

**UNIVERSIDADE ESTADUAL PAULISTA – UNESP
CÂMPUS DE JABOTICABAL**

**INSIGHTS ON THE INTERACTION BETWEEN RUTACEAE
GENOTYPES AND THE ‘*Candidatus Liberibacter asiaticus*’
BACTERIUM**

**Mônica Neli Alves
Biotechnologist**

2022

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Ma. Mônica Neli Alves

Advisor: Prof. Dr. Jesus Aparecido Ferro

Co-Advisor: Prof. Dr. Leandro Antonio Peña Garcia

**Tese apresentada à Faculdade de Ciências
Agrárias e Veterinárias – Unesp, Câmpus de
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a obtenção do título de Doutor em
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UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Jaboticabal



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TÍTULO DA TESE: INSIGHTS ON THE INTERACTION BETWEEN RUTACEAE GENOTYPES AND THE *Candidatus Liberibacter asiaticus* BACTERIUM

AUTORA: MÔNICA NELI ALVES

ORIENTADOR: JESUS APARECIDO FERRO

COORIENTADOR: LEANDRO ANTONIO PEÑA GARCIA

Aprovada como parte das exigências para obtenção do Título de Doutora em Agronomia (Genética e Melhoramento de Plantas), pela Comissão Examinadora:

Prof. Dr. JESUS APARECIDO FERRO (Participação Presencial)
Departamento de Biotecnologia Agropecuária e Ambiental / FCAV - UNESP - Jaboticabal

Prof. Dr. JOSE BELASQUE JÚNIOR (Participação Presencial)
Departamento de Fitopatologia e Nematologia / ESALQ USP Piracicaba/SP

Prof. Dr. DANIEL GUARIZ PINHEIRO (Participação Presencial)
Departamento de Biotecnologia Agropecuária e Ambiental / FCAV UNESP Jaboticabal

Dra. ELIANE CRISTINA LOCALI FABRIS (Participação Presencial)
Fundo de Defesa da Citricultura - FUNDECITRUS / Araçatuba/SP

Prof. Dr. ANTONIO DE GÓES (Participação Presencial)
Departamento de Ciências da Produção Agrícola / FCAV / UNESP - Jaboticabal

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Impact record

The confirmation of HLB-resistant genotypes in citrus relatives, as well as the sequencing and transcriptomics informations generated on this work, opens promising perspectives for a more effective and sustainable control of the main disease that affects citrus culture worldwide.

AUTHOR'S INFORMATION

MÔNICA NELI ALVES - Born on October 15th of 1994, in Unaí-MG. Graduated in Biotechnology (Federal University of Uberlândia/2013-2016), where she participated on the scientific initiation program (IC) “Young Talents for Science” (CAPES/CNPq) conducting studies in the Soybean Breeding Program. She also did an IC by FAPEMIG (Fundação de Apoio à Pesquisa de Minas Gerais), working on the characterization of secreted proteins and outer membrane vesicles of the bacterium *Xylella fastidiosa*. She performed an internship at EMBRAPA Cenargen, Brasília-DF, working with tissue culture and plant transformation by *Agrobacterium tumefaciens* and Bioballistics. After the internships at this institution, she did an IC by FAPDF (Fundação de Apoio à Pesquisa do Distrito Federal) working with Plant Virology. Still at graduation, she was a member of the academic directory DAMAB (Diretório Acadêmico Malcom Antônio Brandeburgo), Director of Research and Development and Director of Projects at the Junior Company TriBiotec and monitors in the disciplines “Plant Breeding” and “Molecular Markers”. In 2017, she joined the Professional Master's Degree in Plant Health at Fundecitrus, Araraquara-SP. During her master's degree, she worked on identification of sources of resistance to ‘*Candidatus Liberibacter asiaticus*’ (Las). At the time, she worked with biochemical characterization by HPLC and qPCR of false blood oranges. After completing her master's degree in 2018, she received the award for “Best MasterCitrus Student”. Then, she started the PhD in Agronomy (Genetics and Plant Breeding) at Unesp/FCAV (Capes) studying the gene expression of species with different susceptibility/resistance responses to Las, during which he stayed for 3 months at the CSIC – Valencia, Spain. In addition, she continues in partnership with Fundecitrus in the activities involving the generating of Las resistant x Las susceptible Rutaceae population that will be used for genetic mapping and QTLs identifications of the resistant trait.

DADOS CURRICULARES DO AUTOR

MÔNICA NELI ALVES – Nascida em 15/10/1994, em Unaí-MG. Graduada em Biotecnologia (Universidade Federal de Uberlândia/2013-2016), onde participou do programa de iniciação científica (IC) “Jovens Talentos para a Ciência” (CAPES/CNPq) realizando estudos no Programa de Melhoramento de Soja. Também realizou IC pela FAPEMIG (Fundação de Apoio à Pesquisa de Minas Gerais), trabalhando na caracterização de proteínas secretadas e de vesículas de membrana externa da bactéria *Xylella fastidiosa*. Realizou estágio na EMBRAPA Cenargen, Brasília-DF, trabalhando com cultura de tecidos e transformação de plantas via *Agrobacterium tumefaciens* e Biobalística. Após os estágios nesta instituição, realizou IC pela FAPDF (Fundação de Apoio à Pesquisa do Distrito Federal) trabalhando com Virologia Vegetal. Ainda na graduação, foi membro do diretório acadêmico DAMAB (Diretório Acadêmico Malcom Antônio Brandeburgo), Diretora de Pesquisa e Desenvolvimento e Diretora de Projetos da Empresa Júnior TriBiotec e monitora nas disciplinas “Melhoramento de Plantas” e “Marcadores Moleculares”. Em 2017, ingressou no Mestrado Profissional em Fitossanidade do Fundecitrus, Araraquara-SP. Durante o mestrado, trabalhou na identificação de fontes de resistência à ‘*Candidatus Liberibacter asiaticus*’ (Las). Na época, trabalhou também com caracterização bioquímica via HPLC e expressão gênica (qPCR) de laranjas falsas sanguíneas. Após concluir o mestrado em 2018, recebeu o prêmio de “Melhor Aluna MasterCitrus”. Em seguida, iniciou o doutorado em Agronomia (Genética e Melhoramento de Plantas) na Unesp/FCAV (Capes) estudando a expressão gênica de espécies com diferentes respostas de suscetibilidade/resistência a Las, período em que permaneceu por 3 meses no CSIC – Valência, Espanha. Além disso, continua em parceria com o Fundecitrus nas atividades que envolvem a geração de população de Rutaceae Las resistente x Las suscetível que serão utilizadas para mapeamento genético e identificação de QTLs relacionados a característica de resistência.

I dedicate,
To my parents, Maurício and Ivete.

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INSIGHTS ON THE INTERACTION BETWEEN RUTACEAE GENOTYPES AND THE '*Candidatus Liberibacter asiaticus*' BACTERIUM

