela receptividade, passeios pelo país e ajuda durante os onze meses de estadia em Omã.

À Sultan Qaboos University por disponibilizar toda a sua infraestrutura para a execução do trabalho.

Aos produtores rurais em Omã por cederem suas fazendas para condução de parte do projeto.

À minha amada esposa Elaine Nascimento por todo amor, companheirismo, paciência e dedicação à nossa família e, principalmente, por me presentar com o bem mais precioso da minha vida, nosso filho Miguel. TE AMO!

Ao Miguel por todo amor e por me ensinar cada dia mais o valor de ser pai. Obrigado também por cuidar da mamãe quando o papai esteve ausente. Filho, TE AMO PRA LÁ DE MARRAKECH!

À minha família por sempre acreditar e torcer por mim, em especial à minha mãe Leine e ao meu pai Diosmar, que me ensinaram sempre seguir o caminho do bem.

À minha sogra Dona Lia e às minhas cunhadas Li, Fabiana e Flávia por todo carinho.

A todos os amigos pelos momentos de alegria e pela torcida.

Finalmente, a todos aqueles que direta ou indiretamente contribuíram para a realização deste trabalho.

Shukran!

BIOGRAFIA

RENAN BATISTA QUEIROZ, filho de Diosmar Gabriel de Queiroz e Leine Rose Batista Queiroz, nasceu em São Francisco, MG, no dia 22 de novembro de 1985.

No período de 1997 a 1999, cursou o ensino fundamental na Escola Estadual José Bernardino, Icaraí de Minas-MG. Em 2000, concluiu o ensino fundamental na Escola Estadual Dona Alice Mendonça, São Francisco-MG e de 2001 a 2003, cursou o ensino médio no Colégio Indyu, Montes Claros-MG.

Em março de 2004 ingressou no curso de Agronomia na Universidade Federal de Viçosa, onde iniciou seus trabalhos na área de Entomologia Agrícola em 2005 sob a orientação do Prof. Marcelo Coutinho Picanço. Foi bolsista de iniciação científica do PIBIC/CNPq durante dois anos, desenvolvendo pesquisas na área de manejo integrado de pragas de hortaliças, grandes culturas, fruteiras e ornamentais. Foi monitor da disciplina de Manejo Integrado de Pragas do curso de Agronomia durante dois anos.

Em março de 2009, ingressou no curso de Mestrado em Entomologia na UFV, o concluindo em fevereiro de 2011. Em seguida ingressou no curso de Doutorado em Entomologia nesta mesma instituição sob a orientação do Prof. Simon Luke Elliot, desenvolvendo pesquisas na área de interações inseto-microrganismo. Conduziu o projeto "Implementation and sustainability of citrus disease management", no qual a maior parte dele foi desenvolvida em Omã durante o período de 11 meses de doutorado sanduíche. Sua tese foi defendida em 27 de maio de 2014, com nove meses de antecedência, o qual iniciará os trabalhos como Pós-Doutor pelo PNPD (Capes).

SUMÁRIO

RESUMO

QUEIROZ, Renan Batista. D.Sc., Universidade Federal de Viçosa, maio de 2014. **Interações entre o patógeno dos citros '*Candidatus* Phytoplasma aurantifolia' e vetores hemípteros**. Orientador: Simon Luke Elliot. Coorientadores: Claudine Márcia Carvalho e Ângelo Pallini Filho.

O objetivo principal deste trabalho foi obter as informações e ferramentas necessárias para implantar medidas de controle contra a vassoura de bruxa do limão galego (WBDL-sigla em inglês), doença causada por '*Candidatus* Phytoplasma aurantifolia', examinando em especial o papel de insetos vetores. Aqui, nós consideramos os fitoplasmas de limão galego (*Citrus aurantifolia*) no Brasil e em Omã, que estão intimamente relacionados, mas que têm sintomatologias muito diferentes - no Brasil, plantas infectadas são assintomáticas, enquanto em Omã e no Oriente Médio em geral, os sintomas são aparentes, constituindo uma grave ameaça para a citricultura. Em ambos os casos, a infecção de plantas de *Citrus aurantifolia* por '*Ca*. Phytoplasma aurantifolia' teve um efeito positivo no inseto vetor *Diaphorina citri*, como é esperado para patógenos transmitidos de maneira persistente. Isto foi baseado em um estudo sobre o crescimento populacional e a capacidade reprodutiva usando parâmetros obtidos a partir de tabelas de vida de fertilidade, uma vez que estes parâmetros ajudam a entender o crescimento populacional de insetos. A taxa intrínseca de crescimento (r_m) de *D. citri* foi quase o dobro em plantas infectadas por fitoplasma do que plantas não infectadas. Este aumento foi devido a maior taxa de oviposição, uma vez que o tempo de geração e de sobrevivência não foram afetados. Além disso, também investigamos vários aspectos deste sistema em Omã. Nós identificamos duas espécies, *D. citri* e *Hishimonus phycitis*, como insetos vetores de '*Ca.* Phytoplasma aurantifolia' e ambas as espécies estão presente no campo durante todo o ano. Finalmente, também identificamos duas plantas hospedeiras alternativas deste fitoplasma em Omã.

ABSTRACT

QUEIROZ, Renan Batista. D.Sc., Universidade Federal de Viçosa, May 2014. **Interactions between the citrus pathogen '*Candidatus* Phytoplasma aurantifolia' and hemipteran vectors**. Adviser: Simon Luke Elliot. Co-advisers: Claudine Márcia Carvalho and Ângelo Pallini Filho.

The main goal was to obtain the information and tools necessary to implement control measures against Witches' Broom Disease of Lime (WBDL), disease caused by '*Candidatus* Phytoplasma aurantifolia' in Oman, examining in particular the role of vectors. We consider phytoplasmas of acid lime (*Citrus aurantifolia*) in Brazil and in Oman, that are closely related yet that have very different symptomatology – in Brazil, the infected plants are asymptomatic while in Oman and the Middle East in general, the symptoms are apparent and represent a severe threat to the citrus industry. In both cases, infection of *Citrus aurantifolia* plants by '*Ca*. Phytoplasma aurantifolia' had a positive effect on the vector *Diaphorina citri,* as is expected for pathogens transmitted in a persistent manner. This was based on a study of the population growth and reproductive capacity using parameters obtained from fertility life tables, which are an important aid for understanding the insect's population growth. The intrinsic rate of increase (r_m) of this insect was almost doubled on phytoplasma-infected plants than uninfected plants. The increase was due to a tripling of oviposition rates, while generation and survival times were unaffected. Furthermore, we also investigated several aspects of this system in Oman. We identified two species, *Diaphorina citri* and *Hishimonus phycitis*, as insect vectors of '*Ca* Phytoplasma aurantifolia ' and found both species to be present in the field all year round, in four field sites. Finally, we identified two alternative host plants of this phytoplasma in Oman.

GENERAL INTRODUCTION

The transmission and dispersal of plant-pathogenic viruses and bacteria largely depends on arthropod vectors (Stout et al., 2006). This interaction is essential not only for the pathogen's fitness but also for the reproductive success of the insect vector (Elliot et al., 2003; Sugio et al., 2011). The plant-pathogen-vector system may involve direct interactions between the pathogen and the vector, including transmission and dispersal of the pathogen by the vector, the pathogen's replication within the vector, shared host plant and even the pathogen's presence within the vector, which could activate the vector's immune defenses (Jiu et al., 2007). These interactions can significantly affect disease spread and insect vector population dynamics. These effects can be very important from an applied point of view, especially when we consider invasive organisms, asymptomatic infected plants or both. It is known, for example, that phytoplasmas (wall-less bacterial plant pathogens) can cause morphological and physiological changes to their host plants that improve the fitness of the insect vector (MacLean et al., 2011; Sugio et al., 2011; MacLean et al., 2014). Here we consider phytoplasmas of acid lime (*Citrus aurantifolia*) in Brazil and in Oman, which are closely related yet that have very different symptomatology – in Brazil infected plants are asymptomatic, while in Oman and the Middle East in general, symptoms are apparent and represent a severe threat to the citrus industry. Among the potential insect vectors of this phytoplasma is *Diaphorina citri* (Hemiptera: Psyllidae), an invasive insect that acts as a vector for the bacteria *Candidatus* Liberibacter spp. that cause another devastating citrus disease, Huanglongbing.

Phytoplasmas are transmitted in a persistent manner, as occurs with some plant viruses (see below), by hemipterans (leafhoppers, planthoppers and psyllids)

(Weintraub & Beanland, 2006). Phytoplasma are phytopathogenic bacteria without cell walls that inhabit the phloem vessels of plants and can cause a range of crop diseases (Hogenhout et al., 2008b; Sugio et al., 2011). Symptoms of infection can include production of small shoots in so-called "witches' broom", floral organs changes structures such as leaves, leaf or shoots chlorosis, sterility of flowers, shortening of internodes and total necrosis of the plant (Bertaccini, 2007; Sugio et al., 2011). These morphological changes may attract more insects that may prefer to feed and lay their eggs in parts infected by the pathogen (Hogenhout et al., 2008b; Sugio et al., 2011).

Phloem-feeding insects acquire phytoplasmas passively during feeding on the phloem of infected plants. The time required to acquire the phytoplasma is known as the acquisition access period. This period is quite variable and it may be a few minutes to many hours - the longer this period, the greater the chance of acquisition (Purcell, 1982). The time between acquisition and the ability to transmit the pathogen is the latent period and can vary between 7 and 80 days (Powell et al., 1995; Murral et al., 1996). During the latent period the phytoplasmas circulate in the hemolymph, where they may infect other tissues and replicate within the vector's body (Lherminier et al., 1990; Lefol et al., 1994; Nakashima & Hayashi, 1995; Kawakita et al., 2000). The insect vector is only capable of transmitting the phytoplasma when the pathogen reaches to the salivary gland (Hogenhout et al., 2008b).

As phytoplasmas, the majority of described plant viruses are transmitted by hemipteroid insects that include aphids, whiteflies, leafhoppers, planthoppers and thrips (Hogenhout et al., 2008a). In the plant-virus-vector system it is more common to have a beneficial effect on the insect vector when the virus is transmitted in a persistent manner (Mauck et al., 2012). In persistent transmission (PT), insects can inoculate the acquired virus for much longer periods (days/week), transmitting the virus after molting and

often for their entire lifespan (Hogenhout et al., 2008a). For the pathogen, benefits arise through the enhanced reproduction of the vectors (or their accelerated juvenile development), on infected plants. This eventually leads to large numbers of infected vectors (Gildow, 1980; Gildow, 1983; Zhang et al., 2000; Muller et al., 2001).

Meanwhile, in non-persistent transmission (NPT), insects are able to inoculate the virus into uninfected plants only a few minutes after acquisition and the insect loses the virus within a few minutes or after molting (Hogenhout et al., 2008a). The dissemination of the viruses in a non-persistent manner takes place when the vectors quickly examine infected plants and may then disperse before beginning the long-term feeding. These viruses can reduce host quality and palatability to the insect, meaning that vectorborne dispersal can occur rapidly after acquiring the virus (Mauck et al., 2012).

Initially, the relationship between these vectorborne pathogens and their vectors was generally considered to be commensal (Wijkamp et al., 1996; Roca et al., 1997), although some studies did show harm to vectors arising from infection (Costa et al., 1993; DeAngelis et al., 1993). From an evolutionary point of view, the pathogen's use of the vector as a resource may damage the vector, in a virulence-transmission trade-off (Elliot et al., 2003; Alizon et al., 2009). More recently, it has become clear that in many systems this relationship is beneficial not just for the pathogen, but also for the vector (Belliure et al., 2005; Colvin et al., 2006; Stout et al., 2006). The literature has been synthesized by Mauck et al. (2012), who hypothesized that persistently and non-persistently transmitted viruses will exhibit different effects on aspects of host phenotypes that can mediate vector attraction to, arrestment on and dispersal from infected plants. In a metanalysis of the literature, they found that vectors prefer to settle on plants infected with persistently-transmitted viruses, while non-persistently

transmitted viruses generally either have no effect on vector settling and feeding or cause infected plants to be less preferred than healthy plants.

Here, we hypothesize that infection of *Citrus aurantifolia* plants by '*Ca*. Phytoplasma aurantifolia' has a positive effect on its insect vector since that this pathogen is transmitted via persistent manner. The study of the population growth and fitness was based on parameters obtained from fertility life tables as these are an important aid for understanding the insect's population dynamics (Carey, 1993). We also investigated various aspects of this system in Oman, for applied reasons we now discuss.

Applied aspects of the study systems

Invasive species are one of the greatest challenges for most ecosystems in the world causing major economic losses (Bennett, 2013). Biological invasions can increase the possibility of novel species interactions, notably between plants, pathogens and insects (Richardson et al., 2000). The Asian citrus psyllid (*Diaphorina citri*) (Hemiptera: Psyllidae) is an invasive species considered nowadays as the main pest of citrus in the world. This is because this insect is the vector of phloem-limited bacteria *Candidatus* Liberibacter spp. that causes citrus greening or Huanglongbing (HLB) (Halbert & Manjunath, 2004; Bové, 2006).

Meanwhile in the Middle East (specifically Iran, UAE and Oman), the phytoplasma '*Ca*. Phytoplasma aurantifolia' causes Witches' Broom Disease of Lime (WBDL) (see below). The main insect vector candidate is the leafhopper *Hishimonus phycitis* (Hemiptera: Cicadellidae: Deltocephalinae). This was identified first in 1991 in Oman, then in 1993 in the UAE, and in 2000 in Iran (Bové et al., 2000). However, there

are no conclusive studies demonstrating whether it is, in fact, a vector of this phytoplasma.

The management methods most commonly used to control insect vectors are insecticide spraying and removal of symptomatic plants (Hogenhout et al., 2008b; Weintraub & Wilson, 2010). However, if asymptomatic plants can increase vector fitness (and thus its population growth and the number of potential future vectors), these methods may be ineffective for disease control. Additionally, conventional insecticides, even when frequently used will not control the appearance of disease as there is often a constant influx of new vectors from surrounding habitats (Wally et al., 2004). For example, Mori et al. (2008) studied the effects of insecticides applied to the central canopy of grapevines in 18 vineyards in reducing *H. obsoletus* populations and found that there was no significant reduction in vector populations. This was probably due to the fact that *H. obsoletus* prefers other plants and is only incidentally found on grapevine.

The use of pathogen-free propagation material is the first step for the effective strategy to control vectorborne diseases. For this it is necessary to acquire certified seedlings produced from protected cultivation. Plant resistance is the most promising approach for phytoplasmas control. However, there are few studies with consistent data on the response of the host, on the host-pathogen interaction and on the anatomical, physiological and molecular bases of resistance (Seemüller & Harries, 2010).

Although some methods of phytoplasma control do exist, the management of both the disease and the insect vectors is in an early phase since most studies of phytoplasmas concern their identification and characterization. Thus, an aim of the present study is to obtain some of the information and tools necessary to implement control measures against WBDL in Omani citrus. This disease is responsible for the

decline in acid lime (*Citrus aurantifolia*) production in Oman and is estimated to affect 98% of acid lime trees in the country (Chung et al., 2006), so it a challenging problem.

Oman is characterized by a coastal plain along the Arabian Sea and Gulf of Oman. The interior of the country has a mountainous region and also an area with sand, a typical desert. Oman is comprised of five distinct regions and three governorates (Figure 1), of which Al-Batinah region (south and north) and Dhofar Governorate (Salalah) stand out in terms of agriculture.

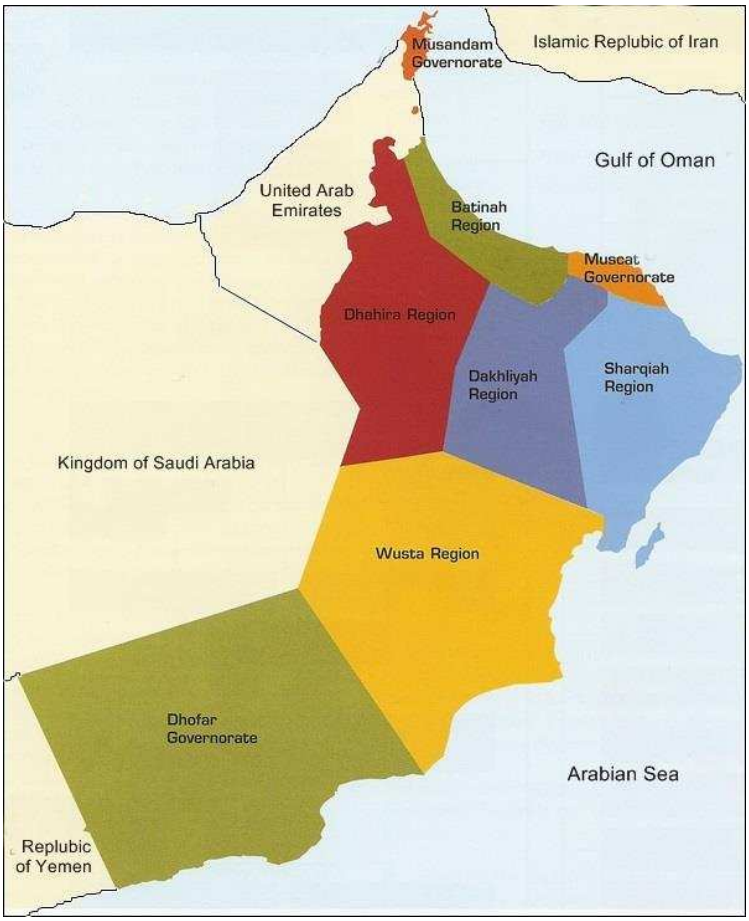

Figure 1. Map of the Regions and Governorates in Oman.

Farm holdings vary from less than 0.4 ha to more than 84 ha. Those less than 1.26 ha are about 11% of total farm holdings; those between 1.26 to 12.60 ha are 65%, while those greater than 12.6 ha are about 24% (MAF, 2011) (Figure 2).

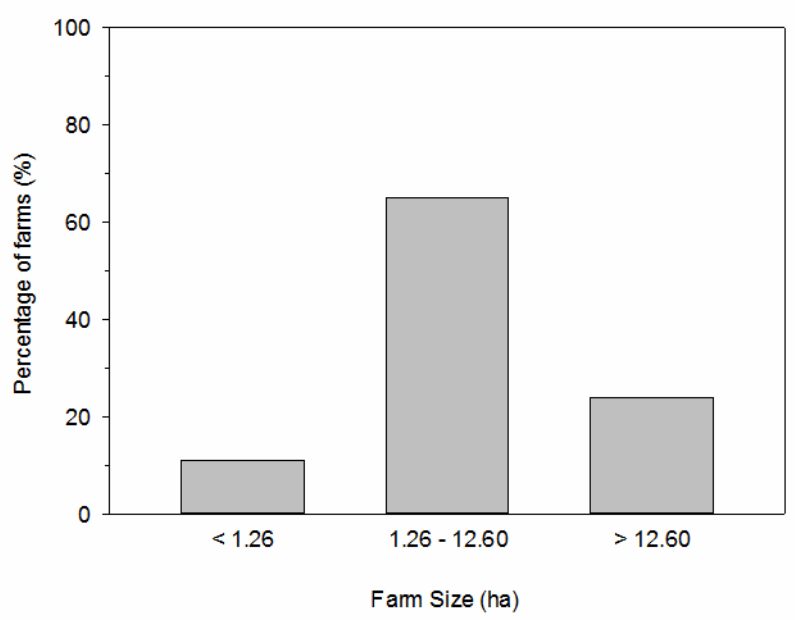

Figure 2. Percentage of farms (%) according to their size in hectares (ha).

The farms are classified as "traditional" and "conventional". The former are characteristically under mixed cropping regimes (mango, dates, citrus, vegetables and pasture/fodder crops). The latter have these same crops, but grown separately, with each one occupying a different space in the farm. All the farms are irrigated and most of the water for irrigation is obtained by Falaj system, in which a vertical shaft is dug from the surface to reach water in porous rock. Currently, most of the agricultural products are imported because the country does not have favorable conditions for agriculture, mainly due an increase of the salinity, significantly reducing the water quality. Thus, agriculture in Oman currently is more for subsistence.

Aims

Nascimento et al. (*submitted paper*) recently found Brazilian acid lime to be infected by a phytoplasma closely related with the Omani '*Ca*. Phytoplasma aurantifolia'. The most interesting observation is that the infected trees in Brazil showed no WBDL typical symptoms. We thus wished to test the hypothesis that *D. citri*, besides being able to vector '*Ca*. Liberibacter asiaticus', would also be affected by these asymptomatic infections. In Oman, we wished to test the same hypothesis but in symptomatic trees. Further, we wished to recognize the vector capacity of both *D. citri* and *H. phycitis*. The development of management strategies could be made feasible if there were periods in the year where vector abundance was very low, or when there was very little transmission. We thus examined these possibilities, and also the potential for weeds to act as field reservoirs for the pathogen in Oman.

The following is the structure of my thesis.

Chapter One is an empirical study whose aim was to test the hypothesis that the performance of the invasive species *D. citri* is greater on phytoplasma-infected, yet asymptomatic, plants of *C. aurantifolia* than on healthy plants. We show that phytoplasma infection increases *D. citri* reproduction, specifically increasing oviposition (*versus* survival for example). This is the first time that this phenomenon has been shown on asymptomatic plants and we discuss its practical implications. These include potentially favor the spread of the invasive (both insects and pathogens) and the suggestion that the increase in herbivorous insects on the plant is itself a disease symptom.

Chapter Two was carried out in Oman. We examined the potential for transmission of '*Ca*. Phytoplasma aurantifolia' to *C. aurantifolia* by *H. phycitis* and *D. citri*. Both species were able to transmit this pathogen to acid lime plants, although *H. phycitis* was an order of magnitude considerably more efficient. The practical importance of this difference may be limited, however, as we show that, as in Brazil, *D. citri* reproduction is also greatly increased on infected (here, symptomatic) plants. (It was not possible to test this on *H. phycitis* for logistical reasons).

In **Chapter Three,** we monitored populations of both insect vectors in the field. We also sought to understand the inoculum pressure in the field, using uninfected "sentinel" plants and to determine if weed species could act as reservoirs for the phytoplasma. The results were not particularly encouraging for phytoplasmas management as there were no periods in the year when fields were free of the vectors. Sentinel plants were also infected in all study areas, although these data are still being collected. Finally, we identified two weed species that can act as reservoirs for the phytoplasma.

In a final section, I list some perspectives on the study systems and disease management, including the role of entomological studies in this.

References

Alizon S, Hurford A, Mideo N & van Baalen M (2009) Virulence evolution and the trade-off hypothesis: history, current state of affairs and future. J. Evol. Biol. 22: 245-259.

Belliure B, Janssen A, Maris PC, Peters D & Sabelis MW (2005) Herbivore arthropods benefit from vectoring plant viruses. Ecol Lett 8: 70-79.

Bennett AE (2013) Can plant–microbe–insect interactions enhance or inhibit the spread of invasive species? Functional Ecology 2013, 27, 27: 661-671.

Bertaccini A (2007) Phytoplasmas: diversity, taxonomy, and epidemiology. Front. Biosci. 12: 673-689.

Bové JM (2006) Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. J Plant Pathol 88: 7-37.

Bové JM, Danet JL, Bananej K, Hsaanzadeh N, Taghizadeh M, Salehi M & Garnier M (2000) Witches' Broom Disease of Lime (WBDL) in Iran: Proceeding 14th conference of IOCV (ed., Riverside, CA, pp. 207-212.

Carey JR (1993) Applied demography for biologists with special emphasis on insects. Oxford University Press, New York.

Chung KR, Khan IA & Brlansky RH (2006) Citrus diseases exotic to Florida: Witches' Broom Disease of Lime (WBDL). EDIS publication, University of Florida, Gainesville.

Colvin J, Omongo CA, Govindappa MR, Stevenson PC, Maruthi MN & al. e (2006) Host-plant viral infection effects on arthropod-vector population growth, development and behaviour: management and epidemiological implications. Adv Virol Res 67 67: 419-452.

Costa HS, Brown JK, Sivasupramaniam S & Bird J (1993) Regional distribution, insecticide resistance, and reciprocal crosses between the A and B biotypes of *Bemisia tabaci*. Insect Sci Appl 14: 255-266.

DeAngelis JD, Sether DM & Rossignol PA (1993) Survival, development, and reproduction in western flower thrips (Thysanoptera: Thripidae) exposed to *Impatiens necrotic spot virus*. Environ Entomol 22: 1308-1312.

Elliot SL, Adler FR & Sabelis MW (2003) How virulent should a parasite be to its vector? Ecology 84: 2568-2574.

Gildow FE (1980) Increased production of alatae by aphids reared on oats infected with Barley yellow dwarf virus. Annals of the Entomological Society of America 73: 343-347.

Gildow FE (1983) Influence of Barley yellow dwarf virus-infected oats and barley on morphology of aphid vectors. Phytopathology 73: 1196-1199.

Halbert SE & Manjunath KL (2004) Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: A literature review and assessment of risk in Florida. Fla Entomol 87: 330-353.

Hogenhout SA, Ammar E, Whitfield AE & Redinbaugh MG (2008a) Insect vector interactions with persistently transmitted viruses. Annual Review of Phytopathology 46: 327-359.

Hogenhout SA, Oshima K, El-Desouky A, Kakizawa S, Kingdom HN & Namba S (2008b) Phytoplasmas: bacteria that manipulate plants and insects. Molecular Plant Pathology 9: 327-359b.

Jiu M, Zhou X-P, Tong L, Xu J, Yang X, Wan F-H & Liu S-S (2007) Vector-virus mutualism accelerates population increase of an invasive whitefly. PLoS ONE 2: e182.

Kawakita H, Saiki T, Wei W, Mitsuhashi W & Watanabe K (2000) Identification of mulberry dwarf phytoplasmas in the genital organs and eggs of leafhopper *Hishimonoides sellatiformis*. Phytopathology 90: 909-914.

Lefol C, Lherminier J, Boudon-Padieu E, Larrue J & Louis C (1994) Propagation of Flavescence dorée MLO (mycoplasmalike organism) in the leafhopper vector *Euscelidius variegatus* Kbm. J. Invertebr. Pathol. 63: 285-293.

Lherminier J, Prensier G, Boudon-Padieu E & Caudwell A (1990) Immunolabeling of grapevine Flavescence dorée MLO in salivary glands of *Euscelidius variegatus*: a light and electron microscopy study. Histochem. Cytochem 38: 79-86.

MacLean AM, Orlovskis Z, Kowitwanich K, Zdziarska AM, Angenent GC, Immink RGH & Hogenhout SA (2014) Phytoplasma Effector SAP54 Hijacks Plant Reproduction by Degrading MADS-box Proteins and Promotes Insect Colonization in a RAD23-Dependent Manner. PLoS Biol 12: e1001835.

MacLean AM, Sugio A, Makarova OV, Findlay KC, Grieve VM, Tóth R, Nicolaisen M & Hogenhout SA (2011) Phytoplasma Effector SAP54 Induces Indeterminate Leaf-Like Flower Development in *Arabidopsis* Plants. Plant Physiolog 157: 831-841.

MAF (2011) The state of animal genetic resources in the Sultanate of Oman: DGLR (Al-Rumais, Oman), Oman.

Mauck K, Bosque-Pérez NA, Eigenbrode SD, De Moraes CM & Mescher MC (2012) Transmission mechanisms shape pathogen effects on host-vector interactions: evidence from plant viruses. Functional Ecology 26: 1162-1175.

Mori N, Pavan F, Bondavalli R, Reggiani N, Paltrinieri S & Bertaccini A (2008) Factors affecting the spread of 'Bois Noir' disease in north Italy vineyards. Vitis 47: 65-72.

Muller CB, Williams IS & Hardie J (2001) The role of nutrition, crowding and interspecific interactions in the development of winged aphids. Ecological Entomology 26: 330-340.

Murral DJ, Nault LR, Hoy CW, Madden LV & Miller SA (1996) Effects of temperature and vector age on transmission of two Ohio strains of aster yellows phytoplasma by the aster leafhopper (Homoptera: Cicadellidae). J. Econ. Entomol. 89: 1223-1232.

Nakashima K & Hayashi T (1995) Multiplication and distribution of rice yellow dwarf phytoplasma in infected tissues of rice and green rice leafhopper *Nephotettix cincticeps*. Phytopathol. Soc. Jpn. 61: 451-455.

Powell KS, Gatehouse AMR, Hilder VA, van Damme EJM & Peumans WJ (1995) Different antimetabolid effects of related lectins towards nymphal stages of *Nilaparvatha lugens*. Entomol. Exp. Appl. 75: 61-65.

Purcell AH (1982) Insect vector relationships with prokaryotic plant pathogens. Annu. Rev. Phytopathol. 20: 397-417.

Richardson DM, Allsopp N, D'Antonio CM, Milton SJ & Rejmanek M (2000) Plant invasions – the role of mutualisms. Biological Reviews 75: 65-93.

Roca E, Aramburu J & Moriones E (1997) Comparative host reactions and *Frankliniella occidentalis* transmission of different isolates of tomato spotted wilt tospovirus from Spain. Plant Pathol. 46: 407-415.

Seemüller E & Harries H (2010) Plant Resistance: Phytoplasmas: Genomes, Plant Hosts and Vectors (ed. by PG Weintraub & P Jones) CABI, Wallingford, UK, pp. 147-169.

Stout MJ, Thaler JS & Thomma BPHJ (2006) Plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods. Annu Rev Entomol. 51: 663-689.

Sugio A, Kingdom HN, MacLean AM, Grieve VM & Hogenhout SA (2011) Phytoplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis. PNAS 108: 1254-1263.

Wally O, Daayf F, Khadhair AH, Adam L, Elliott B, Shinners-Carnelley T, Iranpour M, Northover P & Keyworth S (2004) Incidence and molecular detection of yellows-type disease in carrots, associated with leafhoppers in southern Manitoba, Canada. Canadian Journal of Plant Pathology 26: 498-505.

Weintraub PG & Beanland L (2006) Insect vectors of phytoplasmas. Annu Rev Entomol 51: 91-111.

Weintraub PG & Wilson MR (2010) Control of Phytoplasma Diseases and Vectors: Phytoplasmas: Genomes, Plant Hosts and Vectors (ed. by PG Weintraub & P Jones) CABI, Wallingford, UK, pp. 233-249.

Wijkamp I, Goldbach R & Peters D (1996) Propagation of tomato spotted wilt virus in *Frankliniella occidentalis* does neither result in pathological effects nor in transovarial passage of the virus. Entomol Exp Appl 81: 285-292.

Zhang XS, Holt J & Colvin J (2000) A general model of plant-virus disease infection incorporating vector aggregation. Plant Pathology 49: 435-444.

CHAPTER 1

Enhanced Population Growth of the Invasive Psyllid *Diaphorina citri*, Vector of Huanglongbing, on Asymptomatic Phytoplasma-Infected Citrus

Renan B. Queiroz[1], Gabriel A. Vieira[1], Fábio N. Silva[2], Felipe V. Prado[1], Issa H. Al-Mahmooli[3], Abdullah M. Al-Sadi[3], Claudine M. Carvalho[2], Simon L. Elliot[1,4]

[1]Department of Entomology, Universidade Federal de Viçosa, Viçosa, MG, 36570–900, Brazil

[2]Department of Phytopathology, Universidade Federal de Viçosa, Viçosa, MG, 36570–900, Brazil

[3]Department of Crop Sciences, College of Agricultural and Marine Sciences, Sultan Qaboos University, PO Box 34, Al-Khod 123, Oman

[4]E-mail: selliot@ufv.br

Abstract

It is known that prokaryotic and viral plant pathogens can indirectly enhance population growth of their vectors via effects on the host plants. Where the pathogen and/or its vector are invasive, this may increase their potential to spread on a geographical scale. Here we show for the first time that a completely asymptomatic infection (in this case *Candidatus* Phytoplasma aurantifolia 16SrII-C in acid lime, *Citrus aurantifolia*, in Brazil) can also cause an increase in vector population growth. The psyllid *Diaphorina citri* (Hemiptera: Psyllidae) is a major pest of citrus mainly due to its status as vector of the phloem-limited bacteria *Candidatus* Liberibacter spp. that cause Huanglongbing. This insect is invasive on more than one continent. With fertility life tables, we show that the intrinsic rate of increase (r_m) of *D. citri* is almost doubled on asymptomatic phytoplasma-infected plants versus uninfected plants. This was due to a tripling of oviposition rates, while generation and survival times were unaffected. Given the importance of the citrus industry in Brazil and the threat posed by *D. citri* and Huanglongbing, the consequences of this latent infection on the spread of vector and disease are of critical importance and we propose that there may be many other latent infections of plants that affect herbivore population dynamics.

Keywords: Invasive species, latent infections, plant pathogens, phytoplasmas, insect vectors, *Citrus aurantifolia*, fertility life table, intrinsic rate of increase.

Introduction

Invasive species represent one of mankind's greatest challenges, causing severe problems in natural and managed ecosystems, including agroecosystems, where economic losses can be severe (Bennett, 2013). Invasive insect vectors can be particularly problematic if they carry disease with them, even more so if they generate new host-pathogen associations (as in emergent diseases, for example). Notable examples of invasive vector insects are *Aedes aegypti* (Diptera: Culicidae), that vectors dengue virus (Gubler, 1989; Kyle & Harris, 2008) and the whitefly *Bemisia tabaci* (Hemiptera: Aleyrodidae) biotype B, now classified as a species/group Middle East-Asia Minor 1 species (MEAM1) (Dinsdale et al., 2010). The latter has spread across tropical and subtropical regions of the world since the 1980's (Oliveira et al., 2001; Perring, 2001) and is a particular problem as geminiviruse vector between plants – the polyphagous nature of this insect facilitates rapid dissemination of geminiviruses and their evolution through recombination (Power, 2000).

It has been proposed that plant pathogens that have a persistent relationship with their host plant would be selected to favor their vector's development on the plant, ultimately leading to many more vectors for the pathogen's dispersal (Elliot et al., 2003; Mauck et al., 2012). Evidence to support this is accumulating (Hogenhout et al., 2008a; Mauck et al., 2010; Mann et al., 2012; McMenemy et al., 2012) and some of the mechanisms are being elucidated (MacLean et al., 2011; Sugio et al., 2011; MacLean et al., 2014). In the case of whiteflies, the link between this phenomenon and the geographical spread of plant diseases has also been made (Jiu et al., 2007; Guo et al., 2010; Guo et al., 2012).

Here, we study the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), an invasive species and a major pest of citrus. Its pest status is largely because it is a vector of the phloem-limited bacteria *Candidatus* Liberibacter spp., which causes the Huanglongbing (HLB) disease (Bové, 2006; Hall et al., 2012). Until recently, the Americas were free of this disease, but in March 2004 and August 2005, symptoms of the disease were observed in two of the main citrus growing regions in the world, in the São Paulo State, Brazil, and in Florida, USA, respectively (Bové, 2006). HLB represents a threat to world production of citrus, especially in areas in which *D. citri* is prevalent. Moreover, in 2008 a new agent was identified in orange trees with HLB symptoms that were negative for the presence of *Ca*. Liberibacter spp; it was a phytoplasma with a 99% similarity with Pigeon pea witches'-broom phytoplasma belonging to the 16Sr IX group (Teixeira et al., 2008).

Phytoplasma are wall-less prokaryotes that survive and replicate intracellularly inside both insect and plant hosts (MacLean et al., 2011). They cause serious diseases in different crops, including citrus. '*Candidatus* Phytoplasma aurantifolia' is the causal agent of witches' broom disease of lime (WBDL), which is the most serious disease of acid lime (*Citrus aurantifolia* Swingle) in the Middle East. Although the phytoplasma belonging to 16SrII-C, closely related with '*Ca*. Phytoplasma aurantifolia', has been recently observed in acid lime trees in Brazil (Nascimento et al., *submitted paper*), no symptoms of WBDL were observed in Brazil until now. The main means of phytoplasma dispersal is by its insect vector which acquires this pathogen passively during feeding in infected plants (Weintraub & Beanland, 2006). Recent studies made in Oman have also provided evidence that *D. citri* is a potential vector of '*Ca.* Phytoplasma aurantifolia' (Chapter 2). These data are from BLAST analysis with

amplified PCR products using the primers R16F2n/R16R2 (Deng & Hiruki, 1991), which relative clones showed 99% identity with '*Ca*. Phytoplasma aurantifolia'.

Diaphorina citri has a high reproductive capacity, and can reach up to nine generations in a year (Liu & Tsai, 2000). Thus, studies of life table are important because they enable us to elucidate with more details the main parameters related with insect fitness (Morais et al., 2010). Constructing life tables is an important aid for understanding an insect's population growth (Carey, 1993). Fertility life tables are able to express the main characteristics of the specific mortality in each age (Rabinovich, 1978), and the reproductive capacity of a population (Price, 1997).

The aim of this study was to test the hypothesis that performance of the invasive species *D. citri* is greater in phytoplasma-infected *C. aurantifolia* (Swingle) than on healthy plants. In Brazil, even phytoplasma-infected citrus plants do not show Witches' broom disease of lime (WBDL), characteristic symptoms of '*Ca.* Phytoplasma aurantifolia' which is closely related with the phytoplasma found in Brazil (Nascimento et al., *submitted paper*). Thus, we also evaluated the morphological and physiological characteristics of plants in an effort to detect symptoms that may not be immediately apparent.

Materials and Methods

Plant material

Acid lime plants (*C. aurantifolia*) were acquired from a citrus nursery located in Dona Euzébia, Minas Gerais, southeastern Brazil. The seedlings had been produced in an unprotected environment and were grafted on Rangpur (*C. limonia* Osb.). They were transplanted to 10 liter pots of soil. After transplanting, each pot received 15g of 10-10-10 mineral fertilizer (N-P-K) every 15 days and these seedlings were irrigated daily.

Leaf samples were collected from 40 seedlings of *Citrus aurantifolia*, acquired from a citrus nursery, showing no typical symptoms of phytoplasma infection. Total DNA was extracted from fresh leaf midribs using the NucleoSpin Plant II Kit (Macherey-Nagel) according to the manufacturer's recommendation. Extracted nucleic acids were used as templates for direct PCR with the primers IMP3F/IMP3R. This primer is specific because it amplifies DNA fragments of the gene encoding the immune-dominant membrane protein of WBDL phytoplasma (Askari et al., 2011). The temperature profile for the direct PCR consisted of a first denaturation at 94ºC for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. This was followed by a final extension cycle at 72°C for 3 min.