ABSTRACT – In Brazil, the *Citrus* disease Huanglongbing (HLB) is mostly associated with the presence of the phloem-limiting and unculturable '*Candidatus Liberibacter asiaticus*' which is transmitted by the insect vector *Diaphorina citri*. Although all *Citrus* species are susceptible, Las resistant *Microcitrus* and *Eremocitrus* have recently been reported. Aiming to gain more insights on the resistant phenotype, a set of five experiments were done through which we evaluated: (1) Las acquisition by *D. citri* fed onto the resistant genotypes; (2) Las infection in sweet orange plants grafted with bark or budwood from them; (3) Las infection in sweet orange plants top grafted onto them; (4) Las infection in new shoots from rooted plants of them; and (5) Las infection in new shoots of them after drastic back pruning. Results confirmed the full-resistance of those materials and a morphological and genotyping by sequencing analysis confirmed the genetic background informations that are essential to map properly genomic regions for a citrus breeding program. Apart from those genotypes, *Murraya paniculata* and *Berberis koenigii* have been described as transient and immune hosts to the HLB bacterium, respectively. However, differently from *Eremocitrus* and *Microcitrus*, they are neither graft nor cross-compatible with *Citrus*. Aiming to gain more information of the Las-plant host interaction on these two species and sweet orange, we studied the time course of Las multiplication and whole-plant colonization immediately after the inoculation by infected psyllids feeding onto them for 2 days. We observed that soon after psyllids removal, Las titers dropped until the 12th days in all three species. After that, Las increased exponentially in *C. × sinensis* and *M. paniculata*. In *C. × sinensis*, Las reached a stationary phase from the 40th day onward, while in *M. paniculata*, Las titer increased at a lower rate between the 40th and 60th days, decreasing gradually thereafter. In *B. koenigii*, Las titer decreased from the start and remained undetectable along the experiment. Furthermore, we observed that it was required 23.6 days for Las to move from the flush where inoculation was done and colonize the entire plant of *C. × sinensis*. Following those observations, we proposed an RNA-seq analysis on the key-time points that we selected as most crucial for either resistance or susceptibility responses. When comparing plants exposed to Las+ insects vs healthy insects a low number of significant differently expressed genes were observed. However, when

comparing challenged-inoculated samples with samples not exposed to insects it was observed that *B. koenigii* did not undergo any change in expression and largely arrested their transcriptome due to infection until 10 days after being exposed to Las+ insects. GSEA analysis suggested that photosynthesis was restricted when samples were exposed to Las+ insects, being these decrease stronger in *B. koenigii* than in sweet orange. Taken together, our results allowed us to observe that plant cell defense response to Las infections was only active after few days of bacterial infection. However, more analysis should be done to try to gain more insight on those interactions.

Keywords: Aurantioideae, citrus breeding, greening, HLB, RNA-seq

CHAPTER 1 – General considerations

1. INTRODUCTION

Huanglongbing (HLB) is the most devastating *Citrus* disease and is associated with the presence of the Gram-negative, α -proteobacteria '*Candidatus Liberibacter*' spp., which colonize the phloem of their host plants (Jagoueix et al., 1994; Bové, 2006). In Brazil, two species of this bacterium were described, '*Ca. L. asiaticus*' (Las) and '*Ca. L. americanus*' (Lam), being both transmitted by the Asian citrus psyllid *Diaphorina citri* Kuwayama (Sternorrhyncha: Liviidae) (Capoor et al., 1967; Coletta-filho et al., 2004; Teixeira et al., 2005). Las is the most destructive one and it is present in more than 99% of the samples analyzed in the diagnostic laboratory of Fundecitrus (Bassanezi et al., 2020). Besides Brazil, Las is present in Central America, Caribe, Colombia, Bolivia, Paraguay, North Argentina, Iran, Oman, Pakistan, India, China, South Asia, South Japan, Philippines, Papua New Guinea and Indonesia (Pre-HLB, 2022), being responsible for the devastation of orange orchards in Florida-USA (Gottwald, 2010).

In Brazil, the control is based on the use of healthy trees from certified nurseries, the monitoring and chemical control of *D. citri* to reduce the population of the insect vector and eradication of symptomatic trees. Those strategies have been used since the discovery of the disease (Bassanezi et al., 2020). However, they are not environment neither economically sustainable. Also, despite their use, the disease is still increasing, reaching 24.42% of the plants in 2021 in the citrus park which encompasses the state of São Paulo and the Triângulo Mineiro (Fundecitrus, 2022). Therefore, researchers all over the world keep maintaining efforts on trying to improve knowledge on the host-pathogen pathosystem to try to elucidate a more attractive and functional solution.

The use of resistance varieties would be of great interest to try to mitigate the disease. However, there is no resistance species on the *Citrus* genera (Folimonova et al., 2009). Recently, species more or less phylogenetically distant from *Citrus* were irrefutably described as being source of Las-resistance (Ramadugu et al., 2016; Beloti et al., 2018; Cifuentes-Arenas et al., 2019; Alves, et al., 2021a; 2021b; 2022).

Phylogenetically closer to the *Citrus*, there are *Eremocitrus glauca* and some species of the genera *Microcitrus* and hybrids among them that were described as fully

resistant to Las (Ramadugu et al., 2016; Alves, et al., 2021b). After propagation onto the susceptible 'Rangpur lime' rootstock and two years of graft-inoculation, accession from those genus remained without the presence of Las even being the rootstock colonized by Las (Alves et al., 2021b). However, Las was detected in their stem bark above the scion-rootstock graft union.

Eremocitrus glauca and *Microcitrus* are monoembryonic species and compatible with *Citrus* for grafting and pollination, opening possibilities of breeding until now unimaginable (Swingle and Reece, 1967). Las resistant and graft-compatible varieties can be evaluated to be used directly as rootstock or inter-stock or introduced in rootstock breeding programs aiming to generate resistant materials for Brazil's orchards. On the other hand, Las-resistant cross-compatible species can be used in breeding programs as source of resistance to generate new canopies or as parents in contrasting crosses aiming to obtain hybrids populations that can be used in genetic mapping. Genetic mapping is a useful tool to overview the complex relationships between genes and the environment. It is an interesting approach to understand the inheritance of the resistance or susceptibility to biotic stress and to map important genes in the genome (Huang et al., 2018).

In this study, aiming to gain an understanding of the full-resistance phenotype, new experiments were carried out with the challenge-inoculated genotypes through which we evaluated: (1) Las acquisition by *D. citri* fed onto them; (2) Las infection in sweet orange plants grafted with bark or budwood from them; (3) Las infection in sweet orange plants topgrafted onto them; (4) Las infection in new shoots from rooted plants of them; and (5) Las infection in new shoots of them after drastic backpruning.

Regarding more distant phylogenetically genus, *Murraya paniculata* and *Bergera koenigii*, were described as partially resistant and immune to the HLB bacterium. However, they are both more attractive to the insect vector than the *Citrus* species (Damsteegt et al., 2010; Teck et al., 2011; Westbrook et al., 2011; Beloti et al., 2018; Cifuentes-Arenas et al., 2019; Alves et al., 2021a). They are species from the same subfamily as the *Citrus*, the Aurantiodeae, and does not have a good compatibility for grafting and neither for pollination with *Citrus* (Swingle and Reece, 1967; Raiol-Junior et al., 2022). Because of it, the use of those species in breeding

programs are more limited to bioinformatics and biotechnological approaches (Oliveira et al., 2013).

As *M. paniculata* and *B. koenigii* are not compatible for graft neither pollination, transcriptomic studies came as a possible tool for studying the resistant trait of those species. When studying plant-pathogen interactions, researchers should follow standards for methods. The bioassay design for tissue collection over which transcript profiles will be monitored is critical and should be chosen based on observations of the plant-insect-pathogen interaction. Other important aspects to be standardized for genomics studies include the developmental stages of the plant, insect, and inoculum level used (Thompson et al., 2006). Plant responses may differ according with the age and kind of tissue sampled (Liao and Burns, 2012; Martinelli et al., 2012; Ribeiro et al., 2021) and the amount of Las in the specific tissue evaluated, which is a critical issue taking in consideration the already known irregular distribution of Las *in planta* (Tatineni et al., 2008; Li et al., 2009).

Because of it, in this work, we first identified the time course of Las multiplication and whole-plant colonization immediately following inoculation by infected psyllids feeding for 2 days in new shoots (NS) of *Citrus × sinensis* (susceptible), *Murraya paniculata* (partially resistant), and *Bergera koenigii* (fully resistant). Following this, we proposed an RNA-seq comparative study between these three species on the previous identified key-time point of the early stages of Las colonization.

2. LITERATURE REVIEW

2.1 The Citriculture

The species belonging to *Citrus* and related genera are native to the tropics and sub-tropics of Southeast Asia – northeast India, Southern China, the Indochinese peninsula – and the Malay Archipelago. From there, citrus fruits spread to other parts of the world, reaching the Americas at the end of the 15th century (Webber, 1967).