The amplification products were analyzed by 0.8% agarose gel electrophoresis in 0.50X TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.3) buffer followed by staining with GelRed™ Nucleic Acid Stain and visualized with a UV transilluminator L-Pix (Loccus Biotecnologia, São Paulo, Brazil).

After PCR analyses, we mounted two treatments, which one consisted of ten phytoplasma-infected and another of ten uninfected plants of *C. aurantifolia* based on results from the use IMP3F/IMP3R primers. The experiment was carried out in randomized blocks.

Insect rearing

The rearing of the Asian citrus psyllid (*Diaphorina citri* Kuwayama) was initiated one year prior to the experiments, using adult insects collected from a citrus orchard of the Department of Crop Science at the Federal University of Viçosa, Minas Gerais, Brazil. The insects were reared on 30-40cm tall acid lime seedlings in gauze

cages (50 x 50 x 50 cm). These seedlings were produced from pathogen-free seeds in the greenhouse. Note that these seedlings used for insect rearing were different from those acquired from a citrus nursery.

Fertility life table

The life table studies were carried out in a greenhouse. The air temperature and relative humidity were not controlled but were monitored daily during the experiment. Mean environmental conditions were: relative humidity 61.3±6.1 % and maximum, minimum and mean temperature at 40.3±5.0, 18.2±1.7 and 29.3±2.7 °C, respectively.

Five couples of *D. citri* were placed on each plant in gauze bags (45 x 23 cm) for an oviposition period of 24 hours. After that, adults were removed and the eggs were counted on each plant. Eggs and nymphs of first and second instars were counted with a hand magnifying glass at 10x magnification. Individual insects were evaluated daily for development and survival. The exuviae were used to determine moulting.

Newly emerged adults were collected and sexed individually under the stereomicroscope (the female's ovipositor is visible). The couples were separately placed on the plants for oviposition as described above. Evaluations were made daily until the last female died (approximately 100 days).

To construct the fertility life table we used the following components:

x = age (days);

m_x = fecundity (number of female offspring per female in interval of age x);

l_x = survival (number of surviving females in the beginning of each x);

With these components we calculated the following parameters as described by Carey (1993):

r_m = Intrinsic rate of increase ($r_m = \ln(R_0)/T$);

R_0 = Net reproductive rate ($R_0 = \sum l_x m_x$);

T = Generation time (days) ($T = \ln(R_0)/r_m$);

DT = Necessary time for the population doubling (days) ($DT = \ln(2)/r_m$).

All data obtained of the life table were from nine phytoplasma-infected and seven healthy *C. aurantifolia* plants. This was because in some plants the insects did not complete their life cycle, not allowing, in this way, the calculation of the parameters for the life table from these plants.

Morphological and physiological characteristics of *Citrus aurantifolia*

We evaluated some morphological and physiological characteristics those 20 plants used on the life table experiment since they had no symptoms of phytoplasma infection. To estimate the leaf area, we counted all leaves of the plants and measured the length (L) and largest width (W) of 10% them. The measurements of L and W were multiplied by the coefficient 0.72 and then by the total number of leaves, as described by Coelho Filho et al. (2005). The numbers of flowers and fruits were counted, while the stalk diameter was measured just above the grafting union. Chlorophyll contents (*a*, *b* and *total*) were obtained using the portable meter SPAD-502 (Minolta Camera Co., Osaka, Japan). The measurements were carried out between 9 and 11a.m. from five mature leaves in different parts of each plant.

Data analyses

The survival curves (l_x) were obtained using Kaplan-Meier survival distributions (Crawley, 2007) with the aid of the 'survival' package (Lumley, 2011). Fertility tables were constructed according to the procedure described by Carey (1993). Analyses of variance (ANOVA) were conducted for the biological variables from the life tables,

morphological and physiological characteristics in phytoplasma-infected and healthy plants. The cumulative number of eggs/female of *D. citri* in phytoplasma-infected and healthy plants was analyzed using linear mixed-effects models with repeated measures. In this model was considered the treatments (phytoplasma-infected and healthy plants) as fixed effects and the time as random effect. This data were transformed by log(x+1) and fitted to negative binomial because they showed overdispersion. The assumptions of normality and homoscedasticity were tested before data analyses using Shapiro-Wilk's normality test and Bartlett's test, respectively. Fertility life table was constructed in randomized blocks. However, preliminary analyses did not show blocks effect. Thus, all the posterior analyses were done without blocks. All analyses were performed using software R version 2.13.0 (R Development Core Team, 2014).

Results

Survival analyses

The survival of *D. citri* were not different in phytoplasma-infected and healthy plants (survival curves obtained using Kaplan-Meier estimators; Log-rank test: χ^2 =0.06, df=1, p=0.80). The survival time mean was approximately 77 and 80 days to phytoplasma-infected and healthy plants, respectively (Figure 1). This species showed a low mortality rate in the early stages and it had a higher mortality around the 80th day in both treatments.

Fertility life table

The intrinsic rate of increase of *D. citri* (r_m) ($F_{1,14}$=26.37, p<0.001) was approximately higher in phytoplasma-infected plants than on healthy plants (Figure 2).

This is the main parameter from fertility life tables because it represents how much the population increased during a generation.

The net reproductive rate (R_0) ($F_{1,14}$=42.87, p<0.001) was higher in phytoplasma-infected plants than on healthy plants (Figure 3A). This parameter represents the number of females generate by a single female in a determined period of time. As we evaluated the life table daily, these values represent the number of females generated per day.

There was significant difference among the curves of cumulative number of eggs/female (χ^2 =379.02, df=1, p < 0.0001) (Figure 3B). Oviposition started around 30 days after the beginning the experiment, until around 70 days. The average number of eggs per female of *D. citri* in phytoplasma-infected plants was around 270 during the life cycle, while the mean in healthy plants was about 80 eggs.

There was no significant difference in the generation time (T) ($F_{1,14}$ =0.10, p=0,753) of *D. citri* on healthy and phytoplasma-infected plants of *C. aurantifolia* (Figure 4A). The generation time was around 45 days in both treatments. However, the population doubling time (DT) was two times higher on healthy plants than on phytoplasma-infected plants ($F_{1,14}$ =18.43, p<0.001) (Figure 4B).

Morphological and physiological characteristics of *Citrus aurantifolia*

There was no significant difference among the morphological (leaves/branch, leaf area, stalk diameter, fruits/branch and flowers/branch) and physiological (chlorophyll *a*, *b* and *total*) characteristics in phytoplasma-infected and healthy plants of *C. aurantifolia* (Table 1).

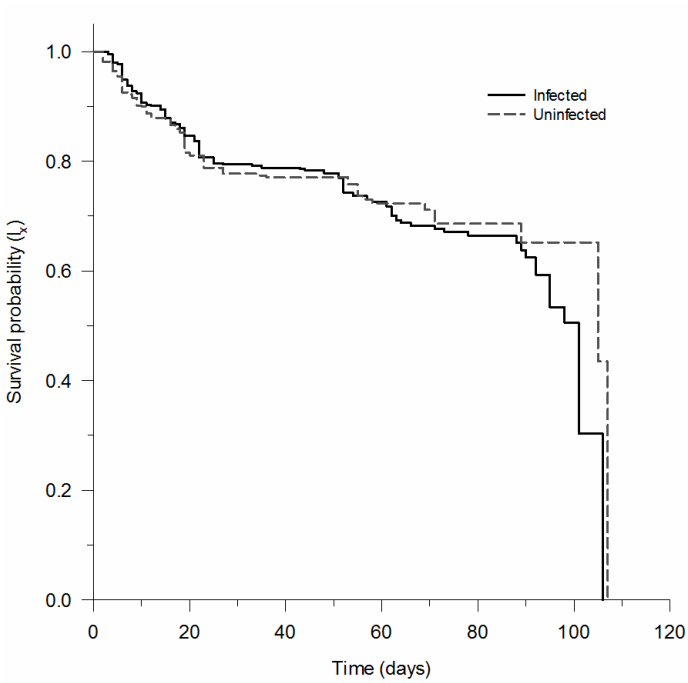

Figure 1. Survival rate of *Diaphorina citri*. Survival rate (l_x) of *D. citri* (Hemiptera: Psyllidae) in phytoplasma-infected (solid black line) and uninfected (dash gray line) plants of *Citrus aurantifolia*.

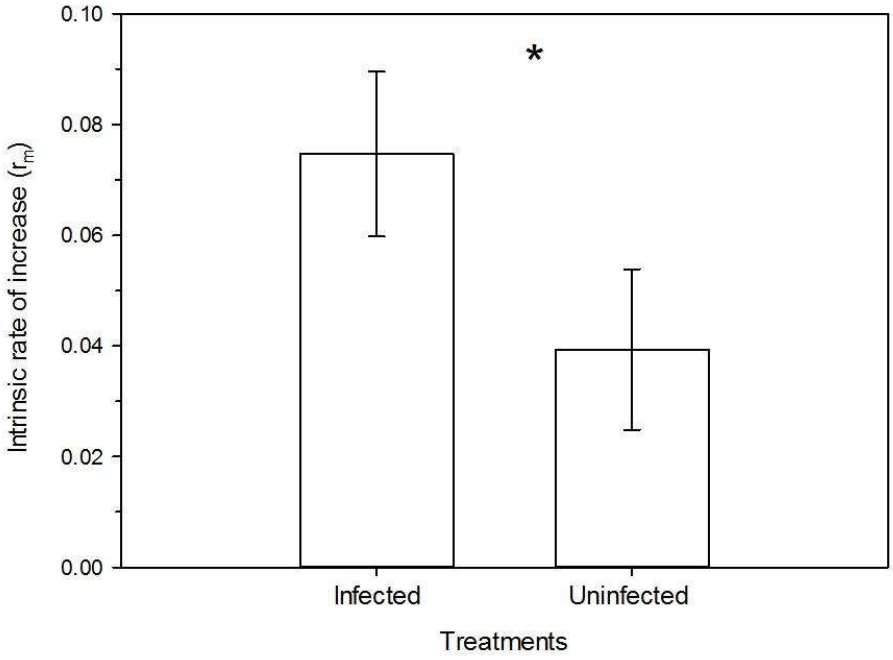

Figure 2. Intrinsic rate of increase of *Diaphorina citri*. Mean ± SE of intrinsic rate of increase (r_m) of *Diaphorina citri* (Hemiptera: Psyllidae) in phytoplasma-infected and uninfected plants of *Citrus aurantifolia*. The value followed by the asterisk (*) represents a significant difference ($P<0.05$, *F* test).

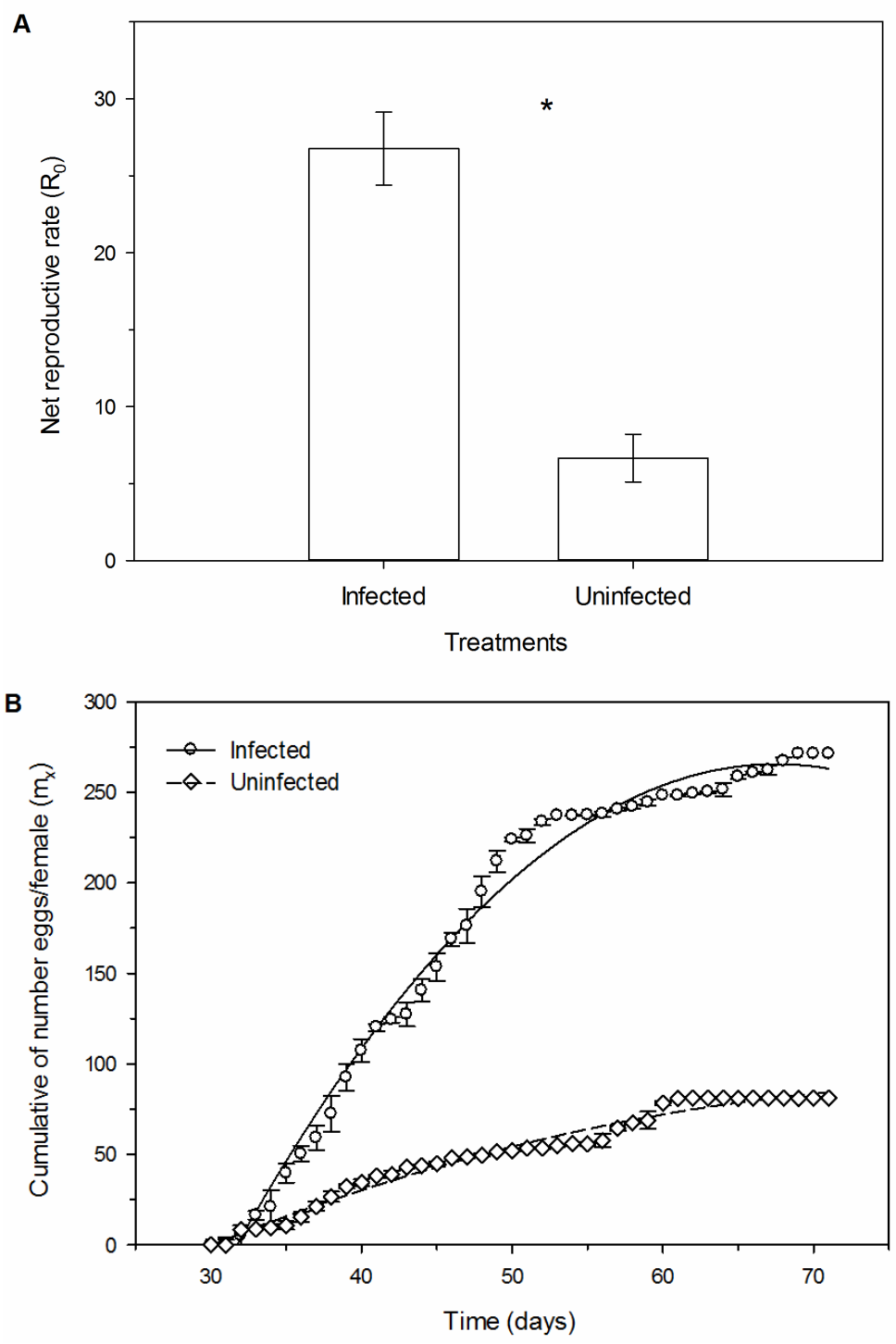

Figure 3. Net reproductive rate of *Diaphorina citri*. Mean ± SE of net reproductive rate (R_0) (**A**) and curve of cumulative number of eggs/female (m_x) (**B**) of *D. citri* (Hemiptera: Psyllidae) in phytoplasma-infected and uninfected plants of *Citrus aurantifolia*. Values followed by the asterisk (*) represent significant differences ($P<0.05$, *F* test). The symbols represent means and standard error.

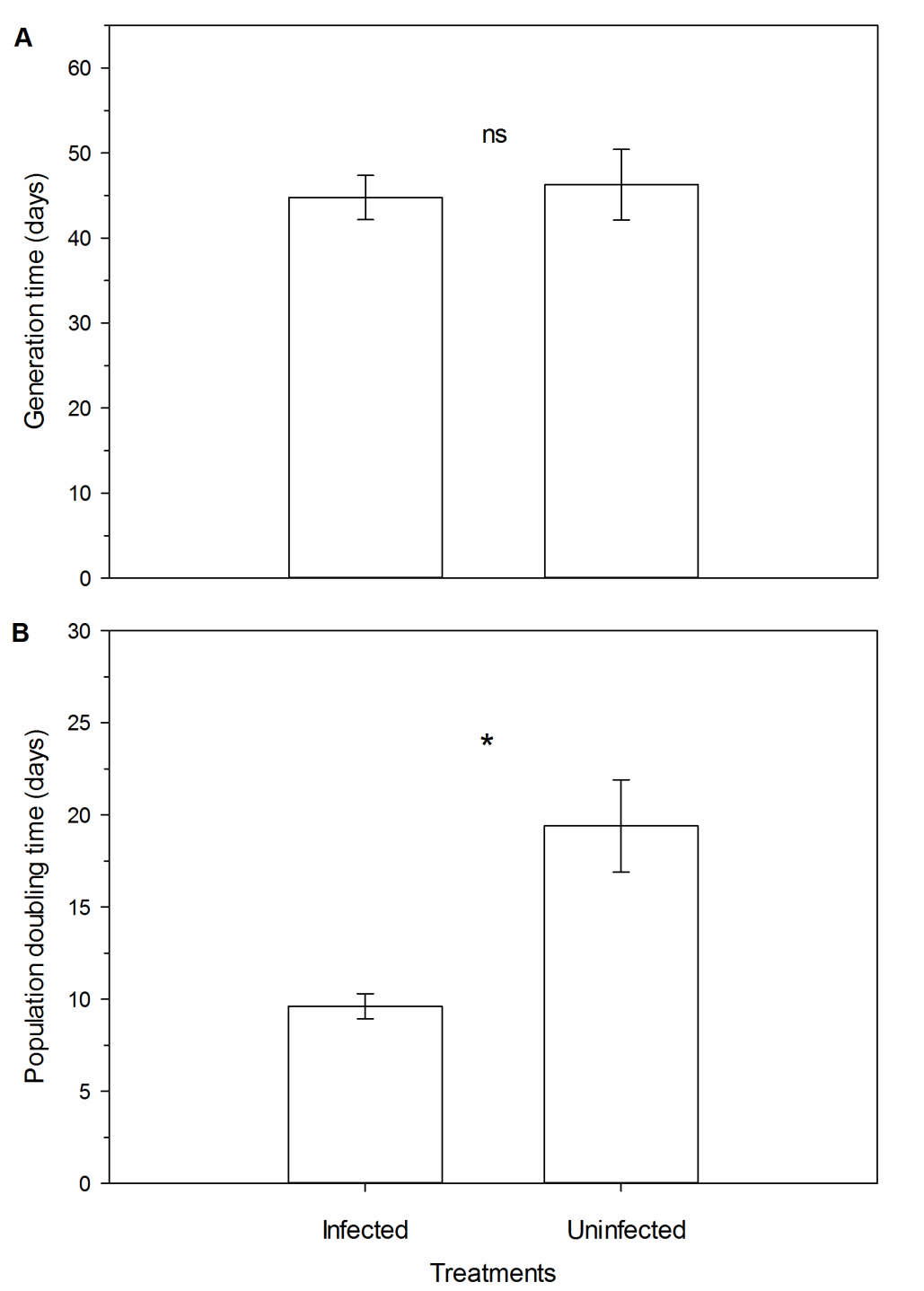

Figure 4. Generation time and population doubling time of *Diaphorina citri*. Mean ± SE of generation time (days) (**A**) and population doubling time (days) (**B**) of *D. citri* (Hemiptera: Psyllidae) on uninfected and phytoplasma-infected plants of *Citrus aurantifolia*. Values followed by the asterisk (*) represent significant differences ($P<0.05$, *F* test).

Table 1. Mean ± SE of the morphological and physiological characteristics in phytoplasma-infected and uninfected plants of *Citrus aurantifolia*.

Measurement	Plants of *Citrus aurantifolia*			
	Infected	Uninfected	$F_{(1,18)}$	*p*
Leaves/branch	16.04 ± 0.60	18.46 ± 1.58	2.06	0.17 n.s.
Leaf area (m^2)	0.36 ± 0.01	0.34 ± 0.02	0.65	0.43 n.s.
Stalk diameter (cm)	1.56 ± 0.06	1.52 ± 0.06	0.19	0.66 n.s.
Fruits/branch	18.23 ± 1.04	20.31 ± 1.57	1.23	0.28 n.s.
Flowers/branch	35.24 ± 2.05	34.92 ± 1.88	0.01	0.91 n.s.
Chlorophyll *a* (mg/m^2)	548.64 ± 15.57	550.45 ± 15.53	0.01	0.94 n.s.
Chlorophyll *b* (mg/m^2)	225.20 ± 7.23	226.38 ± 7.32	0.01	0.91 n.s.
Chlorophyll *total* (mg/m^2)	770.78 ± 22.56	773.64 ± 22.57	0.01	0.93 n.s.

n.s. means no significant differences ($P<0.05$, *F* test).