Over the years, an expressive agro-industrial activity has been installed, leading citrus to be cultivated in all tropical areas, with the main commercial areas being the subtropical regions, at latitudes above 20°N and 20°S (Soost and Roose, 1996). According to the latest update from FAO, in 2019 world citrus production reached

143755.6 thousand tonnes, with Asia holding almost 50% of citrus fruit production, followed by Americas (35%), Africa (10%), Europe (7%), Caribbean (0.4%), and Oceania (0.1%). Regarding specifically the production of oranges, Brazil, China, India, USA, Mexico, Spain and Egypt are the largest producers in the world, respectively.

In Brazil, since the 1980s, the citrus sector has shown significant importance in the national economy with a PIB contribution of US\$ 6.5 billion in 2009, with Brazil being the largest orange producer and the largest world exporter of concentrated and frozen orange juice (CFOJ) (USDA, 2021). Fruit production in the citrus belt, which encompasses regions of the state of São Paulo, the Triângulo Mineiro and South-west of Minas Gerais reached 262.97 million boxes of 40.8 kilograms in the 2020/2021 season's harvest, with an estimated production for the 2022/2023 season's harvest of 316.95 million boxes (Fundecitrus, 2022). According to the USDA (2021), in the 2019/2020 season's harvest Brazil produced 32.6% of the world's total sweet oranges and was responsible for 80.42% of the total exported CFOJ.

However, pests and diseases severely affect Brazilian orchards, which is reflected in spending on agricultural inputs, representing approximately 20% of production costs in orange, tangerine and lemon orchards up to 3 years of age and about 40% in orchards 4 years old, in the year 2020 (Agriannual, 2021).

With the presence of the disease called Huanglongbing (HLB) in Brazilian orchards, the use of insecticides increased. In addition to the use of these products being directly related to the rise in production costs, an important alert regarding sustainability issues were generated. Furthermore, producers who choose to export, whether fresh fruit or concentrated juice, must be aware of the phytosanitary barriers imposed by the importing countries (Neves et al., 2010).

2.2 The Citrus genus and its relatives

The oldest register of a citrus fruit came from the golden apples of the Hesperides Garden. Thousands of years of citrus cultivation and natural or even controlled crosses have generated a complex mixture of species very difficult to classify, creating controversies among the principals' taxonomies (Talon et al., 2020). As an example, Tanaka (1954) classified 36 different species of mandarins, while

Swingle and Reece (1967) only three. The possibility to sequence the genome of citrus species has provided good insights into the phylogeny, origin, evolution and dispersal.

According to Swingle and Reece (1967), the *Citrus* genus is one of the 160 genera that are part of the Rutaceae family which with 21 other families form the Geraniales order. Those 160 genera include more than 1600 species splitted in 7 subfamilies and 12 tribes (Talon et al., 2020). One of those 7 subfamilies is the Aurantioideae composed by trees or shrubs with persistent leaves (except in the genera *Poncirus*, *Aegle*, and *Feronia* and in three species of *Clausena* and one of *Murraya*). Normally, flowers are white and with a characteristic sweet fragrance and fruits composed with numerous oil glands (Swingle and Reece, 1967).

The number of chromosomes in the Aurantioideae are $n = 9$, been dominated by the diploidia $2n = 2x = 18$, occasionally, intraspecific autopolyploids ($3x$ and $4x$) occur. Stabilized polyploid are rarely found and the stability in the Aurantioideae Karyotype is apparently linked to high interspecific hybridization capacity (Guerra et al., 1997; 2000).

The Aurantioideae subfamily is composed of two tribes, Clauseneae and Citreae, each of one composed by 3 subtribes. The Clauseneae is formed by the Micromelinae (very remote citroid fruit trees), Clauseninae (remote citroid fruit trees) and Merrillinae (large-fruited remote citroids fruit trees) subtribes and the Citreae tribe is composed by the Triphasiinae (minor citroids fruit trees), Citrinae (citrus fruit trees) and Balsamocitrinae (hard-shelled citroid fruit trees) (Swingle and Reece, 1967).

In the Clauseneae tribe, the Micromelinae and Merrillinae subtribes are both compose only be one genus (*Micromelum* and *Merrilia*, respectively). In the other hand, the Clauseninae subtribe is formed by three genera, *Glycosmis*, *Clausena* and *Murraya*. Among the three subtribes of the Citreae tribe, the Triphasiinae is composed by eight genera, the Balsamocitrinae by 7 genera and the Citrinae by 13 genera, which comprises the A) Primitive Citrus fruit trees (*Severinia*, *Pleiospermium*, *Burkillanthus*, *Limnocitrus* and *Hesperethusa*), B) Near-citrus fruit trees (*Citropsis* and *Atalantia*), and C) True citrus fruit trees (*Fortunella*, *Eremocitrus*, *Poncirus*, *Clymenia*, *Microcitrus* and *Citrus*) (Swingle and Reece, 1967).

In the Aurantioideae subfamily, the most promising citrus rootstocks genera are from the Tribo Citreae Subtribe Citrinae: *Atalantia* (with some species classified in the

Severinia genus), *Naringi* (called as *Hesperethusa*), *Citropsis* and *Limonia* (called as *Feronia*); and from the Tribo Citreae Subtribe Balsamocitrinae the genus *Swinglea* (Bitters et al., 1964, 1969, 1977; Raiol-Junior et al., 2022).

According to Swingle and Reece, 1967, the genus *Murraya* is composed of eleven species and considered *Merrillia* related to an ancestor of *Murraya*, however, molecular systematic research showed that *Murraya koenigii* (L.) Spreng has shown to be *Bergera koenigii* L., as originally described, and much more closely related to *Clausena* than it is to *Murraya* (Bayer et al., 2009). In general, *Murraya* shows stems ellipsoidal and red fruits, large petals, and straw to light greyish-yellow stems, whereas *Bergera* shows globular purplish-black fruits, petals are smaller, and stems are dark brown (Bitters, 1986).

Commonly known as orange jasmine and curry leaf, respectively, *M. paniculata* (L.) Jack and *B. koenigii* are used as ornamental plants in tropical and subtropical regions around the world (Swingle and Reece, 1967). Furthermore, *M. paniculata* has been used in folk medicine for different purposes (Aziz et al., 2010) and in breeding programs as source of nematode tolerance (Bitters, 1964).

Recently, *M. paniculata* and *B. koenigii*, became of specially interest in citriculture. They were described as been susceptible to the insect vector of HLB, the *Diaphorina citri* (Kuwayama) (Sternorrhyncha: Liviidae) (Damsteegt et al., 2010; Teck et al., 2011; Westbrook et al., 2011), being much more attractive to insects' females than sweet orange under laboratory-controlled conditions and in field experiments (Beloti et al., 2017; Tomaseto et al., 2019). However, *M. paniculata* was described as a transient host, and *B. koenigii* immune to the associated HLB bacterium '*Candidatus Liberibacter asiaticus*' (Las) (Damsteegt et al., 2010; Beloti et al., 2018; Cifuentes-Arenas et al., 2019; Alves et al., 2021a).

Previously reported as incompatible with *Citrus*, *M. paniculata* showed low-graft-take with intrageneric and intergeneric species having better performance when used as a scion in graft compatibility experiments (Bitters et al., 1964, 1969; Raiol-Junior et al., 2022). Otherwise, *B. koenigii* demonstrated to be more compatible for autograft and intergeneric combinations than the orange jasmine (Raiol-Junior et al., 2022).

The six genera from the true citrus fruit trees of the Citreae tribe, Citrinae subtribe, have orange or lemon-like fruits, pulp-vesicles more or less fusiform, and for

times more stamens than petals. Apart from *Poncirus* which has trifoliate and deciduous leaves, all the other five genera have persistent and unifoliate or simple leaves. The exclusively pulp-vesicles of the subtribe Citrinae reached their highest level of development in this group C, they are never conical in shape, being narrowed at the base (except in *Clymenia*) and full of oil droplets (Swingle and Reece, 1967).