Discussion

The reproductive parameters obtained from fertility life tables of *D. citri* ($r_m>0$ and $R_0>1$) showed a doubling in population growth when the insects were fed on phytoplasma-infected plants. This contributes to the growing number of systems in which vector fitness has been shown to increase on plants infected with persistent pathogens (Gomes et al., 2000; Jiu et al., 2007; Nava et al., 2007; Guo et al., 2010; Sugio et al., 2011). It is distinct, however, in that this is the first study, to the best of our knowledge that has shown this on a completely asymptomatic (yet infected) plant. While this is of biological interest (and we discuss this below), it is of particular practical concern for several reasons.

Firstly, our results imply that a 'silent' infection, such as that caused by the phytoplasma we studied here, may spread very effectively due to increases in vector fitness, perhaps reaching regions where the infection becomes pathogenic. Note that this

phytoplasma is a close relative of the aetiological agent of the devastating Witches' Broom Disease of Lime in the Middle East – it is not difficult to conceive of the pathogen that we found in the humid southeast of Brazil, spreading northwards and reach semi-arid regions of Brazil where climatic factors and the accumulation of salts in the soil approach conditions in the Middle East – if these factors are important in WBDL etiology, they may cause the asymptomatic phytoplasma-infected plant to become symptomatic.

A second implication is that a 'silent' infection may contribute to the spread of a more aggressive pathogen such as the agent of Huanglongbing, where plants are co-infected (or even in regions where both microoganisms occur) as described by Teixeira et al. (2008). They found that some samples from orange plants were positive for both the phytoplasma and '*Ca*. Liberibacter asiaticus'. The third practical implication is that a microorganism may infect a plant and not, of itself, be pathogenic. If it increases the burden of herbivorous insect vectors via the phenomenon observed here, however, then the increased insect population itself becomes an indicator of the disease symptoms. Allied to a recent study that gives very strong evidence that phytoplasma-infected plants may largely be to benefit the insect vector (MacLean et al., 2014), it becomes apparent that the vectors are even a key component of the virulence of some plant pathogens. We have shown that the *C. aurantifolia*-Lime witches' broom symptomless phytoplasma-*D. citri* system had a positive effect in this invasive species. This type of positive influence on insects has been rarely explored in the context of invasive species (Bennett, 2013). One example this effect has occurred with the glassy winged sharpshooter *Homalodisca vitripennis* (Hemiptera: Cicadellidae) in California. This species increases the spread of the native bacterial pathogen, *Xylella fastidiosa* that causes Pierce's disease in grapevine (Gomes et al., 2000). Another invasive species, the thrips *Frankliniella occidentalis*, has

a mutualistic interaction with plant tospoviruses (Belliure et al., 2005) and this may well have contributed to its spread. The combination of invasive insects and their pathogens associates can therefore cause significant economic damage to agricultural systems (Bennett, 2013).

The invasive species *D. citri* is native to Southeast Asia, where citrus originated (Halbert & Manjunath, 2004; Beattie et al., 2008). This invasive insect has been recorded in Brazil for more than 70 years (Costa Lima, 1942), which it is able to transmit the two different forms of '*Ca*. Liberibacter' in Brazil: '*Ca*. L. americanus' and '*Ca*. asiaticus'. Both were discovered at the same location in two orchards in São Paulo State in Brazil, and are spreading from that point (Beattie et al., 2008). The two forms were introduced simultaneously in the mid-1990s to Brazil (Halbert & Núñez, 2004). However, the problem with HLB disease has been observed only since 2004 (Bové, 2006). This type of association could also occur between the invasive species *D. citri* and the phytoplasma closely related to '*Ca*. Phytoplasma aurantifolia' found in Brazil, because ours results showed that *D. citri* had a positive effect when fed on phytoplasma-infected plants. Co-evolution between insects and plant pathogens can occur quickly due to the large number of generations both organisms complete in a short period of time and this may be occurring with our study organisms. It is also possible that in other cases, invasive insects acquire new associations or incidentally transfer pathogens (Bennett, 2013). Thereby, this process may enhance the fitness of the invasive insect *D. citri* and thus increase the ability of phytoplasma dispersal by this species.

There are many studies that have evaluated insect fitness only by survival or fertility. One of these studies has been with the major invasive insect *B. tabaci*. This species had increased longevity and fecundity when fed on tobacco plants infected with two begomoviruses, *Tobacco curly shoot virus* (TbCSV) and *Tomato yellow leaf curl*

China virus (TYLCCNV) (Jiu et al., 2007). In another study, Guo et al. (2010) showed that tobacco infected with TYLCCNV increased egg production and realized fecundity in biotype B. Moreover, whiteflies indirectly benefitted from interactions with TYLCCNV through accelerated ovarian development (Guo et al., 2012). Similarly, the survival and fecundity of the leafhopper *Macrosteles quadrilineatus* was increased after exposure to a strain of aster yellows phytoplasma (Beanland et al., 2000). A life table approach allows a deeper understanding of effects on the insects and also provides estimates of fitness, which direct isolated studies of fecundity or survival, for example, do not.

Here, the total number of eggs per female (until the end of life cycle) was three times higher on phytoplasma-infected than on healthy plants. In plant-pathogen-vector systems such as this, the pathogen needs the arthropod vector for transmission and dispersal (Jiu et al., 2007). Therefore, the higher production of eggs will increase vector population and this can facilitate the spread of phytoplasma in the field. Sugio et al. (2011) showed that *M. quadrilineatus* leafhopper laid more eggs on plants infected with Aster Yellows phytoplasma strain Witches' Broom (AY-WB) than on uninfected plants. However, the oviposition and female survival rates were not affected, indicating that increased oviposition is predominantly responsible for the higher *M. quadrilineatus* progeny yield from infected plants.

The high transmission capacity of *Ca.* Liberibacter spp. by *D. citri* along with an increased reproductive rate caused by phytoplasma may result in a greater dispersion of HLB. Adults of *D. citri* feed on young stems and on leaves of all stages of development. However, they prefer to feed on shoots of citrus plants and eggs are laid exclusively on new flush shoots (Aubert, 1990; Hall & Albrigo, 2007). Phytoplasmas are restricted to the phloem and may accumulate in greater amounts in shoots, mainly in source petioles

and leaves (Christensen et al., 2004). This behavior of *D. citri* can increase its chance of acquiring phytoplasma. In symptomatic plants an accumulation of soluble carbohydrates and amino acids can occur in the infected parts due to phytoplasmas adhering to the host cells (Lefol et al., 1993). Thus, these plants can have an increase in their carrying capacity and allow a higher spread of the pathogen by the insect vector.

We observed that the generation time (T) of *D. citri* was not influenced by phytoplasma. However, the population doubling time (DT) was lower in phytoplasma infected plants. This parameter is important because it showed that *D. citri* is able to double the population every 10 days. Liu & Tsai (2000) showed that *D. citri* reared on orange jessamine plants (*Murraya paniculata*) had population doubling time of 3.5 days at a temperature of 28 °C. The mean *D. citri* generation time (T) was shorter, 36.2 days, on orange jessamine (Nava et al., 2007). The survival rate and developmental stages of *D. citri* were not affected by phytoplasma-infected plants. *Diaphorina citri* had a high reproductive rate in the first days after adult emergence. Thus, a change in their survival due to phytoplasma infection would alter little their total reproductive output. Furthermore, greater survival can increase exposure of *D. citri* to predators and weather. Nava et al. (2007) showed that the peak *D. citri* oviposition is in the first 10 days and it has extended until around day 26, according to the host plant.

The symptoms of Witches' Broom Disease of Lime (WBDL) caused by '*Ca.* Phytoplasma aurantifolia' are severe in countries with high temperatures, such as Oman (Al-Sadi et al., 2012), United Arab Emirates (Garnier et al., 1991), India (Ghosh et al., 1999), Iran (Bové et al., 1999), and Saudi Arabia (Alhudaib et al., 2009). Temperature was found to have an influence on Flavescence dorée phytoplasma multiplication, which was nearly twice as fast in broad beans incubated at 25°C than these incubated at 20°C. Furthermore, plants at 25°C expressed symptoms one week earlier than at 20ºC

(Salar et al., 2013). In another study there was an increase in the proportion of *M. quadrilineatus* leafhoppers that became vectors after feeding on plants with aster yellows phytoplasma-infected incubated at high temperatures (Murral et al., 1996). Thus, temperature may be one reason why infected acid lime plants with that phytoplasma closely related in the Middle East show no symptoms of witches' broom in Brazil. Other potential causes include the possible low amount of phytoplasma on infected citrus plants in Brazil and the absence of the main insect vector, *Hishimonus phycitis*, which is common only in the Middle East (Salehi et al., 2007; Chung et al., 2009).

Our results showed that *D. citri* was able to produce more offspring on phytoplasma-infected plants of *C. aurantifolia*. Moreover, healthy and phytoplasma-infected plants presented similar values for morphological and physiological variables (Table 2). Although plants did not show symptoms, increased infestations of insects can be regarded as a symptom itself as discussed above. Although symptoms of WBDL are not visible, other physiological changes that take place in the infected plants may be responsible for manipulating behavior of the insect vectors. Phytoplasmas are able to manipulate both insect vectors and plants by effector proteins that target host pathways to increase host susceptibility to the pathogen (Hogenhout et al., 2008b). The leafhopper vector *M. quadrilineatus* produced more offspring in *Arabidopsis thaliana* plants infected with (AY-WB). This occurred because protein effectors secreted from AY-WB phytoplasma (SAP11) decrease the expression of lipoxygenase (LOX), a key component in the jasmonic acid (JA) biosynthesis. Jasmonic acid is an important component in response to herbivory, and its accumulation is decreased in plants with reduced expression of LOX (Sugio et al., 2011). Besides these protein effectors, another (SAP54) induced by the same phytoplasma, modified flowers into leaves (leaf-like

flowers) in *A. thaliana* plants, effectively castrating the plant. This modification makes the plants more attractive to *M. quadrilineatus* and then there was a greater oviposition by this phytoplasma insect vector than in infected plants (with non-leaf-like flowers) (MacLean et al., 2014).

Our study provided evidence that this phenomenon can occur even in asymptomatic plants. It will be interesting to elucidate the mechanisms for this because it may be of considerable practical importance to investigate the factors suppressing witches' broom symptoms in phytoplasma-infected *C. aurantifolia* plants in Brazil due the importance of the citrus industry in this country, or potentially, to use this knowledge to suppress symptoms in the Omani system.

Acknowledgments

This research was principally funded by the Vale Technological Institute. We also thank CNPq for financial support and Sultan Qaboos University for financial support and facilitating the research progress through project # EG/AGR/CROP/12/01; we are grateful to D.L. Siqueira for help in citrus seedling acquisition. Furthermore, we wish to thank A. Pallini, E. Pereira and R. Guedes for comments on an earlier version of the manuscript.

References

Al-Sadi AM, Al-Moqbali HS, Al-Yahyai RA & Al-Said FA (2012) AFLP data suggest a potential role for the low genetic diversity of acid lime (*Citrus aurantifolia*) in Oman in the outbreak of witches' broom disease of lime. Euphytica 188: 285-297.

Alhudaib K, Arocha Y, Wilson M & Jones P (2009) Molecular identification, potential vectors and alternative hosts of the phytoplasma associated with a lime decline disease in Saudi Arabia. Crop Protection 28: 13-18.

Askari N, Jouzani GS, Mousivand M, Foroutan A, Nazari AH, Abbasalizadeh S, Soheilivand S & Mardi S (2011) Evaluation of Anti-Phytoplasma Properties of Surfactin and Tetracycline Towards Lime Witches' Broom Disease Using Real-Time PCR. J. Microbiol. Biotechnol. 21: 81-88.

Aubert B (1990) Integrated activities for the control of huanglongbing-greening and its vector *Diaphorina citri* Kuwayama in Asia: Proceedings of the Fourth FAO-UNDP International Asia Pacific Conference on Citrus Rehabilitation (ed. by B Aubert, S Tontyaporn & D Buangsuwon), Chiang-Mai, Thailand, pp. 133-144.

Beanland L, Hoy CW, Miller SA & Nault LR (2000) Influence of aster yellows phytoplasma on the fitness of the aster leafhopper (Homoptera: Cicadellidae). Ann. Entomol. Soc. Am. 93: 271-276.

Beattie GAC, Holford P, Haigh AM & Broadbent P (2008) On the origins of Citrus, huanglongbing, *Diaphorina citri* and *Trioza erytreae*: Proceedings of the International Research Conference on Huanglongbing (ed. by TR Gottwald & JH Graham) Plant Management Network, Orlando, FL, USA, pp. 23-56.

Belliure B, Janssen A, Maris PC, Peters D & Sabelis MW (2005) Herbivore arthropods benefit from vectoring plant viruses. Ecol Lett 8: 70-79.

Bennett AE (2013) Can plant–microbe–insect interactions enhance or inhibit the spread of invasive species? Functional Ecology 27: 661-671.

Bové JM (2006) Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. J Plant Pathol 88: 7-37.

Bové JM, Danet JL, Bananej K, Hassanzadeh N, Taghizadeh M, Salehi M & Garnier M (1999) Witches' broom disease of lime (WBDL) in Iran: Proceedings of the 14th Conference of the International Organization of Citrus Virologists (IOCV) (ed. by JV da Graça, RF Lee & RK Yokomi), Riverside, CA, pp. 207-212.

Carey JR (1993) Applied demography for biologists with special emphasis on insects. Oxford University Press, New York.

Christensen NM, Nicolaisen M, Hansen M & Schulz A (2004) Distribution of Phytoplasmas in Infected Plants as Revealed by Real-Time PCR and Bioimaging. Molecular Plant-Microbe Interactions 17: 1175-1184.

Chung KR, Khan IA & Brlansky RH (2009) Citrus diseases exotic to Florida: witches' broom disease of lime (WBDL): EDIS Publications, University of Florida, Gainesville, pp. 1-3.

Coelho Filho MA, Angelocci LR, Vasconcelos MRB & Coelho EF (2005) Estimativa da área foliar de plantas de lima ácida 'Tahiti' usando métodos não-destrutivos. Rev. Bras. Frutic. 27: 163-167.

Costa Lima AM (1942) Insetos do Brasil: Homoptera. Escola Nacional de Agronomia, Rio de Janeiro

Crawley MJ (2007) The R Book. John Wiley & Sons Ltd, Chichester.

Deng S & Hiruki C (1991) Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. J Microbiol Methods 14: 53-61.

Dinsdale A, Cook L, Riginos C, Buckley Y & De Barro PJ (2010) Refined global analysis of *Bemisia tabaci* (Gennadius) (Hemiptera: Sternorrhyncha: Aleyrodoidea) mitochondrial CO1 to identify species level genetic boundaries. Ann Entomol Soc America 103 196-208.

Elliot SL, Adler FR & Sabelis MW (2003) How virulent should a parasite be to its vector? Ecology 84: 2568-2574.

Garnier M, Zreik L & Bové JM (1991) Witches' broom, a lethal mycoplasmal disease of lime trees in the Sultanate of Oman and the United Arab Emirates. Plant Disease 75: 546-551.

Ghosh DK, Das AK, Singh S, Singh SJ & Ahlawat YA (1999) Occurrence of witches' broom, a new phytoplasma disease of acid lime (*Citrus aurantifolia*) in India. Plant Disease 83: 302.

Gomes WR, Cooksey DA, Dahlsten DL, Kirkpatrick B, McBride JR & Morse JG (2000) Report of the Pierce's Disease Research and Emergency Response Task Force: Office of the President, University of Florida, Oakland, USA, p. 80.

Gubler DJ (1989) Dengue: The Arboviruses: epidemiology and ecology (ed. by TP Monath) CRC Press, Boca Raton, FL, pp. 223-260.

Guo J-Y, Dong S-Z, Yang X-l, Cheng L, Wan F-H, Shu-Sheng L, Xue-ping Z & Gong-Yin Y (2012) Enhanced Vitellogenesis in a Whitefly via Feeding on a Begomovirus-Infected Plant. PLoS ONE 7: e43567.

Guo J-Y, Ye G-Y, Dong S-Z & Liu S-S (2010) An Invasive Whitefly Feeding on a Virus-Infected Plant Increased Its Egg Production and Realized Fecundity. PLoS ONE 5(7): e11713.

Halbert SE & Manjunath KL (2004) Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: A literature review and assessment of risk in Florida. Fla Entomol 87: 330-353.

Halbert SE & Núñez CA (2004) Distribution of the Asian citrus psyllid, *Diaphorina citri* Kuyayama (Sternorrhyncha: Psyllidae) in the Caribbean basin. Florida Entomologist 87: 401-402.

Hall DG & Albrigo LG (2007) Estimating the relative abundance of flush shoots in citrus, with implications on monitoring insects associated with flush. HortScience 42: 364-368.

Hall DG, Richardson ML, Ammar ED & Halbert SE (2012) Asian citrus psyllid, *Diaphorina citri*, vector of citrus huanglongbing disease. Entomologia Experimentalis et Applicata 146: 207-223.

Hogenhout SA, Ammar E, Whitfield AE & Redinbaugh MG (2008a) Insect vector interactions with persistently transmitted viruses. Annual Review of Phytopathology 46: 327-359.

Hogenhout SA, Oshima K, Ammar e-D, Kakizawa S, Kingdom HN & Namba S (2008b) Phytoplasmas: bacteria that manipulate plants and insects. Mol Plant Pathol. 9: 403-423.

Jiu M, Zhou X-P, Tong L, Xu J, Yang X, Wan F-H & Liu S-S (2007) Vector-virus mutualism accelerates population increase of an invasive whitefly. PLoS ONE 2: e182.

Kyle JL & Harris E (2008) Global Spread and Persistence of Dengue. Annu. Rev. Microbiol. 62: 71-92.

Lefol C, Caudwell A, Lherminier J & Larrue J (1993) Attachment of the Flavescence doree pathogen (MLO) to leafhopper vectors and other insects. Ann. Appl. Biol. 123: 611-622.

Liu YH & Tsai JH (2000) Effects of temperature on biology and life table parameters of the Asian citrus psyllid, *Diaphorina citri* Kuwayama (Homoptera: Psyllidae). Annals of Applied Biology 137: 201-206.

Lumley T (2011) survival: Survival analysis, including penalised likelihood: R package version 2, pp. 36-39.

MacLean AM, Orlovskis Z, Kowitwanich K, Zdziarska AM, Angenent GC, Immink RGH & Hogenhout SA (2014) Phytoplasma Effector SAP54 Hijacks Plant Reproduction by Degrading MADS-box Proteins and Promotes Insect Colonization in a RAD23-Dependent Manner. PLoS Biol 12: e1001835.

MacLean AM, Sugio A, Makarova OV, Findlay KC, Grieve VM, Tóth R, Nicolaisen M & Hogenhout SA (2011) Phytoplasma Effector SAP54 Induces Indeterminate Leaf-Like Flower Development in *Arabidopsis* Plants. Plant Physiolog 157: 831-841.

Mann RS, Ali JG, Hermann SL, Tiwari S, Pelz-Stelinski KS & al. e (2012) Induced Release of a Plant-Defense Volatile 'Deceptively' Attracts Insect Vectors to Plants Infected with a Bacterial Pathogen. PLoS Pathog 8: e1002610.

Mauck K, Bosque-Pérez NA, Eigenbrode SD, De Moraes CM & Mescher MC (2012) Transmission mechanisms shape pathogen effects on host–vector interactions: evidence from plant viruses. Functional Ecology 2013, 27, 26 1162-1175.

Mauck KE, De Moraes CM & Mescher MC (2010) Deceptive chemical signals induced by a plant virus attract insect vectors to inferior hosts. Proceedings of the National Academy of Sciences 107 3600-3605.

McMenemy LS, Hartley SE, MacFarlane SA, Karley AJ, Shepherd T & Johnson SN (2012) Raspberry viruses manipulate the behaviour of their insect vectors. Entomol. Exp. Appl. 144: 56-68.

Morais EGF, Picanço MC, Barreto RW, Silva NR & Campos MR (2010) Biological performance of *Diclidophlebia smithi* (Hemiptera: Psyllidae), a potential biocontrol agent for the invasive weed *Miconia calvescens*. Biocontrol Science and Technology 20: 107-116.

Murral DJ, Nault LR, Hoy CW, Madden LV & Miller SA (1996) Effects of temperature and vector age on transmission of two Ohio strains of aster yellows phytoplasma by the aster leafhopper (Homoptera: Cicadellidae). J. Econ. Entomol. 89: 1223-1232.

Nava DE, Torres MLG, Rodrigues MDL, Bento JMS & Parra JRP (2007) Biology of *Diaphorina citri* (Hemiptera: Psyllidae) on different hosts and at different temperatures. Journal of Applied Entomology 131: 709-715.

Oliveira MRV, Hanneberry TJ & Anderson P (2001) History, current status and collaborative research projects for *Bemisia tabaci*. Crop Prot 20: 709-723.

Perring TM (2001) The *Bemisia tabaci* species complex. Crop Prot 20: 725-737.

Power AG (2000) Insect transmission of plant viruses: a constraint on virus variability. Curr. Opin. Plant Biol. 3: 336-340.

Price PW (1997) Insect Ecology. John Wiley & Sons, New York.

R Development Core Team (2014) R: A Language and Environment for Statistical Computing: R Foundation for Statistical Computing, Vienna, Austria.

Rabinovich JE (1978) Ecologia de Poplaciones Animales. Secretaria General de la Organización de los Estados Americanos, Washington, DC.

Salar P, Charenton C, Foissac X & Malembic-Maher S (2013) Multiplication kinetics of Flavescence dorée phytoplasma in broad bean. Effect of phytoplasma strain and temperature. Eur J Plant Pathol 135: 371-381.

Salehi M, Izadpanah K, Siampour M, Bagheri A & Faghihi SM (2007) Transmission of '*Candidatus* phytoplasma aurantifolia' to Bakraee (*Citrus reticulata* Hybrid) by Feral *Hishimonus phycitis* leafhoppers in Iran. Plant Disease 91: 466.

Sugio A, Kingdom HN, MacLean AM, Grieve VM & Hogenhout SA (2011) Phytoplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis. PNAS 108: 1254-1263.

Teixeira DC, Wulff NA, Martins EC, Kitajima EW, Bassanezi R, Ayres AJ, Eveillard S, Saillard C & Bové JM (2008) A phytoplasma closely related to the Pigeon pea witches'-broom phytoplasma (16SrIX) is associated with citrus Huanglongbing symptoms in the state of São Paulo, Brazil. Phytopathology 98: 977-984.

Weintraub PG & Beanland L (2006) Insect vectors of phytoplasmas. Annu Rev Entomol 51: 91-111.

CHAPTER 2

Transmission of '*Candidatus* Phytoplasma aurantifolia' to acid lime *Citrus aurantifolia* (Swingle) by *Hishimonus phycitis* and *Diaphorina citri* in Oman

Abstract

In recent decades, the acid lime production in Oman has been severely affected by diseases, to the point where the country, once an exporter of limes, now imports the fruit. The principal disease responsible for the acid lime decline is the Witches' Broom Disease of Lime (WBDL), caused by '*Ca*. Phytoplasma aurantifolia'. The transmission of this phytoplasma is carried out in a persistent propagative manner by leafhoppers, planthoppers, and psyllids. Thus, the aim of this study was investigate the transmission of '*Ca*. Phytoplasma aurantifolia' to acid lime *C. aurantifolia* by *H. phycitis* and *D. citri* in Oman. Moreover, we also evaluated the effects of '*Ca*. Phytoplasma aurantifolia' in the psyllid *D. citri* in Oman, which were similar those found in Brazil. Even in asymptomatic phytoplasma-infected plants, *D. citri* showed a populational increase higher than uninfected and healthy plants. Both species were able to transmit this pathogen to acid lime plants, which five of nine *C. aurantifolia* seedlings were phytoplasma-infected by *H. phycitis* and three of nine seedlings by *D. citri*. The probability of '*Ca*. Phytoplasma aurantifolia' transmission by *H. phycitis* was around 11 times higher than by *D. citri*. Therefore *H. phycitis* is more efficient on the WBDL transmission than *D. citri*.

Keywords: Invasive species, transmission efficiency, plant pathogens, phytoplasmas, insect vectors, fertility life table, intrinsic rate of increase.

Introduction

In recent decades, acid lime production in Oman has been severely affected by diseases, to the point where the country, once an exporter of limes, now imports the fruit. Since the 1970's, over half a million acid lime trees (*Citrus aurantifolia* Swingle) have been lost in Oman. Declining incomes in what were traditional farming systems have led to the abandonment of many farms, or conversion of these farms to other uses (Garnier et al., 1991; Al-Sadi et al., 2004). Although some progress has been made in identifying the causal agents of the lime diseases, there is at present no means of control.

The principal disease responsible for the decline in acid lime production is Witches' Broom Disease of Lime (WBDL), now estimated to infect 98% of acid lime trees in the country (Chung et al., 2006). It was first detected in the northern coastal plain of Oman, near to the United Arab Emirates (UAE), in the 1970's (Bové, 1986; Bové et al., 1988; Garnier et al., 1991). It has since been detected in the UAE (Garnier et al., 1991) and Iran (Bové et al., 2000), and possibly in India (Ghosh et al., 1999; Bové et al., 2000). The disease is caused by '*Candidatus* Phytoplasma aurantifolia' (Zreik et al., 1995).

Phytoplasma are phytopathogenic wall-less bacteria that inhabit plant phloem vessels and can cause several diseases in crops (Firrao et al., 2007; Sugio et al., 2011a). Phytoplasma-infected plants can present diverse symptoms such as the production of small shoots in the so-called "witches' broom", floral organs changing into leaves, leaf or shoot chlorosis, sterility of flowers, shortening of internodes and total necrosis of the plant (Bertaccini, 2007; Sugio et al., 2011a).

In an extensive report on the WBDL situation of acid lime in Oman, Bové (1995) compiled several years of his personal observations of the situation in the field at

that time. He expressed the strong opinion that an insect vector is responsible for the dynamics of the disease in the field. He based this on observations of comparatively rapid local spread of the disease within orchards, a phenomenon typical of local vector-borne plant disease dissemination. Phytoplasmas are transmitted in a persistent propagative manner by leafhoppers, planthoppers, and psyllids. All known vector species are in the order Hemiptera. Within Hemiptera, the family containing the largest number of vector species is Cicadellidae, and 75% of all confirmed phytoplasma vector species are found in the subfamily Deltocephalinae (Weintraub & Beanland, 2006). Up to now, only two psyllid genera are recognized as phytoplasma insect vectors (Weintraub & Beanland, 2006).

Of the insects that have been found in citrus orchards in Oman, the key candidate to act as a vector is the leafhopper *Hishimonus phycitis* (Hemiptera: Cicadellidae: Deltocephalinae). Early reports indicated the presence of a phytoplasmas within field-collected insects, but at that point in time, the techniques were not available to confirm that it was the same pathogen strain (Bové et al., 1993). Another important insect vector of plant disease is the Asian citrus psyllid *Diaphorina citri* (Hemiptera: Psyllidae). This specie is the vector of phloem-limited bacteria *Candidatus* Liberibacter spp. that causes citrus greening or Huanglongbing (HLB) (Halbert & Manjunath, 2004; Bové, 2006). The first record of *D. citri* was in Barka (Al-Batinah region, Oman), in 2005 (Al-Zadjali et al., 2008).

The objective of this work was to investigate the capacity of *H. phycitis* and *D. citri* to transmit '*Ca*. Phytoplasma aurantifolia' to *C. aurantifolia* seedlings. Knowing the main vector species of the phytoplasma is indispensable for the WBDL disease management. Further, we tested the hypothesis that performance of the invasive species *D. citri* is greater in asymptomatic phytoplasma-infected plants of *C. aurantifolia*

(Swingle) than in uninfected ('blank-inoculated') and healthy plants (control). For this, we used fertility life table parameters. We also investigated the possible transovarial transmission of '*Ca*. Phytoplasma aurantifolia' by the psyllid *D. citri* as this trait is very important to the management strategy.

Material and Methods

Plant Material

Citrus aurantifolia seedlings were acquired from the Agricultural Extension Station, localized in Barka (Al-Batinah area) and belonging to the Ministry of Agriculture, Oman. The seedlings were produced from seeds and maintained inside a greenhouse. Ten leaves samples were collected from each seedling. Leaf midribs and petioles were cut and then macerated using liquid nitrogen and a mortar and pestle. Total DNA was extracted using the NucleoSpin Plant II Kit (Macherey-Nagel) according to the manufacturer's recommendation. Extracted nucleic acids were used as templates for direct PCR with universal primers P1/P7 (Deng & Hiruki, 1991; Schneider et al., 1995). Amplification was carried out with an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 40 sec, extension at 72°C for 1.5 min and a final extension cycle at 72°C for 7.5 min. To increase the sensitivity of assays, products from the direct PCR were diluted 20 times and then used in nested reactions as templates for amplification with universal primers R16F2n/R16R2 (Lee et al., 1998). The thermocycling program consisted of an initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1.5 min and a final extension cycle at 72°C for 7.5 min.

The amplification products (5 µL) were analysed by 1.0% agarose gel electrophoresis in 0.50 TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.3) buffer followed by staining with ethidium bromide and visualized with a UV GeneFlash (Syngene Bio Imaging).

The same process for the PCR was carried out with the seedlings after ending the transmission experiment. Only the phytoplasma-free seedlings were used to begin this experiment.

Transmission Experiment

Adults of *D. citri* and *H. phycitis* were collected from infected orchards using a motorized suction trap *D-vac*. Adult *D. citri* were kept for one month in phytoplasma-infected (WBDL) parts of the canopy of *C. aurantifolia*. After that, 30 adults of *D. citri* were placed inside an insect-proof cage (100 x 70 x 70 cm) each containing one healthy seedling of *C. aurantifolia* (absence of phytoplasma infection confirmed previously by nested PCR). We used a total of nine cages. Sub-samples from adults *D. citri* were tested for the presence of the phytoplasma before starting the experiment. After eight weeks the insects were collected from the cages with an insect mouth-operated pooter and the acid lime seedlings were tested for the presence of the phytoplasma by nested PCR. Some adults were also tested for the presence of the phytoplasma after ending the transmission experiment.

The same transmission experiment was carried out with *H. phycitis*. However, we used five adult insects by cage as it was more difficult to collect this species from the orchard. Moreover, adult *H. phycitis* were transferred directly to the healthy seedlings to begin the experiment. Some samples from adults of *H. phycitis* collected in the field were tested before and after the transmission experiment. For both experiment

we had a control with nine seedlings but with no insects. All the experiments were carried out in a glasshouse (24±1 ºC) localized at the Agricultural Experimental Station from the Sultan Qaboos University, Muscat, Al-Khoud, Oman.

As both phytoplasma transmission experiments were carried out using groups of insects, the actual proportion of infectious insects was estimated using the maximum likelihood estimator of p, $\hat{p} = 1 - H^{1/k}$, where H is the observed fraction of healthy or uninfected plants and k is the number of insects per plant, assuming that the vectors acted independently (Swallow, 1985; Bosco & Tedeschi, 2013).

Transovarial transmission

Fifteen couples of *D. citri* were collected from phytoplasma-infected acid lime seedlings and other 15 from healthy seedlings. Each couple was placed on a new branch of a healthy seedling for oviposition. After 24 hours, the insects were removed and the females were tested individually for nested PCR. Only those females positive for phytoplasma by nested PCR were considered for continuance of the experiment as described by Tedeschi et al. (2006). The eggs laid by each infected and healthy female were removed from the branch and tested by nested PCR in groups of 20. After 20 days, five nymphs (IV–V instar) were collected from each seedling for DNA extraction and molecular analysis to test for phytoplasmas. All newly emerged adults were collected from each seedling and analyzed in groups of five. Total DNA was extracted from adults and all stages of *D. citri* following a procedure described below.

Insect material

The dead insects from of the traps and transmission experiment were kept at -20 prior to preserve the DNA. Total DNA was extracted from adults of *D. citri* and *H.*

phycitis according to the protocol described by Marzachi et al. (1998). Individualized insects were macerated in Eppendorf tubes (1.5 ml) containing 100 µl buffer with CTAB using a sterile micropestle. The suspension was incubated for 30 min at 60°C, then 200 µl of chloroform-isoamyl alcohol (24:1) was added and it was centrifuged at 13,000 rpm for 20 min. The supernatant was collected and transferred to another Eppendorf tube (1.5 ml) and same volume of isopropanol was added. The tubes were kept at 4 °C overnight and then they were centrifuged at 13,000 rpm for 5 min. After that, the supernatant was discarded and the tubes were washed with 70% ethanol and centrifuged again at 13,000 rpm for 5 min. Finally, the tubes were dried at room temperature and resuspended in 50 µl of sterile milli-Q water.

Sequencing

All the products of nested PCR positive from *H. phycitis*, *D. citri* and *C. aurantifolia* plants were purified using the kit (GeneJet^{TM} PCR Purification Kit) according to the manufacturer's recommendations. The purified samples were sent to Macrogen (Macrogen Sequencing Servive, South Korea) for sequencing. The clones were subjected to BLAST analysis and aligned with 99% similarity to those relatives of '*Ca*. Phytoplasma aurantifolia'.

Fertility life table of *Diaphorina citri*

The life table studies were carried out in a glasshouse as described in the previous chapter, however with some differences listed below. The air temperature was controlled at 24±1.0 ºC. Twenty 2nd instar nymphs of *D. citri* from the rearing (on healthy *C. aurantifolia*) were transferred to each *C. aurantifolia* seedling inside the cage (100 x 70 x 70 cm) and there were three seedlings in each cage. This experiment was

carried out with three treatments: (i) seedlings that had been infected with phytoplasma via infected vectors *D. citri*, (ii) uninfected seedlings that had been 'blank-inoculated' with uninfected *D. citri* and (iii) uninfected and uninfested controls. Individual insects were evaluated every two days for development and survival. The exuviae were used to determine moulting. Newly emerged adults were collected and sexed individually under the stereomicroscope (the female's ovipositor is visible). The couples were placed again on the same seedlings for oviposition. The eggs were also counted on each plant every two days. To construct the fertility life table we used the same components as describe on previous chapter.

Data analyses

The survival curves (l_x) were obtained using Kaplan-Meier survival distributions (Crawley, 2007) with the aid of the 'survival' package (Lumley, 2011). Fertility tables were constructed according to the procedure described by Carey (1993). One-way analyses of variance (ANOVA) were also conducted with biological traits from the life tables of the three treatments. The cumulative number of eggs/female of *D. citri* in phytoplasma-infected, uninfected and healthy plants was analyzed using linear mixed-effects models with repeated measures. In this model was considered the treatments (phytoplasma-infected, uninfected and healthy plants) as fixed effects and the time as random effect. This data were transformed by log(x+1) and fitted to negative binomial because they showed overdispersion. The assumptions of normality and homoscedasticity were tested before data analyses using Shapiro-Wilk normality test and Bartlett test, respectively. All analyses were conducted using software R version 2.13.0 (R Development Core Team, 2014).

Results

'*Candidatus* Phytoplasma aurantifolia' was present in most samples of tested insects, independently of the area where they were collected (Table 1). In *H. phycitis*, 65% of the analyzed insects were infected with phytoplasma according to the PCR analyses, while *D. citri* had 45% of the samples positive.

Table 1. Presence of '*Candidatus* Phytoplasma aurantifolia' in *Hishimonus phycitis* (Hemiptera: Cicadellidae) and *Diaphorina citri* (Hemiptera: Psyllidae) collected from different area in Oman.

Area	*Hishimonus phycitis*		*Diaphorina citri*	
	Collected specimes	PCR positive/ analyzed insect[a] (%)	Collected specimes	PCR positive/ analyzed insect[a] (%)
AES/SQU	76	4/9 (44)	6	4/6 (66)
Al-Suwaiq	59	11/19 (58)	76	2/9 (22)
Barka	12	8/12 (66)	3	2/3 (66)
Musanah	40	6/8 (75)	184	11/28 (39)
Samael	36	8/9 (88)	3	3/3 (100)
Mean	44.6	7.4/11.4 (65)	54.4	4.4/9.8 (45)

[a]Insects were submitted individually to PCR analysis for the presence of the phytoplasma.

We tested by nested PCR 27 individuals of *H. phycitis* collected from the field before beginning the transmission experiment. Of these, 11 were infected by '*Ca*. Phytoplasma aurantifolia'. After finishing the experiment, we tested all the 45 individuals used as inoculum source for the phytoplasma transmission and 20 of these were infected. The same procedures were carried out with *D. citri* and all the samples, before and after the experiment, were infected with the same phytoplasma. This took place because *D. citri* adults were kept on infected-plytoplasma *C. aurantifolia* for one month. Both species were able to transmit '*Ca*. Phytoplasma aurantifolia' to the acid lime seedlings. In the experiment with *H. phycitis*, five of the nine seedlings were shown to be infected with phytoplasma by nested PCR after ending the experiment. The

same was true with *D. citri*, however, only three of nine seedlings turned infected. All the acid lime seedlings without insects used as a negative control were plytoplasma-free before and after the experiment (Table 2). The likelihood of '*Ca*. Phytoplasma aurantifolia' transmission was 1.4% and 15.1% by *D. citri* and *H. phycitis*, respectively (Table 3). The efficiency of phytoplasma transmission was higher for *H. phycitis* even using the small group of insects.

Table 2. Transmission of '*Candidatus* Phytoplasma aurantifolia' by *Hishimonus phycitis* (Hemiptera: Cicadellidae) and *Diaphorina citri* (Hemiptera: Psyllidae) in *Citrus aurantifolia* seedlings.

Specie	PCR positive/analyzed sample	
	Before[a]	After[b]
Hishimonus phycitis	11/27	20/45
Diaphorina citri	20/20	24/24
Citrus aurantifolia with *H. phycitis*	0/9	5/9
Citrus aurantifolia with *D. citri*	0/9	3/9
Control (plants without insects)	0/9	0/9

[a]PCR analysis before beginning of the transmission experiment.
[b]PCR analysis after ending of the transmission experiment.