Among the genera in group C, *Clymenia*, *Eremocitrus* and *Poncirus* are monospecific, and the two other, *Fortunella* and *Microcitrus*, have four and six species respectively. Four of those six genera are adapted to grown in conditions not good for the genus *Citrus*. *Eremocitrus* can grow in semiarid regions of Australia, been classified as xerophyte while *Microcitrus* is semi-xerophytic, supporting long periods of drought. *Poncirus* is deciduous and resistance to cold winters and *Fortunella* has the capacity to be long periods of time without new growth to escape from danger and injury from spring frosts (Swingle and Reece, 1967). More recently, the *Eremocitrus* and *Microcitrus* genera become of more importance after the description of species resistant to Las, the α -proteobacteria associated with the HLB disease (Ramadugu et al., 2016; Alves et al., 2021b; Alves et al., 2022).

The genus *Eremocitrus* and *Microcitrus* are believed to be the result of a progressive evolution over years from a primitive ancestor that came under speciation in Australia and New Guinea as these islands were separated. From this ancestor, a lineage that gave rise to the *Microcitrus* and the genus *Eremocitrus* was obtained (Swingle and Reece, 1967; Hashemian et al., 2010). This ancestor has similarities with the New Guinea species, *M. warburgiana*, which has small leaves and almost spherical fruits. Evolutionarily, a new lineage generated *M. australis*, which constitutes large trees with long, thin, pointed and larger fruits than those of *M. warburgiana*. Another evolutionary line was responsible for the formation of *M. inodora* and *M. maideniana*, species with the largest dense, broad leaves and spines in pairs. Finally, a third evolutionary line led to the species *M. australasica* and *M. garrawayai*, which are semi-xerophilic, with small, elongated fruits (Swingle and Reece, 1967).

Species of the genus *Microcitrus* are classified as compatible by grafting and for crosses with species of the genus *Citrus* and related species. Hybrids between these genera and others are common as the 'Faustmedin' [*M. australasica* $\times$ (*Fortunella* sp. $\times$ *Citrus reticulata*) 'Calamondin', also known as *C. $\times$ oliveri*; 'Sydney', also known

as *M. virgata* (*M. australis* × *M. australasica*), and the blood variety also called 'Purpura', which is a variant of *M. australasica* (Krueger and Navarro, 2007). *Microcitrus* are considered monoembryonic species and have small leaves and flowers, and the flowers have free stamens and a short pistil. The ovary is composed of multiple ovules and the fruits are composed along the lines (Krueger and Navarro, 2007).

The genus *Eremocitrus* is composed exclusively of one species, the *Eremocitrus glauca*, known as Australian desert lime, which refers to its place of origin: the Australian deserts. It is the only xerophytic citrus, having small, thin, elongated, grayish leaves, with parenchyma on both sides and covered by a thin cuticle. Its vegetative growth is very slow until a good root system is formed, but after the presence of a good root volume, the growth is fast and the leaves reach 50-65 mm x 10-20 mm, with a thickness of approximately 2 millimeters. They have long spines throughout the plant, reaching 9 cm (in field plants). The flowers are small and have 3-5 locule ovaries with two ovules on each locule, a character not found in any other true citrus species except *Fortunella*. The fruits are small, subglobose with 15-25 mm x 12-15 mm. The vesicles are thin and poorly aggregated, not sticking together as tightly as in common citrus. Seeds are small and monoembryonic (Swingle and Reece, 1967).

The genus *Eremocitrus* is compatible by grafting and also for crossing with different species of *Citrus* and among other related genera, such as the species of the genus *Microcitrus*. In addition, they are resistant to saline and water stress and, therefore, their use was already present in breeding programs. Hybrids with *E. glauca* have already been described, including Eremoranges (*E. glauca* × *C. sinensis*), Eremoradias (*E. glauca* × *C. aurantium*) and Citrangeremos [*E. glauca* × (*P. trifoliata* × *C. sinensis*)] (Swingle and Reece, 1967).

Several *Microcitrus* species and the *E. glauca* performed well as interstocks, warranting interesting considerations as possible rootstocks (Bitters et al., 1964, 1977). Recently, under greenhouse experiments, *E. glauca*, an *E. glauca* × *Citrus* sp. hybrid, an *E. glauca* × *Microcitrus* sp. hybrid, and some *Microcitrus* as *M. warburgiana*, *M. australasica*, a *M. australis* × *M. inodora* hybrid and others, demonstrated good compatibility when grafted onto the 'Rangpur' lime (*Citrus × limonia* Osbeck) rootstock (Alves et al., 2021b; 2022).

The use of species of the genus *Microcitrus* in genetic plant breeding programs used to be related to resistance to *Phytophthora* and adaptation to low mineral fertilization (Krueger and Navarro, 2007). The species *M. australis*, *M. australasica* and the hybrid *M. virgata* were classified as susceptible to the CTV virus. Furthermore, *M. australasica* and *E. glauca* have been identified as poor viroid hosts, in which replication and accumulation of viroid's did not occur or occur with very low efficiency (Yoshida, 1996; Hashemian et al., 2010).

Classic citrus breeding, until the present time, is based on the identification and/or induction of mutations, inter and intraspecific crosses and clonal selection (Caruso et al., 2020). The objective is to generate varieties whose fruits present high nutritional and production quality and that have greater resistance to different biotic and abiotic stresses.

Classical breeding programs with specific targets proved to be generally inefficient due to the long time and amount of resources that are needed to obtain and evaluate progenies; the lack of genetic information associated with the main citrus characteristics of interest; understanding of the taxonomy of the species and the high heterozygosity of citrus. However, in recent years, advances in biotechnological techniques, including sequencing methodologies, use of molecular markers, genomic mapping, *in vitro* cultivation, and genetic transformation, have benefit the breeding programs (Salonia et al., 2020).

2.3 The Huanglongbing (HLB)

Huanglongbing (HLB), also known as greening, was first observed in 1870 in Southeast Asia (Lin, 1956). Since then, it has been considered the most destructive and treacherous disease in citrus farming due to the damage it causes to production, fruit quality and its rapid spread and difficult control (Bové, 2006).

Their report was done in different regions of the world under various name, 'yellow shoot disease' in China (Reiking, 1919) which is the translation of 'Huanglongbing', 'mottle leaf' in Philippines (Lee, 1921), 'dieback' in India (Raychaudhuri et al., 1969), 'phloem degeneration' in Indonesia (Aubert, 1992) and as 'greening' in South Africa (Oberholzer et al., 1965).

HLB is associated with the presence of the '*Candidatus* Liberibacter asiaticus' (Las), '*Ca. L. americanus*' (Lam) or '*Ca. L. africanus*' (Laf) (Bové, 2006). Las and Lam are transmitted by the Asian citrus psyllid *Diaphorina citri* Kuwayama (Sternorrhyncha: Liviidae) (Capoor et al., 1967) while Laf is transmitted by the African citrus psyllid *Trioza erytreae* Del Guercio (Sternorrhyncha: Triozidae) (McClellan and Oberholzer, 1965).

In Brazil, HLB was first reported in 2004 (Coletta-filho et al., 2004; Teixeira et al., 2005) and is associated with the presence of Las and Lam. Initially, the disease was predominantly associated with the presence of Lam. However, this scenario has changed and, nowadays, Las is present in 99.9% of all positive samples from the Fundecitrus diagnostic laboratory (Bassanezi et al., 2020). The greater tolerance of Las to high temperatures, the fact that it reaches higher titers compared to Lam and that it is more easily transmitted, are factors that can explain this scenario change (Lopes et al., 2009, 2013).

The '*Candidatus*' Liberibacter spp. are a Gram-negative and endopathogenic α -proteobacteria that exclusively inhabits the phloem of host plants (Jagoueix et al., 1994). Since no pathogenic Liberibacter has been isolated in sterile culture medium, the term '*Candidatus*' is used until Koch's postulate is complete (Li et al., 2009).