Table 3. Estimated the probability (*p*) of '*Candidatus* Phytoplasma aurantifolia' transmission by a single vector of *Diaphorina citri* (Hemiptera: Psyllidae) and *Hishimonus phycitis* (Hemiptera: Cicadellidae).

Insect vector	H^a	k^b	p (C.I.)[c]
Diaphorina citri	0.66 (6/9)	30	0.014 (-0.001─0.026)
Hishimonus phycitis	0.44 (4/9)	5	0.151 (0.027─0.275)

[a]Observed fraction of uninfected plants.
[b]Number of insects per plant.
[c]95% confidence interval.

Ten of 15 *D. citri* females were infected by '*Ca*. Phytoplasma aurantifolia', and only these were used in the experiment. Sample batches of 20 eggs were collected from each of these 10 females and analyzed by PCR, but none was positive for '*Ca*.

Phytoplasma aurantifolia'. In eight of the 10 samples, sufficient young stages were obtained to allow testing of nymphs and newly emerged adults, but again none was positive by nested PCR (Table 4). No amplification products were obtained from females and all stages of *D. citri* used as negative control.

Table 4. Proportion of positive samples for '*Candidatus* Phytoplasma aurantifolia' on females, eggs, nymphs and newly emerged adults of *Diaphorina citri* (Hemiptera: Psyllidae).

Diaphorina citri	PCR positive/ analyzed sample
Females	10/15
Eggs	0/10
Nymphs	0/8
Newly emerged adults	0/8

The survival of *D. citri* were not different in asymptomatic phytoplasma-infected, uninfected ('blank-inoculated') and healthy plants (control) (survival curves obtained using Kaplan-Meier estimators; Log-rank test: χ^2 =1.15, df = 2, p = 0.56). The survival time mean was approximately 19, 20 and 21 days to asymptomatic phytoplasma-infected, uninfected and healthy plants (control), respectively (Figure 1). The intrinsic rate of population increase of *D. citri* (r_m) ($F_{2,6}$ = 25.02, p=0.001) was higher in asymptomatic phytoplasma-infected plants than in uninfected and healthy plants (Figure 2). The same was true to the net reproductive rate (R_0) ($F_{2,6}$ = 22.1, p=0.001) (Figure 3).

The curve of cumulative numbers of eggs per female (χ^2 = 69.07, df = 2, p<0.001) was higher in asymptomatic phytoplasma-infected plants than uninfected and healthy plants (Figure 4). The average numbers of eggs per female of *D. citri*, during the

evaluation period, in asymptomatic phytoplasma-infected, uninfected and healthy plants was around 380, 67 and 81, respectively.

There was no significant difference in the generation time (T) ($F_{2,6}$ = 4.07, p = 0,07) of *D. citri* in asymptomatic phytoplasma-infected, uninfected and healthy plants (Figure 5A). The generation time was around 40 days in both treatments. However, the population doubling time (DT) was three times higher in phytoplasma-uninfected and healthy plants than on asymptomatic phytoplasma-infected plants ($F_{2,6}$ = 13.8, p<0.005) (Figure 5B).

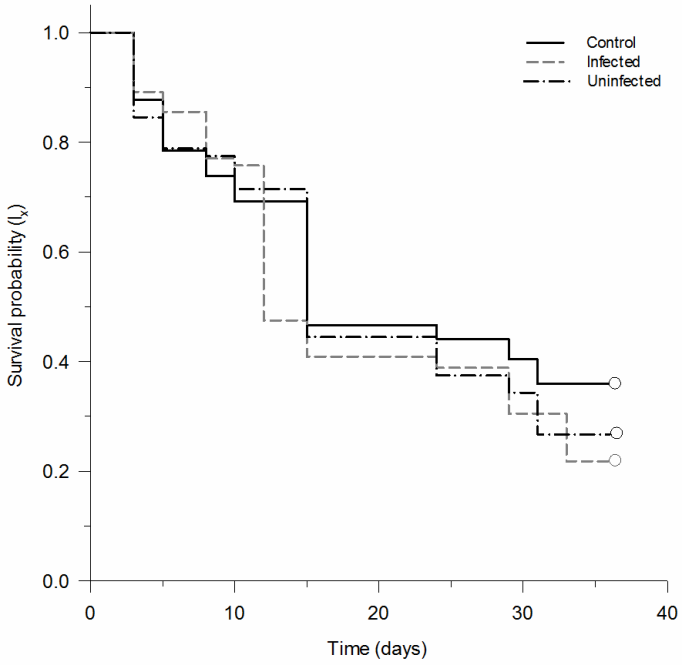

Figure 1. Survival rate (l_x) of *Diaphorina citri* (Hemiptera: Psyllidae) on asymptomatic phytoplasma-infected (dash gray line), uninfected (dash-dot black line) and healthy (solid black line) plants of *Citrus aurantifolia*.

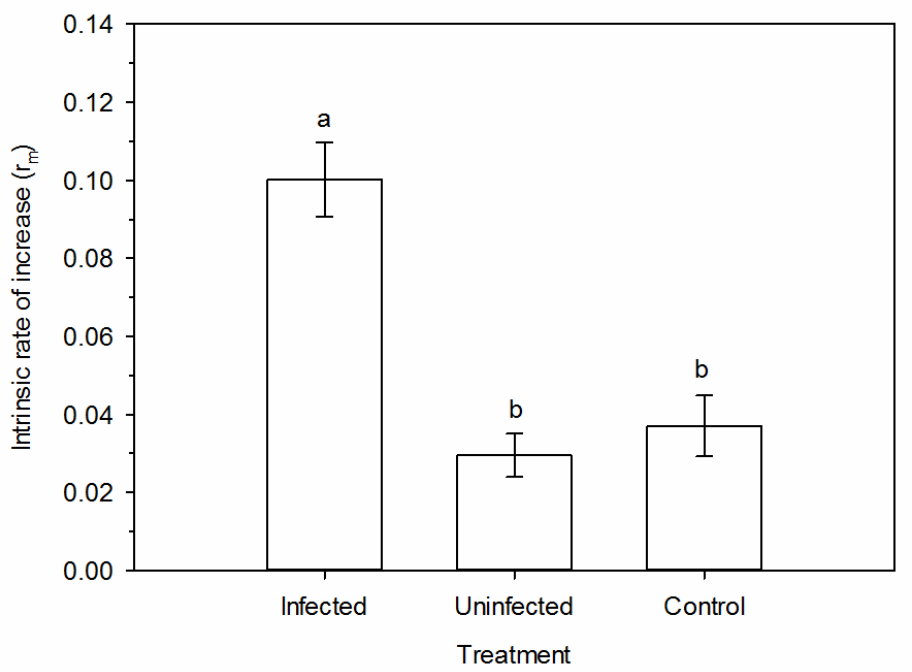

Figure 2. Mean ± SE of intrinsic rate of increase (r_m) of *Diaphorina citri* (Hemiptera: Psyllidae) on asymptomatic phytoplasma-infected, uninfected and healthy plants of *Citrus aurantifolia*. Different small letters represent significant differences (P<0.05) by Tukey's multiple comparison test.

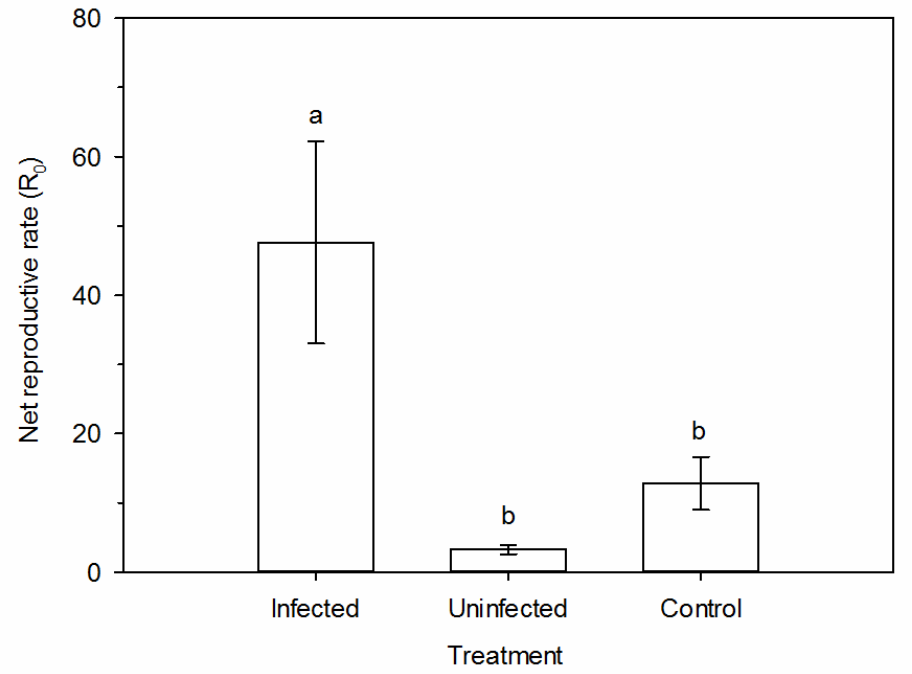

Figure 3. Mean ± SE of net reproductive rate (R_0) of *Diaphorina citri* (Hemiptera: Psyllidae) on asymptomatic phytoplasma-infected, uninfected and healthy plants of *Citrus aurantifolia*. Different small letters represent significant differences (P<0.05) by Tukey's multiple comparison test.

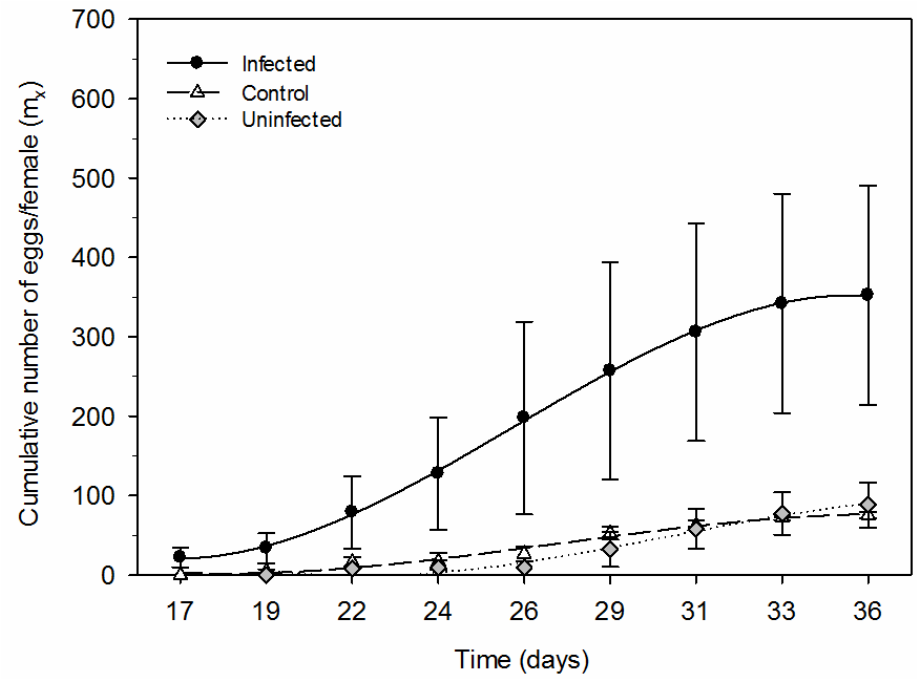

Figure 4. Curve of cumulative number of eggs/female (m_x) of *D. citri* (Hemiptera: Psyllidae) on asymptomatic phytoplasma-infected, uninfected and healthy plants of *Citrus aurantifolia*. The symbols represent means and standard error.

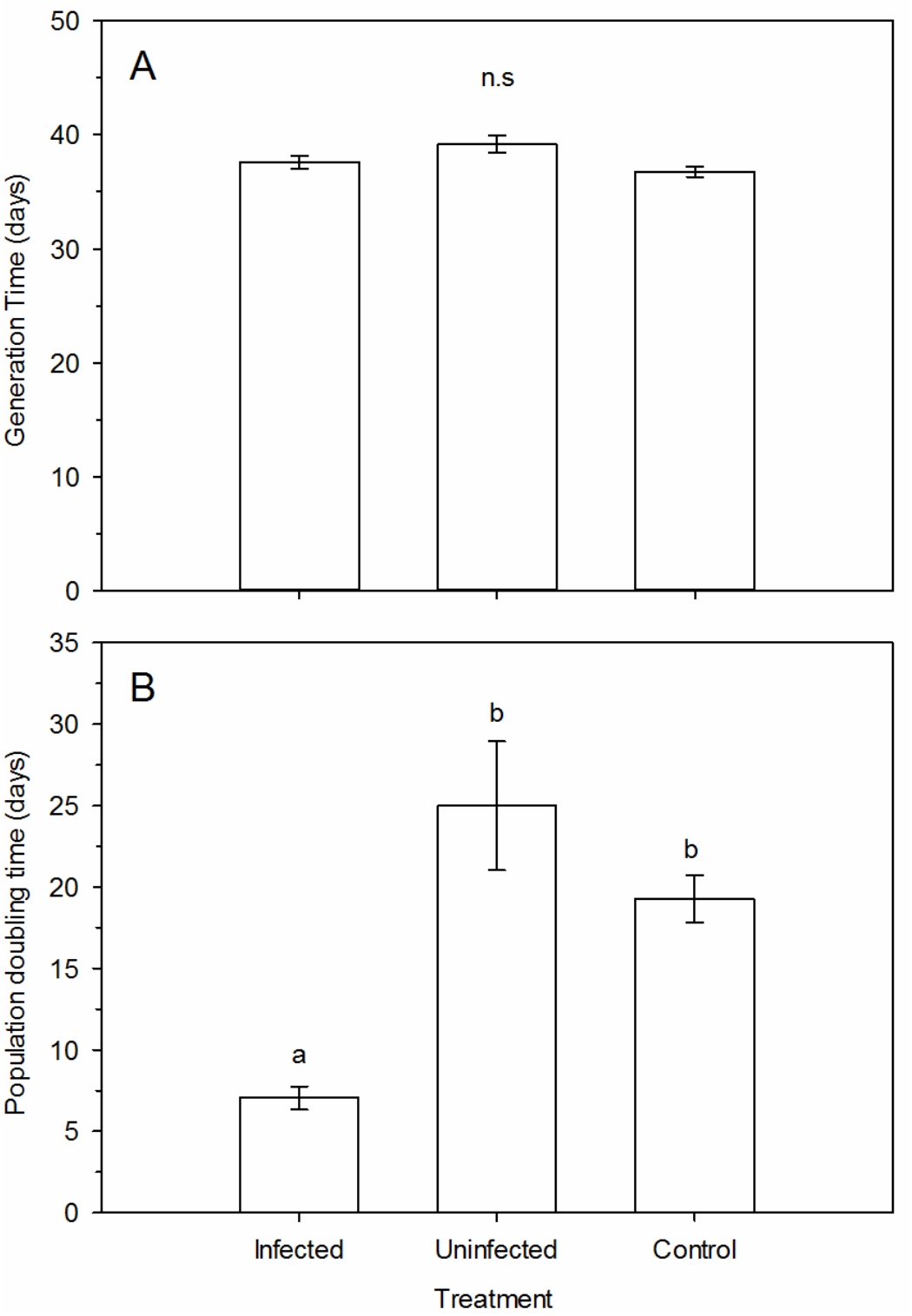

Figure 4. Mean ± SE of generation time (days) (**A**) and population doubling time (days) (**B**) of *D. citri* (Hemiptera: Psyllidae) on asymptomatic phytoplasma-infected, uninfected and healthy plants of *Citrus aurantifolia*. Different small letters represent significant differences (P<0.05) by Tukey's multiple comparison test.

Discussion

We have demonstrated that both *D. citri* and *H. phycitis* are able to transmit '*Ca*. Phytoplasma aurantifolia' to acid lime *C. aurantifolia*. To our knowledge, this is the first experimental confirmation of vectorial capacity of any insect for this system. The probability of '*Ca*. Phytoplasma aurantifolia' transmission was around 11 times greater for *H. phycitis* than for *D. citri*. Psyllids are more efficient at transmitting smaller phytoplasmas, unlike the '*Ca*. Phytoplasma aurantifolia', which it genome size is 1200 kbp. The *Cacopsylla* spp. transmits Apple Proliferation (AP) group (16SrX) phytoplasmas to pome and stone fruit trees. AP phytoplasmas are the smallest, with a genome size of 630 to 690 kbp (Marcone et al., 1999), and it may be the case that psyllids transmit more easily phytoplasmas with small size. The other psyllid genus has one vector species, *Bactericera trigonica* Hodkinson, which transmits a stolbur (Sr16XII) phytoplasma to carrots (Font et al., 1999). Moreover, another explanation for the higher transmission efficiency by *H. phycitis* may be due the '*Ca*. Phytoplasma aurantifolia' is more adapted to this species than to *D. citri*, since *D. citri* was only reported in Oman from 2005 (Al-Zadjali et al., 2008).

Phytoplasmas are transmitted by leafhoppers, planthoppers and psyllids in a persistent propagative manner. In this case, phytoplasma are acquired by vectors from phloem vascular tissues during longer periods of feeding (generally for several hours or days), and infected vectors remain able to transmit it during all their life (Sylvester 1980; Hogenhout et al. 2008). Persistent propagative transmission is advantageous to the insect vector when compared with non-persistent. In a review by Mauck et al. (2012), using virus-plant-insect vector system as a model, they showed that there are greater effects of viruses on vector attraction preference in persistent transmission systems than non-persistent systems. (Mauck et al., 2010) tested attraction based on

volatile cues for two aphid vectors (*Aphis gossypii* and *Myzus persicae*) of *Cucumber mosaic virus* and found that both winged and wingless morphs preferred the cues of virus-infected plants. Moreover, analyses in that same review suggest that vectors frequently show a preference for settling and feeding behavior on virus-infected than healthy plants. For the viruses transmitted in a persistent manner, insect vectors need to settle on the plants for feeding until acquire, for example, those viruses that are within to vascular tissues (Hogenhout et al., 2008) but also for most of those more widely distributed within the plant, including the Geminiviridae and others (Bosque-Pérez, 2000; Ng & Falk, 2006). Finally, they still discuss that study of available experiments indicates that viruses transmitted in a persistent propagative manner have largely positive effects on vector performance, such as survival, fecundity or longevity.

Both *H. phycitis* and *D. citri* collected from the field and tested by PCR nested have shown high proportion of infected insects. However, '*Ca*. Phytoplasma aurantifolia' was more abundant on *H. phycitis* than *D. citri*. Thus, these two species have a high inoculum pressure on the field. As phytoplasmas are persistently transmitted, the pathogen can remain inside the insect vectors and multiply it until they die. This means that the inoculum source by insects will always be present on the field.

'*Candidatus* Phytoplasma aurantifolia' was not found in the eggs, nymphs and newly emerged adults. Phytoplasmas vertical transmission (i.e., to the progeny of infected insects) was not considered for many years. However, recent works have demonstrated the possibility of transovarial transmission. This transmission was confirmed on the leafhopper *Matsumuratettix hiroglyphicus*, vector of the sugarcane white leaf phytoplasma (Hanboonsong et al., 2002) and also in *Hishimonoides sellatiformis*, a vector of the mulberry dwarf phytoplasma (Kawakita et al., 2000). '*Ca*. Phytoplasma prunorum' was also detected in eggs, nymphs and newly emerged adults

of *Cacopsylla pruni* psyllid (Tedeschi et al., 2006). Kawakita et al. (2000) suggested there is a connection among the phytoplasma genome size and the capacity to be transmitted transovarially. These authors noted that, for example, both the agent of aster yellows and the agent of mulberry dwarf belong to the ribosomal group 16SrI (AY), a group while there is a variability in genome size. Thus, they suggested that AY-group phytoplasmas can vary in their phenotypic characteristics, including survival in genital organs and successive phytoplasma transovarial transmission.