Plants with HLB have yellowish leaves or irregular spots on the leaf blade, gradually alternating between green and yellow, a symptom called mottled. The new shoots emerged from an infected plant are small, with yellow leaves and usually with enlarged veins. Fruits are deformed, with a thicker albedo, ripen early and the seeds are aborted (Bové, 2006). All commercial citrus varieties are susceptible to HLB (Folimonova et al., 2009) being the management in citrus production areas based on the use of healthy plants from certified nurseries, extensive application of insecticides to control the population of the insect vector and the elimination of diseased plants (Bassanezi et al., 2020). However, despite the recommended measures, the incidence of the disease continues to grow, reaching 24.42% of the plants in 2021 in the citrus park which encompasses the state of São Paulo and the Triângulo Mineiro (Fundecitrus, 2022). Approximately 55.5 million sweet orange trees were reported with symptoms between January 2005 and June 2019 in the State of São Paulo (Bassanezi et al., 2020).

Due to this, the search for more efficient tools and strategies to contain the spread of the bacterium and the control of the vector has been the subject of intense research aiming to mitigate and/or control the impact of the disease. Furthermore, progress on gaining more insights on '*Candidatus*' Liberibacter spp. movement, -omics analyses of host responses and how this bacterium led to disease development have been the goal of different researchers worldwide.

Las can be carried by the insect in a persistent circulative propagative manner (Inoue et al., 2009; Ammar et al., 2016; Canale et al., 2017) being its acquisition by the insect vector possible at any stage of life and the nymphal stage the more efficient for acquisition and adults more efficient for inoculation (Pelz-Stelinski et al. 2010; Ammat et al., 2020). After immunolocalization studies, Lin et al. (2011) demonstrated that Las enters the gut cells by employing an endo-exocytosis-like mechanism for invasion and aggression. Inside the insect vector, Las was reported by microscopy colonizing the midgut, malpighian tubules, hemocoel, filter chamber, salivary glands, ovaries, vacuoles of the endoplasmic reticulum, muscle, and fat tissues. Las produce an external movement appendage inside the insect; however, how Las move to all these tissues remains unclear (Ammar et al., 2011, 2016, 2019; Ghanim et al., 2016; Hall et al., 2013).

After 2-3 weeks of latent period, infected insects are able to transmit Las to healthy plants (Canale et al., 2017). With its specialized stylets, *D. citri* pierces the plant tissue to access the nutrient-rich phloem sap. The stylet goes through the intercellular spaces (apoplast), and Las is passively delivered into the phloem (Jiang et al., 2019). An electrical penetration graph (EPG) analysis showed that only 8 hours are sufficient for the insect to contact the phloem (Bonani et al., 2010; Wu et al., 2016; Carmo-Souza et al., 2020). However, Las transmission will depend on the plant tissue's developmental stage as well as the environmental conditions (Raiol-Junior et al., 2017; Lopes and Cifuentes-Arenas, 2021; Pandey et al., 2021).

Flushes from citrus plants are the preferred tissue of the insect for feeding and reproduction (Lopes and Cifuentes-Arenas, 2021; Pandey et al., 2021). Inside the plant host, microscopy studies confirmed that Las stays only in the phloem sieve elements (Folimonova and Achor 2010; Hilf 2011). Achor et al. (2020), observed the presence of it in nucleated nonsieve element cells; however, the identity of these cells remains unknown.

After inoculation and Las presence in the phloem, Las remains in the young flush until leaves are mature enough to turn into a source (Pandey et al., 2021; Alves et al., 2021a), preferentially invade new tissues during development (Raiol-Junior et al., 2021a) and irregularly colonizes the plant (Tatineni et al., 2008; Li et al., 2009). Therefore, selection of the tissue sampled for studies and diagnosis is very important (Tatineni et al., 2008; Johnson et al., 2014; Raiol-Junior et al., 2021a).

Las induce callose plugging of the sieve pores, greatly reducing its diameters. However, it is ineffective to restrict Las movement as this bacterium moves in an elongated form to pass through narrowed sieve pores (Achor et al., 2020; Ammar et al., 2019). Las movement is predominantly passive through the phloem sap from sources to sinks, being no evidence of a flagella-like interference well supported (Andrade et al., 2020; De Francesco et al., 2021).

Las has a reduced genome (~1.20Mb) when compared to their closest non-pathogenic cultured relative, *Liberibacter crescens* (~1.50Mb), with a low GC content of approximately 35.3%. Las lost an alternate terminal cytochrome, the stringent response, and multiple two-component regulatory systems during genome reduction, each reflected its ability to adjust to environmental changes. However, as an intracellular pathogen, Las may not be exposed to huge environmental fluctuations (Fagen et al., 2014). Las can synthesize only five amino acids: Glycine, Serine, Threonine, Glutamine, Lysine and Arginine. However, encodes specific amino acid transporter for Proline, Glycine, and Aspartic Acid (Fagen et al., 2014). Furthermore, Las is only able to metabolize a limited number of sugars, and it is supposed to use exogenous molecules from the phloem sap to generate energy (Duan et al., 2009).

Las genome encodes genes necessary for the presence of flagella and a type IV pili, structures usually used by bacterium for swimming and swarming motility, which can also be involved in the perception and initial adherence to surfaces (Kearns, 2010). Although the expression of those genes is upregulated in Las from *D. citri*, low expression in plant host was observed, an indication that flagella and type IV pili machineries may be important for Las colonization and propagation inside the insect but not in plants (Andrade et al. 2019; Andrade and Wang 2019; De Francesco et al. 2021).

In general, the plant immune response to biotrophic pathogens is divided into microbe-associated molecular pattern (MAMP)- triggered immunity (MTI) and effector-triggered immunity (ETI). MAMPs are recognized by the membrane-localized pattern recognition receptors (PRRs), which are commonly receptor-like kinases (RLKs) or receptor-like proteins (Segonzac and Zipfel, 2011). The MTI response elicits the production of reactive oxygen species (ROS), the flux of Ca^{2+} , stomatal closure, deposition of callose, mitogen-activated protein kinase (MAPK)-dependent signal transduction, the expression of defense genes and hormone biosynthesis (Boller and Felix 2009; Macho and Zipfel 2015). In positive interaction, pathogens attenuate the MTI response by delivering an effector to evade or suppress plant immunity (Toruño et al., 2016; Clark et al., 2018). These pathogenic proteins trigger the ETI response (Jones and Dangl, 2006). In Gram-negative bacteria such as Las, six types of protein secretion systems have been described (type's I-VI), being the secretion system type III used to secrete the toxic factors and effectors to extracellular environment or into the host cell to cause infection (Feng et al., 2012; Yu et al., 2017). However, studies have shown that Las has incomplete type III and type IV protein secretion systems and only a complete type I (Duan et al., 2009), demonstrating that the mechanisms of Las to elicit the host defense system may be different from the traditional gram-negative bacteria.

Bioinformatics studies of comparative genomics identified 13 candidates of 'Ca. Liberibacter' spp. MAMPs in *Arabidopsis*, *Nicotiana benthamiana*, and citrus species, including a variety of the *M. australasica*, and suggested that if specific MAMPs are delivered in sufficient quantity to the right tissue, it could increase the MTI response to limit infection (Chen et al., 2020). Other Las genes/proteins have been characterized in different plants and systems as potential proteins of interest in host-pathogen interaction (Clark et al., 2018; Jain et al., 2015, 2018, 2019; Zhang et al., 2019). Recently, stable antimicrobial peptides based on sequences from *Microcitrus* species were reported to be capable of killing *L. crescens* and inhibiting Las infection in plants, reducing Las titer, and inducing innate immunity (Huang et al., 2021). However, no advances were reported in using those proteins/genes to decrease HLB incidence.

Different transcriptomics profiling of host-Las interactions has been extensively studied in both sensitive and less sensitive citrus species. However, plant responses

may differ according to the age and kind of tissue sampled (Ribeiro et al., 2021), amount of bacteria on it, stage of bacterial infection, inoculation procedure, and of course, environmental conditions of experiments (Albrecht and Bowman, 2008; Fan et al., 2012; Liao and Burns, 2012; Martinelli et al., 2012; Wang and Trivedi, 2013; Rawat et al., 2015; da Graça et al., 2016; Martinelli et al., 2016; Wang et al., 2016; Huot et al., 2018; Arce-Leal et al., 2020; Franco et al., 2020; Chin et al., 2021; Wei et al., 2021).

Transcriptomics analysis of tolerant vs. susceptible grapefruit varieties showed a reduced expression of a putative 2OG-FE(II) oxygenase-like genes, beta glucanases, expansins, and DET2 in the tolerant one, which could be involved in the suppression of the host immunity (Wang et al., 2016). Other tolerant varieties showed induced expressions of NPR1, BS-LRR, RLA, cellulases, expansins, pectinesterases, and host defense and pathogenesis-related (PR) genes, but lower effects on starch synthesis and photosynthesis (Arce-Leal et al., 2020; Curtolo et al., 2020; Fan et al., 2012; Hu et al. 2017; Mafra et al. 2013; Wang et al. 2016; Yu et al. 2017). Relatively HLB-tolerant lemon showed less effect on photosynthetic pathways and induced expressions of protease inhibitors when compared with 'Valencia' sweet orange at pre-symptomatic stages (Chin et al., 2021; Ramsey et al., 2020). On the other hand, several susceptible varieties exhibit induced expressions of defense-related genes and high expressions of callose synthase and cell wall breakdown genes (Fan et al., 2012; Fu et al., 2016; Hu et al., 2017; Kim et al., 2009; Koh et al., 2012), which can be associated with a citrus defense response even mostly losing the battle.

All together, we can infer that the most sensitive varieties delayed in their defense response to control Las multiplication or to activate effector-triggered immunity, indicating that the time between Las presence and active defense response plays an important key in this host-pathogen interaction. Because of it, efforts should be directed to elucidate at the gene level the first moments of host-pathogen interaction after an infection. Furthermore, we can highlight that despite all citrus species being susceptible to HLB (Folimonova et al., 2009), the level of susceptibility influences differences in gene expression, demonstrating the importance of doing comparative transcriptomics analysis on the recently described resistance Rutaceae varieties (Ramadugu et al., 2016; Beloti et al., 2018; Cifuentes-Arenas et al., 2019; Alves et al., 2021a; 2021b).

2.4 HLB Resistance and breeding perspectives

Tolerance has been related to the capacity of a specie to support the pathogen's presence without economic loss. On the other hand, resistance is the inability of the pathogen to multiply on a specie (Amorim et al., 2011). Analysis of HLB symptomology to classify levels of tolerance/resistance are of difficult interpretation as they are similar to other diseases symptoms and deficiency nutrition depending on the cultural practices used (Da Graça, 1991; Bové, 2006; Gottwald et al., 2007). Because of it, resistance to HLB has been accessed by a nucleic acid-based molecular diagnosis as qPCR (Li et al., 2006). However, detection of the pathogen at a single time point is not a useful information as some citrus relatives as *Murraya paniculata* and *Limonia acidissima* appear to support the replication of *Liberibacters* for a short period of time (Koizumi et al., 1996; Hun et al., 2020; Cifuentes-Arenas et al., 2019; Alves et al., 2021a). Furthermore, the uneven distributions of Las in an infected tree (Tatineni et al., 2008; Li et al., 2009; Raiol-Junior et al., 2021), its active multiplication quite restricted to new flushes under development (Hilf and Luo, 2018; Raiol-Junior et al., 2021) and the influence of the environment in Las multiplication (Lopes et al., 2009; Gasparoto et al., 2012) have generally rendered confounding and false negative results.

HLB-resistant cultivars were never reported in the genus *Citrus*. Recently, consistent results have demonstrated the existence of varieties, more or less distant from the *Citrus* genus, with total or partial resistance to Las (Ramadugu et al., 2016; Beloti et al., 2018; Cifuentes-Arenas et al., 2019; Alves et al., 2021a; 2021b; 2022). However, in the Rutaceae germplasm family, resistance is discrepant depending on the challenge system chosen for Las inoculation, environmental conditions, age of plants, number of replicates challenged, experiments under field vs. greenhouse, use of seedlings vs. mature plants and grafted vs. rooted plants (Hung et al., 2000; Folimonova et al., 2009; Shokrollah et al., 2009; Feng et al., 2015; Ramadugu et al., 2016; Miles et al., 2017).

The deciduous species *Poncirus trifoliata* was first reported as partially resistant (Folimonova et al., 2009), then as resistant (Albrecht and Bowman, 2012), with different levels of resistance depending on the variety used (Ramadugu et al., 2016)

and as susceptible as a sweet orange when drastically graft-challenged (Alves et al., 2021b; 2022).

Murraya paniculata (L.) Jack and *Bergera koenigii* L. (previously called as *Murraya koenigii*) are Rutaceae species from the subfamily Aurantioideae, tribe Clauseneae, subtribe Clauseninae, commonly known as orange jasmine and curry leaf, respectively and used as ornamental plants in tropical and subtropical regions of the world (Swingle and Reece, 1967). *M. paniculata* was reported as a transient host for Las since infection recovery was observed, and *B. koenigii* was reported as immune (Damsteegt et al., 2010; Beloti et al., 2018; Cifuentes-Arenas et al., 2019; Alves et al., 2021a). However, they were described as good hosts for *D. citri* (Damsteegt et al., 2010; Teck et al., 2011; Westbrook et al., 2011), being even more attractive to females than sweet orange under both laboratory and field conditions (Tomaseto et al., 2019).

The monoembryonic *Microcitrus* and *Eremocitrus* species and hybrids have been considered with more or less capacity to delay the infection varying within the accession used (Ramadugu et al., 2016; Alves et al., 2021b; 2022). As monoembryonic species, the use of zygotic seedlings may justify the genetic differences between repetitions from the same accession (Ramadugu et al., 2016). Furthermore, field experiments under natural infection by the insect are subjected to infections scapes as it is already known that there are some specific colors and volatile compounds that are more or less attractive to *D. citri* (Wenninger et al., 2009; Patt and Sétamou, 2010; Hall et al., 2011) and also some genotypes that are more preferred between the Rutaceae species as *Bergera* and *Murraya*, reported with intermediate preference as *Glycosmis pentaphylla* (Retz.) DC., *Clausena harmandiana* (Pierre) Guillaumin and *Zanthoxylum ailanthoides* (L.) and other *Citrus* relatives as resistant to the insect, such as some *Poncirus trifoliata* accessions or with lower nymphal viability as *E. glauca* (Westbrook et al., 2011; Richardson and Hall, 2013; Hall et al., 2015; Felisberto et al., 2019; Eduardo et al., 2022).

The sexual compatibility of *Eremocitrus* and *Microcitrus* species with the genus *Citrus* opens a range of opportunities that involve obtaining segregating populations and future identification of QTLs associated with resistance to HLB. Furthermore, graft-compatible materials can be used as rootstocks or resistant inter-grafts. Even though phylogenetically more distinct and incompatible, the presence of resistant material

within the same subfamily as the genus *Citrus* becomes the target of molecular studies aiming to better understand the citrus-Liberibacter pathosystem.

3. Aims and Strategy

In a previous study, we determined that the graft and cross-compatible Oceanian citrus relatives *Eremocitrus glauca*, *Microcitrus warburgiana*, *Microcitrus papuana*, and *Microcitrus australis* and three hybrids among them and *Citrus* were full-resistant to Las (Alves et al., 2021b). Aiming to better characterize this phenotype and understand Las movement on them, a set of five new experiments were carried out with challenge-inoculated plants and the genetic background of those seven HLB-resistant genotypes were assessed by genotyping by sequencing (GBS). Experiment procedures, results and discussions are described on **Chapter 2** (doi:10.3389/fpls.2022.1009350).

Moreover, aiming to gain more information on the plant-pathogen interaction of the resistant Rutaceae phylogenetically more distant to *Citrus* species, we present on **Chapter 3** our study describing i) the initial bacterial dynamics once Las is delivered into the phloem by the insect; ii) Las multiplication at the first stages post-inoculation, and ii) the time taken for Las to colonize (or not) the entire plant of the susceptible *Citrus × sinensis* ‘Valencia’, the transient host *Murraya paniculata* and the immune *Bergera koenigii* (doi: 10.3389/fmicb.2021.683923).