The fertility life table of *D. citri* was carried out again in Oman to compare if of the results would be similar with those obtained in Brazil (previous chapter). The pattern of the results were similar, since the life table parameters were higher those in phytoplasma-infected plants. Moreover, the difference between these parameters was higher in Oman than Brazil. Probability, there is a higher concentration of the '*Ca*. Phytoplasma aurantifolia' both insect vectors and plants in Oman than Brazil. More studies are being conducted to compare the phytoplasma DNA concentration in *C. aurantifolia* and insect vectors from Oman and Brazil using a quantitative real time PCR procedure.

Phytoplasma can induce several physiological and morphological changes during infection of their hosts, and phytoplasmas improve vector fitness with these changes. The increase of production of young vegetative tissues, such as witches' broom, can be more attractive to phytoplasma insect vectors. Indeed, phytoplasmas need insect vectors for transmission to other plants and, consequently, an increase in insect vector fitness would also result in an increase in phytoplasma fitness (Sugio et al., 2011b). MacLean et al. (2014) find that *Arabidopsis thaliana* plants Aster Yellows Phytoplasma-infected produce a novel effector protein (SAP54), which is able to interact with regulators of the floral development. SAP54 transforms flowers into leaves

(leaf-like flowers) and converts plants into more attractive hosts for oviposition and reproduction. The leafhopper *M. quadrilineatus* was more attractive and produced more progeny on infected plants (leaf-like flowers) than infected (non-leaf-like flowers) plants.

We concluded that both *H. phycitis* and *D. citri* are able to transmit '*Ca.* Phytoplasma aurantifolia' to acid lime (*C. aurantifolia*). *H. phycitis* was more efficient to transmit this pathogen than *D. citri*. Even in asymptomatic infected-phytoplasma plants, *D. citri* showed a populational increase higher than phytoplasma-uninfected and healthy plants. To know the main insect vectors of '*Ca*. Phytoplasma aurantifolia' is one of the first steps to plan some control measures, since after identification of these species is possible to direct the disease management.

References

Al-Sadi AM, Khan IA & Deadman ML (2004) Economic losses caused by witches' broom disease of lime and some management aspects in Shinas area of Oman. 10th International Citrus Congress, Morroco: 817-818.

Al-Zadjali TS, Ibrahim R & Al-Rawahi AK (2008) First record of *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae) from the Sultanate of Oman. Insecta Mundi 39: 1-3.

Bertaccini A (2007) Phytoplasmas: diversity, taxonomy, and epidemiology. Front. Biosci. 12: 673-689.

Bosco D & Tedeschi R (2013) Insect Vector Transmission Assays: Phytoplasma: Methods and Protocols (ed. by M Dickinson & J Hodgetts) Human Press, pp. 73-85.

Bosque-Pérez NA (2000) Eight decades of maize streak virus research. Virus Research 71: 107-121.

Bové JM (1986) Witches' broom disease of lime. FAO Plant Protection Bulletin 34: 217-218.

Bové JM (1995) Virus and virus-like diseases of citrus in the near east region. FAO Regional Office for the Near East, Rome, Italy.

Bové JM (2006) Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. Journal of Plant Pathology 88: 7-37.

Bové JM, Danet JL, Bananej K, Haasanzadeh N, Taghizadeh M, Salehi M & Garnier M (2000) Witches' broom disease of lime (WBDL) in Iran: Proceeding 14th Conference International Organization of Citrus Virology (ed., Riverside, California, pp. 207-212.

Bové JM, Garnier M, Mjeni AM & Khayrallah A (1988) Witches' broom disease of small-fruited acid lime trees in Oman: First MLO disease of Citrus.: Proceedings of the 10th Conference International Organization of Citrus Virologists (IOCV) (ed., Riverside, California, pp. 307-309.

Bové JM, Zreik L, Danet JL, Bonfils J, Mjeni AMM & Garnier M (1993) Witches' broom disease of lime trees: monoclonal antibody and DNA probes for the detection of the MLO and the identification of a possible vector: Proceedings of the 12th International Organization of Citrus Virologists (ed. Department of Plant Pathology, University of California, Riverside, California, pp. 342-348.

Carey JR (1993) Applied demography for biologists with special emphasis on insects. Oxford University Press, New York.

Chung KR, Khan IA & Brlansky RH (2006) Citrus diseases exotic to Florida: Witches' Broom Disease of Lime (WBDL). EDIS publication, University of Florida, Gainesville.

Crawley MJ (2007) The R Book. John Wiley & Sons Ltd, Chichester.

Deng S & Hiruki C (1991) Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. J Microbiol Methods 14: 53-61.

Firrao G, Garcia-Chapa M & Marzachı C (2007) Phytoplasmas: genetics, diagnosis and relationships with the plant and insect host. Front. Biosci. 12: 1353-1375.

Font I, Abad P, Albinana M, Espino AI & Dally EL (1999) Amarilleos y enrojecimientos en zanahoria: una enfermedad a diagnostico. Bol. San. Veg. Plagas 25: 405-415.

Garnier M, Zreik L & Bové JM (1991) Witches'-broom, a lethal mycoplasmal disease of lime trees in the Sultanate of Oman and the United Arab Emirates. Plant Disease 75: 546-551.

Ghosh DK, Das AK, Singh S, Singh SJ & Agrawal YS (1999) Association of a phytoplasma with witches' broom, a new disease of acid lime (*Citrus aurantifolia*). Current Science 77: 174-177.

Halbert SE & Manjunath KL (2004) Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: A literature review and assessment of risk in Florida. Fla Entomol 87: 330-353.

Hanboonsong Y, Choosai C, Panyim S & Damak S (2002) Transovarial transmission of sugarcane white leaf phytoplasma in the insect vector *Matsumuratettix hirogliphicus* (Matsumura). Insect Molecular Biology 11: 97-103.

Hogenhout S, Ammar E, Whitfield A & Redinbaugh MG (2008) Insect vector interactions with persistently transmitted viruses. Annual Review of Phytopathology 46: 327-359.

Kawakita H, Saiki T, Wei W, Mitsuhashi W, Watanabe K & Sato M (2000) Identification of mulberry dwarf phytoplasmas in the genital organs and eggs of leafhopper *Hishimonoides stellatiformis*. Phytopathology 90: 909-914.

Lee IM, Gundersen-Rindal DE, Davis RE & Bartoszyk IM (1998) Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. Int J Syst Bacteriol 48: 1153-1169.

Lumley T (2011) Survival: Survival analysis, including penalised likelihood: R package version 2. 36-39.

MacLean AM, Orlovskis Z, Kowitwanich K, Zdziarska AM, Angenent GC, Immink RGH & Hogenhout SA (2014) Phytoplasma Effector SAP54 Hijacks Plant Reproduction by Degrading MADS-box Proteins and Promotes Insect Colonization in a RAD23-Dependent Manner. PLoS Biol 12: e1001835.

Marcone C, Neimark H, Ragozzino A, Lauer U & Seemuller E (1999) Chromosome sizes of phytoplasmas composing major phylogenetic groups and subgroups. Phytopathology 89: 805-810.

Marzachi C, Veratti F & Bosco D (1998) Direct PCR detection of phytoplasmas in experimentally infected insects. Ann. Appl. Bid 133: 45-54.

Mauck K, Bosque-Pérez NA, Eigenbrode SD, De Moraes CM & Mescher MC (2012) Transmission mechanisms shape pathogen effects on host-vector interactions: evidence from plant viruses. Functional Ecology 26: 1162-1175.

Mauck KE, De Moraes CM & Mescher MC (2010) Deceptive chemical signals induced by a plant virus attract insect vectors to inferior hosts. Proceedings of the National Academy of Sciences 107: 3600-3605.

Ng JCK & Falk B (2006) Virus-vector interactions mediating non-persistent and semi-persistent transmission of plant viruses. Annual Review of Phytopathology 44: 183-212.

R Development Core Team (2014) R: A language and environment for statistical computing: R Foundation for Statistical Computing, Vienna, Austria.

Schneider B, Seemuller E, Smart CD & Kirkpatrick BC (1995) Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas: Molecular and Diagnostic Procedures in Mycoplasmology (ed. by S Razin) Academic, San Diego, pp. 369-380.

Sugio A, Kingdom HN, MacLean AM, Grieve VM & Hogenhout SA (2011a) Phytoplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis. PNAS 108: 1254-1263.

Sugio A, MacLean AM, Kingdom HN, Grieve VM, Manimekalai R & Hogenhout SA (2011b) Diverse targets of phytoplasma effectors: from plant development to defense against insects. Annu Rev Phytopathol 49: 175-195.

Swallow WH (1985) Group testing for estimating infection rates and probabilities of disease transmission. Phytopathology 75: 882-889.

Tedeschi R, Ferrato V, Rossi J & Alma A (2006) Possible phytoplasma transovarial transmission in the psyllids *Cacopsylla melanoneura* and *Cacopsylla pruni*. Plant Pathology 55: 18-24.

Weintraub PG & Beanland L (2006) Insect vectors of phytoplasmas. Annu Rev Entomol 51: 91-111.

Zreik L, Carle P, Bové JM & Garnier M (1995) Characterization of a mycoplasmalike organism associated with witches'-broom disease of lime and proposition of a *Candidatus* taxon for the organism, "*Candidatus* Phytoplasma aurantifolia". International Journal of Systematic Bacteriology 45: 449-453.

Chapter 3

Population Fluctuation of Insect Vectors and Alternative Plant Hosts of '*Candidatus* Phytoplasma aurantifolia' in Oman

Abstract

Understanding the population fluctuation of these insect vectors in the field can help us to develop an effective pest management program. Thus, the aim of this current work was to evaluate the population fluctuation of the '*Ca*. Phytoplasma aurantifolia' insect vectors in field conditions in Oman. Moreover, we also investigated the incidence of '*Ca*. Phytoplasma aurantifolia' on *C. aurantifolia* seedlings used as a "sentinel" plants in four different areas. Finally, after verifying the presence of the *Ageratum conyzoides* L. (Asteraceae) and *Phyllantus tenellus* (Euphorbiaceae), weeds commonly found in *C. aurantifolia* producing area in Oman, we also examined these two alternative host plants as a possible alternative reservoirs of '*Ca*. Phytoplasma aurantifolia'. In addition, we also investigated the ability of the insect vector, *D. citri*, to feed on *A. conyzoides*. The incidence of '*Ca*. Phytoplasma aurantifolia' on *C. aurantifolia* seedlings used as a "sentinel" plants was higher in the Samael area. We showed that those two alternative hosts are inoculum sources of '*Ca*. Phytoplasma aurantifolia' in Oman, and by which adults of *D. citri* were able to feed in one of these alternative hosts. All these aspects are indispensable to develop management strategies for the insect vectors and WBDL control.

Keywords: "sentinel" plants, weeds, *Diaphorina citri*, inoculum pressure, reservoir source, WBDL.

Introduction

Phytoplasmas are uncultivable mollicutes that lack cell wall and are etiological agents of a number of important plant diseases worldwide (Davis & Sinclair, 1998; Lee et al., 2000). Phytoplasmas have been recognized in more than 100 weed plant species, including gymnosperms, monocots and dicots (Berges et al., 2000; Lee et al., 2000; Schneider et al., 2005). An important component to understand the epidemiology of phytoplasma diseases is knowledge the varied host range of phytoplasmas.

The vector–phytoplasma–host plant three-way interaction shows an important role on the spread of phytoplasmas (Lee et al., 2003). Plant host range for each phytoplasma is determined mainly by the number of natural insect vector species capable of transmitting it and also by the feeding behavior (monophagous, oligophagous, and polyphagous) of these vectors (Lee et al., 1998). These pathogens are transmitted to plants by insect vectors with piercing-sucking mouthparts, especially leafhoppers, planthoppers and psyllids (Weintraub & Beanland, 2006).

Polyphagous insect vectors have the potential to inoculate a wide range of plant species depending on the resistance to infection of each host plant (Weintraub & Beanland, 2006). Experimentally, some phytoplasmas can be transmitted via a polyphagous vector to a wide range of host plants. For example, the North American aster yellows phytoplasmas (16SrI-A, -B) were transmitted mostly by the polyphagous leafhopper *Macrosteles fascifrons* to 191 plant species belonging to 42 families, the eastern X-disease (16SrIII-A) was transmitted by several polyphagous leafhoppers to 59 plant species belonging to 13 families (McCoy et al., 1989). In addition, phytoplasmas such as that of the beet leafhopper transmitted virescence (BLTV, subgroup 16SrVI-A), which is transmitted by a polyphagous beet leafhopper, *Circulifer tenellus*. The California aster yellows (AY, subgroup 16SrI-B), which is transmitted by numerous

polyphagous insect vectors, are capable of causing diseases in a wide variety of plant species. On the other hand, the American elm yellows phytoplasma (subgroup 16SrV-A) is transmitted by the monophagous or oligophagous vector *Scaphoideus luteolus*, causes diseases in only a few plant species, mostly in the genus *Ulmus* (Golino et al., 1989; Lee et al., 1992; Crosslin et al., 2005; Munyaneza et al., 2006; Munyaneza et al., 2007).

'*Candidatus* Phytoplasma aurantifolia' is responsible for the decline in acid lime (*Citrus aurantifolia*) production in Oman due the Witches' Broom Disease of Lime (WBDL) disease, now estimated to infect 98% of lime trees in the country (Chung et al., 2006). Alternative host plants may serve as a reservoir for this phytoplasma and insect vectors may well have contributed to its spread (Garnier et al., 1991). There are several weeds in Oman by which can serve as an alternative host of phytoplasma. These weeds are usually located under the canopy of citrus plants since only this place receives irrigation.

Up to now, the leafhopper *Hishimonus phycitis* (Hemiptera: Cicadellidae) was suspected to be a main vector of '*Ca*. Phytoplasma aurantifolia' (Ghosh et al., 1999; Salehi et al., 2007). In our work it was possible to show that this leafhopper is, indeed, a vector of this phytoplasma. Besides this species, we were also able to identify *Diaphorina citri* as a vector of phytoplasma. To understand the population dynamics of these insect vectors in the field can help us to develop an effective pest management program. Thus, the aim of this current work was to investigate the population dynamics of '*Ca*. Phytoplasma aurantifolia' insect vectors in the field in Oman. We evaluated the *A. conyzoides* L. (Asteraceae) and *P. tenellus* (Euphorbiaceae) weed plants, commonly find in *Citrus aurantifolia* producing area in Oman, as possible alternative reservoirs of

the '*Ca*. Phytoplasma aurantifolia'. In addition, we also investigated the ability of the insect vector, *D. citri*, feeding on *A. conyzoides*.

Material and Methods

Field Sites

This work was carried out in four different areas in Oman as described below.

Table 1. Descriptions of the four different areas located in two different regions in Oman.

Region	Area	Elevation	Temperature[a]	Coordinates
Al-Batinah	Barka	45	31.8	N23°38.684' E058°01.490'
Al-Batinah	Al-Suwaiq	37	31.7	N23°48.130' E057°26.135'
Al-Batinah	Musanah	27	30.8	N23°43.038' E057°33.786'
Dakhliyah	Samael	425	31.7	N23°35.288' E058°08.687'

[a]Temperature (ºC) represents the mean of each field sites during all evaluated period.

Population Fluctuation

Sticky yellow traps (24x12 cm) were placed in the four different areas in Oman to record populational fluctuations of leafhoppers, planthoppers and psyllids, the main vectors of phytoplasma. In each area we monitored three farms producing acid lime (*C. aurantifolia*). The traps were placed in the middle part of the plants and were 10m distant from one another. The number of traps in each farm was in accordance with the number of *C. aurantifolia* plants, ranging from 15 to 20 traps per farm. The traps always were replaced when necessary. Insects of those groups cited above were counted monthly and removed from the traps with aid of forceps for posterior identification and molecular analysis to test for the presence of phytoplasma. The data were collected from June 2013 to March 2014.

Sentinel Plants

Phytoplasma-free acid lime seedlings, confirmed by nested PCR analysis as described above, were placed in the fields as "sentinel" plants to evaluate vectorborne inoculum pressure in the field through the year. In each farm, twenty plants were placed in October 2013 in the field and planted in the ground such that they received irrigation. Twenty leaf samples were removed from each seedling after three months and these were taken to the laboratory for evaluate the presence of phytoplasma by nested PCR as described in the previous chapter. All this process will continue to be done on every three months for a total of twelve months. Here we present data of the first assessment.

Weeds

Leaf samples of *A. conyzoides* and *P. tenellus* plants with and without witches' broom symptoms (Figure 1) were collected, beyond those four areas, in more six different citrus fields in Oman. These fields were chosen according to the highest WBDL incidence in Oman. These samples were tested by nested PCR as described in the previous chapter.

Figure 1. Alternative host weeds *Phyllanthus tenellus* asymptomatic (A) and symptomatic (B); and *Ageratum conyzoides* L. asymptomatic (C) and symptomatic (D).

Survival of *Diaphorina citri* on alternative hosts

Fourteen adults of *D. citri* were placed inside an insect-proof cage (100 x 70 x 70 cm) containing one *A. conyzoides* plant to investigate if *D. citri* was able to feed on this alternative host. The same was done for nymphs but we used 20 second instar nymphs per plant. We used five plants for the nymph survival experiment and other five for adults. The insect survival was evaluated each two days. All the experiment was carried out inside the glasshouse with the same conditions as described on previous chapter.

The survival curves (l_x) were obtained using Kaplan-Meier survival distributions (Crawley, 2007) with the aid of the 'survival' package (Lumley, 2011) as described previously. The analyses were conducted using software R version 2.13.0 (R Development Core Team, 2014).

Results

The incidence of '*Candidatus* Phytoplasma aurantifolia' on *Citrus aurantifolia* seedlings used as a "sentinel" plants was higher in the Samael area, which approximately 94% of the *C. aurantifolia* infected after three months. Al-Suwaiq, Musanah and Barka areas showed 25, 21 and 15% of infected plants, respectively (Table 2). In Samael area all acid lime plants were showing WBDL symptoms. Thus, the '*Ca*. Phytoplasma aurantifolia' transmission is mainly due to the high incidence of WBDL of the plants on the field. Therefore, only a few insects are able to transmit efficiently the pathogen.

Table 2. The incidence of WBDL on acid lime plants on the field and '*Candidatus* Phytoplasma aurantifolia' on *Citrus aurantifolia* seedlings used as a "sentinel" plants planted in four different areas in Oman.

Area	WBDL on the field (%)	Symptomatic seedlings (%)	PCR positive/ analyzed plant	(%) Infected seedlings
Samael	100	0	17/18	94.4
Musanah	7	0	4/19	21
Barka	5	0	3/20	15
Al-Suwaiq	4	0	5/20	25

Hishimonus phycitis and *D. citri* insect vectors were present in all the fields during the evaluated period (Figure 2). In Samael, *D. citri* began to appear after November. The mean number of *D. citri* per trap was mostly higher than *H. phycitis* in Barka (Figure 2A), Musanah (Figure 2B) and Al-Suwaiq (Figure 2D). Only in Samael did *H. phycitis* show a higher density than *D. citri* (Figure 2C). Moreover, the mean number of the *H. phycitis* was also higher than *D. citri* in June and August in Barka; and in December until March in Musanah. We also evaluated the population fluctuation of *H. phycitis* and *D. citri* during 16 weeks on another farm in the Agriculture Experimental Station (AES), belonging to the Sultan Qaboos University (SQU). In this area, the mean number of *D. citri* per trap was also mostly higher than *H. phycitis*.