After selecting the key-time points of Las-plant interaction on the earlier stages of the infection (Chapter 3), we used an RNA-seq approach to study at a transcriptional level the dynamic responses of *C. × sinensis* ‘Valencia’, *M. paniculata* and *B. koenigii* after being challenge inoculated with Las-infected *Diaphorina citri*. Transcriptomic procedures and results are discussed on **Chapter 4**.

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CHAPTER 2 – Insight into resistance to ‘*Candidatus Liberibacter asiaticus*’, associated to Huanglongbing, in Oceanian citrus genotypes^A

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Mônica N. Alves^{1,2}, Laudecir L. Raiol-Junior^{1,3}, Eduardo A. Girardi^{1,3}, Maéva Miranda^{4,5}, Nelson A. Wulff¹, Everton V. Carvalho^{1,3}, Sílvia A. Lopes¹, Jesus A. Ferro², Patrick Ollitrault^{4,5} and Leandro Peña^{1,6*}

¹Fundo de Defesa da Citricultura, Araraquara, Brazil,

²Faculdade de Ciências Agrárias e Veterinárias (FCAV), Universidade Estadual Paulista (UNESP), Jaboticabal, Brazil,

³Empresa Brasileira de Pesquisa Agropecuária, Cruz das Almas, Brazil,

⁴CIRAD, UMR AGAP Institut, Montpellier, France,

⁵AGAP Institut, Univ. Montpellier, CIRAD, INRAE, Institut Agro, Montpellier, France,

⁶Instituto de Biología Molecular y Celular de Plantas – Consejo Superior de Investigaciones Científicas, Universidad Politécnica de Valencia, Valencia, Spain

ABSTRACT

Huanglongbing (HLB), the most destructive citrus disease, is associated with unculturable, phloem-limited ‘*Candidatus Liberibacter*’ species, mainly ‘*Ca. L. asiaticus*’ (Las). Las is transmitted naturally by the insect *Diaphorina citri*. In a previous study, we determined that the Oceanian citrus relatives *Eremocitrus glauca*, *Microcitrus warburgiana*, *Microcitrus papuana*, and *Microcitrus australis* and three hybrids among them and Citrus were full-resistant to Las. After 2 years of evaluations, leaves of those seven genotypes remained Las-free even with their susceptible

rootstock being infected. However, Las was detected in their stem bark above the scion-rootstock graft union. Aiming to gain an understanding of the full-resistance phenotype, new experiments were carried out with the challenge-inoculated Oceanian citrus genotypes through which we evaluated: (1) Las acquisition by *D. citri* fed onto them; (2) Las infection in sweet orange plants grafted with bark or budwood from them; (3) Las infection in sweet orange plants top-grafted onto them; (4) Las infection in new shoots from rooted plants of them; and (5) Las infection in new shoots of them after drastic back-pruning. Overall, results showed that insects that fed on plants from the Oceanian citrus genotypes, their canopies, new flushes, and leaves from rooted cuttings evaluated remained quantitative real-time polymerase chain reaction (qPCR)-negative. Moreover, their budwood pieces were unable to infect sweet orange through grafting. Furthermore, sweet orange control leaves resulted infected when insects fed onto them and graft-receptor susceptible plants. Genomic and morphological analysis of the Oceanian genotypes corroborated that *E. glauca* and *M. warburgiana* are pure species while our *M. australis* accession is an *M. australis* × *M. inodora* hybrid and *M. papuana* is probably a *M. papuana* × *M. warburgiana* hybrid. *E. glauca* × *C. sinensis* hybrid was found coming from a cross between *E. glauca* and mandarin or tangor. *Eremocitrus* × *Microcitrus* hybrid is a complex admixture of *M. australasica*, *M. australis*, and *E. glauca* while the last hybrid is an *M. australasica* × *M. australis* admixture. Confirmation of consistent full resistance in these genotypes with proper validation of their genomic parentages is essential to map properly genomic regions for breeding programs aimed to generate new Citrus-like cultivars yielding immunity to HLB.

Keywords: greening, HLB, Rutaceae, Aurantioideae, *Microcitrus*, *Eremocitrus*, citrus breeding

1. INTRODUCTION

Huanglongbing (HLB), also known as citrus greening, is a ravaging citrus disease that has been concerning citrus growers and researchers across the world, especially during the last two decades, when it spread from South East Asia to West Asia, North Oceania, America and several African countries (Alqu  zar et al., 2022).

HLB is associated with the presence of the unculturable, Gram-negative and phloem-limited α -proteobacteria '*Candidatus Liberibacter africanus*' (Laf), '*Ca. L. asiaticus*' (Las) and '*Ca. L. americanus*' (Lam) (Bové, 2006), being Las the most prevalent and aggressive species (Bassanezi et al., 2020). In the field, Las is transmitted by the insect vector *Diaphorina citri* (Kuwayama) (Hemiptera: Liviidae), commonly known as the Asian citrus psyllid (ACP; Capoor et al., 1967). In Brazil, HLB management relies on the implementation in an area-wide basis of the three-pronged system (TPS) which consists on the elimination of symptomatic trees, use of certified healthy nursery trees for new plantings and resets, and intensive insecticide control of *D. citri* (Bassanezi et al., 2020). This strategy has been executed since the first description of HLB in Brazil and mainly due to these practices, HLB has only reached 22% of the trees in the citriculture belt of São Paulo State and Triangulo Mineiro (Fundecitrus, 2021), a scenario that would be worst without the implementation of the TPS. However, this strategy has not been adopted and maintained by all growers, ACP resistance to insecticides is arising and thus HLB incidence has gradually increased, demanding new approaches to cope with the disease.

Leaves from HLB-affected trees usually show asymmetrical chlorosis or blotchy mottle, and, at later stages of infection, thicker and enlarged veins (Bové, 2006). Fruits are smaller and misshapen while the juice is highly acidic and poor in soluble solids (Bassanezi et al., 2009). Symptomatic leaves and fruits drop prematurely (Moreira et al., 2022). Leaf symptoms can be confusing, especially at early stages of infection, and, thus, not always can be used for diagnosis purposes (Gottwald et al., 2007). The quantitative real-time polymerase chain reaction (qPCR) was then developed for Las, becoming the most important and widely used tool for HLB diagnosis and research (Li et al., 2006). However, qPCR does not distinguish DNA of dead from live bacteria while DNA from dead bacteria is only slowly degraded in plant tissues (Josephson et al., 1993). This can pose problems making difficult the interpretation of results of some works, especially for bacterial titers close to detection limits.

Evaluation by qPCR of citrus genotypes challenge-inoculated with Las in controlled and field conditions has shown that all commercial cultivars are susceptible (Folimonova et al., 2009; Miles et al., 2017). However, some citrus relatives from other genera within the Aurantioideae subfamily have been recently reported as completely

or partially resistant to HLB (Ramadugu et al., 2016; Alves et al., 2021a; Alves et al., 2021b). This includes the Australian desert lime, *Eremocitrus glauca* (Lindl.) Swingle, and several Oceanian *Microcitrus* species. Both genera belong to the true-citrus fruit trees group, are monoembryonic, and cross-compatible with *Citrus* (Swingle and Reece, 1967). Furthermore, *E. glauca*, some *Microcitrus* species, and their hybrids with *Citrus* have shown to be suitable citrus rootstocks (Bitters et al., 1964, 1977), and presented good graft compatibility with the rootstock 'Rangpur' lime (*Citrus × limonia* Osbeck) when used as scions (Alves et al., 2021b).