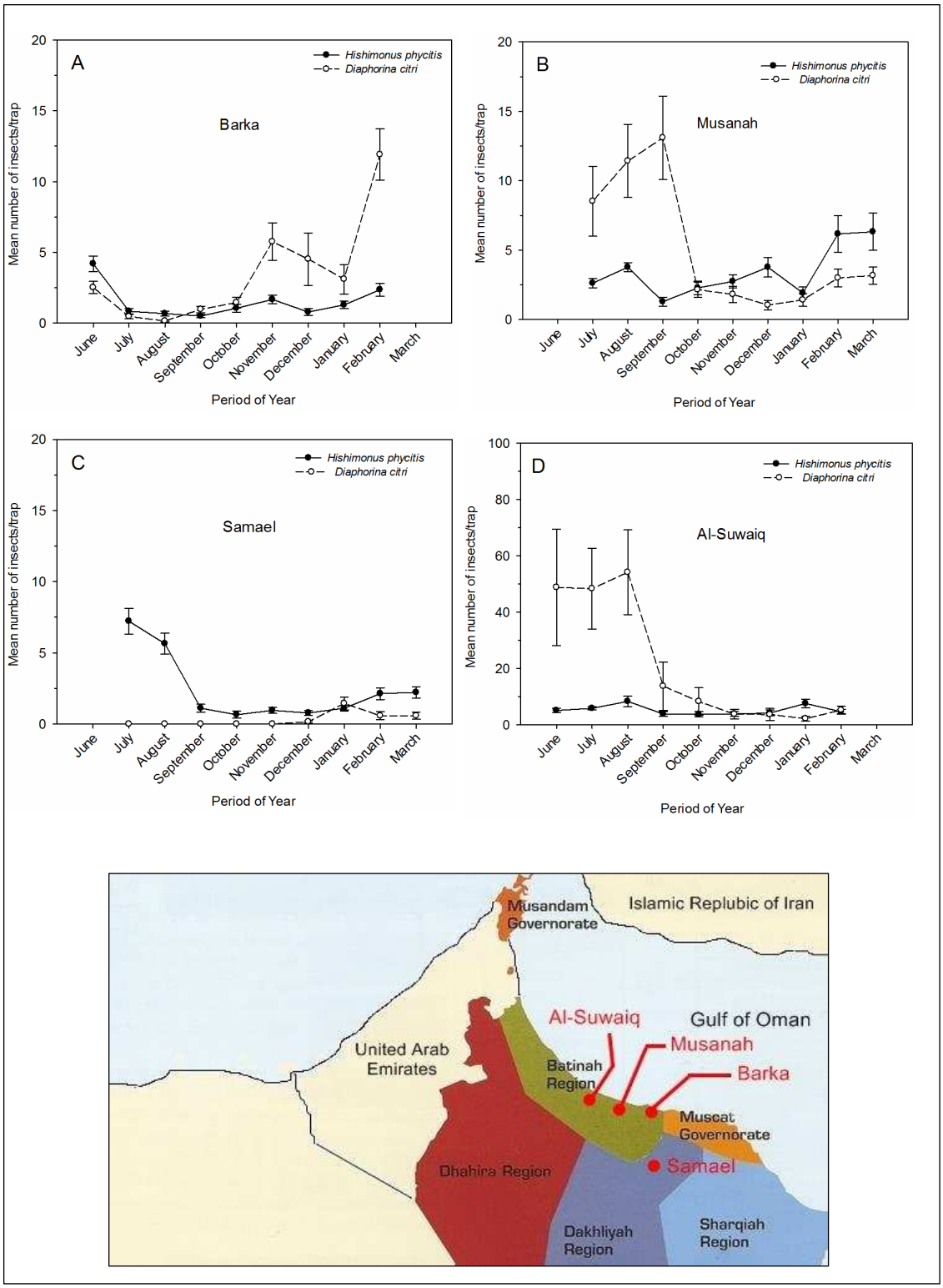

Figure 2. Populational fluctuation of *Hishimonus phycitis* (Hemiptera: Cicadellidae) and *Diaphorina citri* (Hemiptera: Psyllidae) collected by sticky yellow traps in Barka (**A**), Musanah (**B**), Samael (**C**) and Al-Suwaiq (**D**) areas in Oman. Vertical lines on the population density graphs represent the standard error for the means.

All the areas in which we collected samples from *A. conyzoides* and *P. tenellus* were infected by '*Ca*. Phytoplasma aurantifolia' and showing WBDL symptoms. At least one of the *A. conyzoides* plants was infected in each area by '*Ca*. Phytoplasma aurantifolia' in symptomatic plants. Otherwise, even in *P. tenellus* plants without WBDL symptoms also were infected. *P. tenellus* showed more infection rate by '*Ca*. Phytoplasma aurantifolia', around 32%, than *A. conyzoides*, around 21% (Table 3).

Table 3. The incidence of '*Candidatus* Phytoplasma aurantifolia' in symptomatic and asymptomatic alternative host plants, *Ageratum conyzoides* (Asteraceae) and *Phyllantus tenellus* (Euphorbiaceae) collected from different region of Oman. The numbers represent the proportion of the PCR positive/analyzed samples.

Region	*Ageratum conyzoides*		*Phyllantus tenellus*	
	Symptomatic	Asymptomatic	Symptomatic	Asymptomatic
AES/SQU	1/3	0/3	1/3	0/3
Al-Rustaq	1/3	—	—	—
Al-Suwaiq	1/3	0/3	1/7	2/3
Barka	3/6	0/9	5/6	2/6
Mahdah	—	—	1/3	—
Musanah	1/3	0/3	1/9	0/4
Nizwa	4/6	0/3	3/6	—
Samael	1/4	0/6	1/4	0/3
Shinas	1/3	0/3	1/3	—
Sohar	1/3	0/3	2/3	—

Diaphorina citri nymphs were not able to feed on *A. conyzoides*, which the survival time mean of the nymphs was approximately 6 days. On the other hand, the adults were able to feed from the same host until 32 days and the survival time mean was 20 days (Figure 3) (Log-rank test: $\chi^2 = 220.3$, df = 1, $p<0.001$). This period is enough for *D. citri* acquire the '*Candidatus* Phytoplasma aurantifolia' and consequently to be able to transmit it.

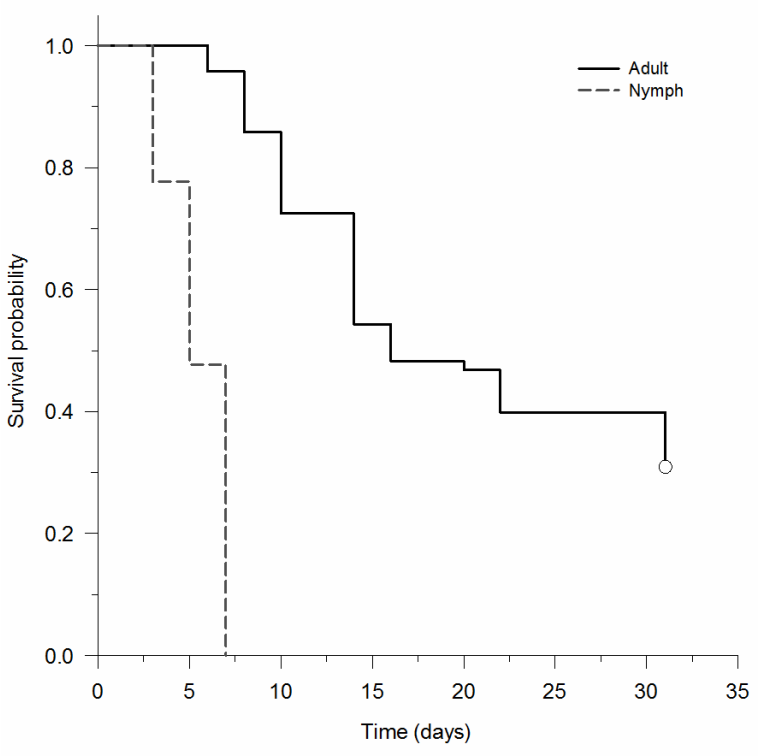

Figure 3. Survival curves of *Diaphorina citri* adults (solid black line) and nymphs (dash gray line) when fed on *Ageratum conyzoides* alternative host plants.

Discussion

The incidence of '*Candidatus* Phytoplasma aurantifolia' on *Citrus aurantifolia* seedlings used as a "sentinel" plants was higher in Samael area. This area is different from the others, in that it has small farms known as "traditional farm" and is located in Oman's interior. This place has milder weather because it is a higher region when compared to the coastal region. "Traditional farms" are characterized by planting different crops in the same area, making it a favorable microclimate for the development of insects. In Samael, all the acid lime plants were showing clear WBDL symptoms and they were also more than 20 years old. Moreover, the farmers use the air layering (or marcotting) method for propagating citrus plants. In this layering method, roots are induced to form on the part of the plant while it remains aerial (aboveground), therefore the term air layering (Lerner & Dana, 2001). Thus, as the citrus plants are 100%

infected in Samael area, the spread of infected plants from this area is huge within the orchards and also by the country.

The populational density of *D. citri* and *H. phycitis*, throughout the study period, was not higher in Samael area but in Al-Suwaiq. Therefore, the population density of the insect vectors was not directly related to the proportion of symptomatic plants. If all the acid lime plants are phytoplasma-infected, even a few insect vectors will be capable of spreading the pathogen efficiently inside and outside the orchards. Therefore, the control of the WBDL disease only via insect vectors is an inefficient method. Thus, together with insect vector control, the first step for efficient WBDL disease control is to use citrus seedlings with certified quality, i.e. free of any pathogens.

As in Brazil, Oman has a structure for certified seedling production. It has a system for production of the several types of seedlings, including citrus, in protected environment (greenhouses). However, these places are only for seedlings for urban forestry and the Sultan's family members or government people. None of these seedlings are provided to the farmers. The seedlings for farmers are available for purchase from nurseries, which they do not manage of the seedlings producing accordingly against pests and diseases, or directly from those other farms by which use the air layering as propagation method.

The period when *D. citri* had a higher population density than *H. phycitis* is directly related with the period after growing of *C. aurantifolia* plants. In this period there was a large formation of new branches and, consequently, greater oviposition of *D. citri*, since this insect only lays eggs in shoots (Hall & Albrigo, 2007). On the other hand, *H. phycitis* population fluctuation was mostly constant and it is able to reproduce actively on acid lime trees. Shabani et al. (2013) analyzed nine microsatellite loci that had been isolated from *H. phycitis*. On the basis of the microsatellite data, populations

from Iran and north Omani were grouped into two separate clades. Thus, they concluded that *H. phycitis* migrated into Iran from Oman, with the transfer probably occurring through contaminated citrus plants. The populations of *H. phycitis* in north Oman became separate and distinct from those in Iran after the species had crossed into Iran. There is also the possibility that populations of *H. phycitis* in different regions vary in terms of their ability to transmit '*Ca*. Phytoplasma aurantifolia', which would affect the status of *H. phycitis* as an acid lime pest in different areas. Furthermore, the preferences for host plants and reproductive behavior of this leafhopper vector might vary and might also affect its status as a pest and the efficiency of control programs.

We have identified two new alternative hosts of '*Ca*. Phytoplasma aurantifolia' in Oman, and by which adults of *D. citri* was able to feed in one of these alternative hosts. Preferential host plants are most frequently visited by polyphagous insect vectors. Occasionally, these kinds of insect vectors may voluntarily feed or they are forced to feed on nonhost plants, in cases in which nonhost plants are available. The nonhost plants will become infected if they are susceptible to the phytoplasma carried by the visiting vectors. Thus, a new ecological niche for the phytoplasma can be created (Lee et al., 1998). A lot of pathogens that can infect multiple hosts can also be transmitted by multiple hosts, and these can be considered as ecological generalists rather than specialists. The evolution of generalists requires that pathogens have both the capability to exploit potential alternative host species and the opportunity to transmit to them. After that, the maintenance of generalists depends on the consequences of an increased host range for pathogen population biology, especially features such as pathogenicity and epidemiology (Woolhouse et al., 2001). When generalist pathogens are dispersed by polyphagous vectors which visit many hosts, the number and diversity of interactions between pathogen and host can be huge.

We show that both *H. phycitis* and *D. citri* were present in the field during all the evaluated period. The most important factor in WBDL disease spread is the high inoculum pressure from the acid lime and some alternative host plants, which many or few insect vectors are able to transmit the '*Ca*. Phytoplasma aurantifolia' efficiently.

Due the characteristics of acid lime production in Oman, by which the farms are up to 5 ha, suggested management of WBDL is eliminate the WBDL symptomatic and asymptomatic plants in the orchards and the subsequent planting of other plants, resistant or tolerant to the phytoplasma and also other pathogens, acquired from certified seedling production. Moreover, the farmers must also manage the alternative host plants inside and around the orchards, eliminating all of them.

References

Berges R, Rott M & Seemuller E (2000) Range of phytoplasma concentrations in various plant hosts as determined by competitive polymerase chain reaction. Phytopathology 90: 1145-1152.

Chung KR, Khan IA & Brlansky RH (2006) Citrus diseases exotic to Florida: witches' broom disease of lime (WBDL): Plant Pathology Department, Florida Cooperative Extension Service, Institute of Food and Agricultural Sciences, University of Florida.

Crawley MJ (2007) The R Book. John Wiley & Sons Ltd, Chichester.

Crosslin JM, Munyaneza JE, Jensen AS & Hamm PB (2005) Association of the beet leafhopper (Hemiptera: Cicadellidae) with a clover proliferation group phytoplasma in the Columbia Basin of Washington and Oregon. Journal of Economic Entomology 98: 279-283.

Davis DL & Sinclair WA (1998) Phytoplasma identity and disease etiology. Phytopathology 88: 1372-1376.

Garnier M, Zreik L & Bové JM (1991) Witches'-broom, a lethal mycoplasmal disease of lime trees in the Sultanate of Oman and the United Arab Emirates. Plant Disease 75: 546-551.

Ghosh DK, Das AK, Singh S, Singh SJ & Agrawal YS (1999) Association of a phytoplasma with witches' broom, a new disease of acid lime (*Citrus aurantifolia*). Current Science 77: 174-177.

Golino DA, Oldfield GN & Gumpf DJ (1989) Experimental hosts of the beet leafhopper-transmitted virescence agent. Plant Disease 73: 850-854.

Hall DG & Albrigo LG (2007) Estimating the relative abundance of flush shoots in citrus, with implications on monitoring insects associated with flush. HortScience 42: 364-368.

Lee IM, Davis RE & Gundersen-Rindal DE (2000) Phytoplasma: phytopathogenic mollicutes. Annual Review of Microbiology 54 221-255.

Lee IM, Gundersen-Rindal D, Davis R & Bartoszyk M (1998) Phytoplasma ecology and diversity. Phytopathology 88: 1359-1366.

Lee IM, Gundersen DE, Davis RE & Chiykowski LN (1992) Identification and analysis of a genomic strain cluster of mycoplasmalike organisms associated with Canadian peach (eastern) X-disease, western X-disease, and clover yellow edge. J. Bacteriol. 174: 6694-6698.

Lee IM, Martini M, Bottner KD, Dane RA, Black MC & Troxclair N (2003) Ecological implications from a molecular analysis of phytoplasmas involved in an aster yellows epidemic in various crops in Texas. Phytopathology 93: 1368-1377.

Lerner BR & Dana MN (2001) New Plants From Layering: Purdue University Cooperative Extension Service, Department of Horticulture, pp. 1-3.

Lumley T (2011) Survival: Survival analysis, including penalised likelihood. R package version 2: 36-39.

McCoy RE, Caudwell A, Chang CJ, Chen TA, Chiykowski LN, Cousin MT, Dale JL, de Leeuw GTN, Golino DA, Hackett KJ, Kirkpatrick BC, Marwitz R, Petzold H, Sinha RH, Sugiura M, Whitcomb RF, Yang IL, Zhu BM & Seemüller E (1989) Plant diseases associated with mycoplasmalike organisms, Vol. 5: The Mycoplasmas (ed. by RF Whitcomb & JG Tully) Academic Press, New York, pp. 545-560.

Munyaneza JE, Crosslin JM & Lee IM (2007) Phytoplasma diseases and insect vectors in potatoes of the Pacific Northwest of the United States. Bulletin of Insectology 60: 181-182.

Munyaneza JE, Crosslin JM & Upton JE (2006) The beet leafhopper (Hemiptera: Cicadellidae) transmits the Columbia Basin potato purple top phytoplasma to potatoes, beets, and weeds. Journal of Economic Entomology 99: 268-272.

R Development Core Team (2014) R: A language and environment for statistical computing: R Foundation for Statistical Computing, Vienna, Austria

Salehi M, Izadpanah K, Siampour M, Bagheri A & Faghihi SM (2007) Transmission of '*Candidatus* Phytoplasma aurentifolia' to Bakraee (*Citrus reticulata* Hybrid) by feral *Hishimonus phycitis* leafhoppers in Iran. Plant Disease 91: 466.

Schneider B, Torres E, Martín MP, Schroder M, Behnke H-D & Seemuller E (2005) '*Candidatus* Phytoplasma pini', a novel taxon from *Pinus silvestris* and *Pinus halepensis*. Int. J. Syst. Evol. Microbiol. 55: 303-307

Shabani M, Bertheau C, Zeinalabedini M, Sarafrazi A, Mardi M, Naraghi SM, Rahimian H & Shojaee M (2013) Population genetic structure and ecological niche modelling of the leafhopper *Hishimonus phycitis*. J Pest Sci 86: 173-183.

Weintraub PG & Beanland L (2006) Insect Vectors of Phytoplasmas. Annu. Rev. Entomol. 51: 91-111.

Woolhouse MEJ, Taylor LH & Haydon DT (2001) Population Biology of Multihost Pathogens. Science 292: 1109.

PERSPECTIVES

- ✓ Characteristics of phytoplasma infection that are able enhance population growth of insect vectors may not be morphological, as we show an improvement in *D. citri* fitness even in phytoplasma-infected asymptomatic plants. This phenomenon can elucidate the mechanisms by which a pathogen affects its plant host and may provide leads to help decrease the effects of this pathogen in citrus industry in Brazil and Oman.
- ✓ As we have demonstrated that two insect species are vector phytoplasma in Oman, management strategies can be focused on these species and, at the same time, we can use the same method to identify other possible vectors of phytoplasma pathogens.
- ✓ If control of the phytoplasma vectors in Oman is to be undertaken, it must be done through the year due the high and constant presence of these insects on the field;
- ✓ The Sultanate of Oman needs to improve the citrus production system aiming acquires pathogen-free seedlings. For this it is necessary to be a cooperation among the government (Ministry of Agriculture), Sultan Qaboos University and rural extension agents so that the results obtained reach the farmers. Furthermore, some techniques for citrus propagation, such as grafting, should be passed to farmers in Oman for the faster seedlings production and with higher quality. The farms also need to avoid or stop to produce seedlings by air layering propagation since this method is completely unusual and harmful for the citrus production.