In field experiments, seedlings of *E. glauca* and some *Microcitrus* genotypes were identified as potential sources of resistance or tolerance to HLB (Ramadugu et al., 2016). In controlled greenhouse experiments, by using clonal plants grafted onto the Las-susceptible 'Rangpur' lime rootstock and upon challenging by graft-inoculation of Las-infected budwood on both the scion and rootstock, Alves et al. (2021b) discerned seven full-resistant citrus genotypes, namely, *E. glauca*, *M. australis*, *M. warburgiana*, *M. papuana* and three hybrids among them and with sweet orange within a collection of Citreae species. Whereas the susceptible rootstock became infected with Las, the bacterium was never detected in the scion leaves of these genotypes thus becoming the most promising germplasm among Oceanian citrus to be used as parents in breeding efforts for generating citrus-like cultivars resistant to Las (Alves et al., 2021b). Because of this, the introgression of HLB resistance into cultivated citrus has become tangible in citrus breeding programs (Alquézar et al., 2021). However, Las was found in the stem bark sampled at 5 cm above the scion-rootstock union of all the seven full-resistant genotypes, and unevenly detected at 30 cm height in two of them (Alves et al., 2021b). Las amplifications at these different distances from the infected rootstock led to query on whether the detected bacterial DNA was viable. Aiming to better characterize the resistance of those genotypes and further understand Las movement within the stem bark of the grafted plant materials, a set of five experiments was carried out. In addition, to support interpretation of the Las resistance phenotype, the genetic background of the seven Oceanian citrus genotypes was assessed by genotyping by sequencing (GBS) analysis.

2. MATERIAL AND METHODS

2.1 Phenotyping of Oceanian citrus genotypes

Morphological analysis of the Oceanian citrus putatively true species was performed with 4-14 healthy plants per genotype propagated onto the 'Rangpur' lime rootstock. The aspect of leaves, spines, flowers, and fruits was compared with their description made by Swingle and Reece (1967), also considering the results obtained from their genome analysis, to ascertain the presumed pure or hybrid nature of each genotype.

2.2 High density SNP genotyping of Oceanian citrus genotypes

Aiming to genetically characterize the Fundecitrus collection used for HLB resistance studies (Alves et al., 2021b), a GBS analysis was performed with the seven genotypes described as full-resistant by Alves et al. (2021b) (*M. warburgiana* (F.M. Bailey) Tanaka, *M. papuana* Winters (revealed to be actually a *M. papuana* hybrid in the present work), *M. australis* (A. Cunn. ex Mudie) Swingle (actually a *M. australis* hybrid), *E. glauca* (Lindl.) Swingle, a *Microcitrus* sp. hybrid (formerly called *Microcitrus* sp. $\times$ *E. glauca*), an *E. glauca $\times$ *Microcitrus* sp. complex hybrid, and a supposed *E. glauca $\times$ *Citrus* $\times$ *sinensis* hybrid (eremorange) and the susceptible sweet orange cultivar 'Tobias' [*C. sinensis* (L.) Osbeck]. Two finger lime (*M. australasica*) accessions of the Fundecitrus germplasm ('True Sanguinea' and a 'Green' one) considered as representative of *M. australasica*, as well as one accession of each *Clymenia polyandra*, *M. inodora* and *M. garrawayae* were included to check their status of pure species representatives and the potential contribution of the different species to the selected resistant germplasm. Five varieties from the CRB-Citrus collection, San Giuliano (Corsica) whose phylogenomic constitution was previously analyzed by GBS or WGS (Wu et al., 2014, 2018; Ahmed et al., 2019) were also added in the GBS analysis as reference to check the quality of the results: Nules clementine ICVN 0100389, Eureka lemon ICVN 0100289, Star Ruby grapefruit ICVN 0100293, eremorange SRA871 and Australian finger lime SRA1002. In addition, published WGS data available in NCBI (Wu et al., 2018; Wang et al, 2017) were used to mine diagnostic SNPs (DSNPs) of ancestral taxa from pure representative accessions: *C. maxima***

(SRR5128240 and SRR3824065), *C. medica* (SRR3938056 and SRR3944139), *C. reticulata* (SRR3747399 and SRR3747527), *M. australis* (SRR5128218 and SRR6188449) and *M. australasica* (SRR5128220).

For GBS analysis, the genomic DNA was isolated using the Plant DNAeasy kit (Qiagen) according to the manufacturer's instructions. The genomic DNA concentration of each sample was adjusted to 20 ng/μL, and ApeKI GBS libraries were prepared following the protocol described by Eslhire et al. (2011) with 96 DNA samples multiplexed per GBS library (the accessions used for this paper were included in another larger experiment). Ten μL of each DNA sample (200 ng) were digested with the ApeKI enzyme (New England Biolabs, Hitchin, UK). Digestion took place at 75 °C for 2 h and then at 65 °C for 20 min for enzyme inactivation. The ligation reaction was completed in the same plate as the digestion, using T4 DNA ligase enzyme (New England Biolabs, Hitchin, UK) at 22 °C for 1 h. Then, the ligase was inactivated prior to pooling the samples by holding it at 65 °C for 20 min. For each library, ligated samples were pooled and PCR-amplified in a single tube. Genome complexity was reduced using PCR primers with one selective base (A) as described by Sonah et al. (2013). Pair-end sequencing was performed on one lane of an Illumina HiSeq4000 platform at Genewiz facilities. RAW sequencing data were cleaned with cutadapt (Martin, 2011) and demultiplexed with GBSX (Herten et al., 2015). Clean demultiplexed sequencing data from GBS are available in the NCBI SRA (Sequence Read Archive), under accession number (Submission ID: PRJNA862483). SNP genotype calling was performed with GATK4 pipeline (Van der Auwera and O'Connor, 2020). The reference genome was constituted by the nuclear scaffolds of the Clementine v1.0 genome assembly (https://phytozome-next.jgi.doe.gov/info/Cclementina_v1_0), the mitochondrial (Yu et al., 2018) and chloroplast (Bausher et al., 2006) reference genomes from *C. × sinensis* (available in NCBI with reference NC_037463 and NC_008334 respectively). Positions with less than 10 reads were considered as missing data. Polymorphic positions were filtered for diallelic SNPs and minor allele frequency greater than 0.01.

2.3 Genetic data analysis

Neighbor-joining analyses for nuclear and mitochondrial data as well as a factorial analysis for nuclear data were performed to describe the genetic diversity organization of the analyzed germplasm. Both analyses are based on a dissimilarity matrix established from SNP genotyping data using the Manhattan dissimilarity index:

$$D_{ij} = \frac{1}{K} \sum_1^K |x_{ik} - x_{jk}|$$

where i and j are the two individuals, k is the locus, K is the total number of loci, and x_{ik} is the frequency of the alternative allele at locus k for the individual i. All analyses were performed with DARwin software version 6.0 (<https://darwin.cirad.fr/>).

Ancestral genome contribution along the genome of the admixed genotype was analyzed based on the distribution of homozygosity and heterozygosity of ancestral diagnostic SNPs (DSNPs). Fourteen accessions were used to select diagnostic SNPs for 10 ancestral species: two accessions for *C. maxima*, *C. reticulata*, *C. medica* and *M. australis* and one for *M. australasica*, *M. inodora*, *M. warburgiana*, *M. garrawayae*, *E. glauca* and *Clymenia polyandra*. For each ancestor, DSNPs that fully differentiated the considered ancestor from all other species (i.e., all accessions of the considered ancestor were homozygous for the same allele and all others accessions homozygous for the second allele) were filtered in Excel. For each ancestor homozygosity and heterozygosity curve along the genome were established with sliding windows of 20 consecutive DSNPs for the considered ancestor (ie: proportion of the 20 markers in homozygosity or heterozygosity for the alleles of the considered ancestor). The medium position of the 20 markers of each sliding window was used to draw the curve in Circos representation (Krzywinski et al., 2009) true Galaxy interface (Rasche and Hiltemann, 2020).

2.4 Plant Materials and Growth Conditions for Las resistance assessment

In this work, the plants of the Oceanian citrus genotypes were the same used in the previous work by Alves et al. (2021b), and included *M. warburgiana* (F.M. Bailey) Tanaka, *M. papuana* Winters (revealed to be actually a *M. papuana* hybrid in the present work), *M. australis* (A. Cunn. ex Mudie) Swingle (actually a *M. australis* hybrid),

E. glauca (Lindl.) Swingle, a *Microcitrus* sp. hybrid (formerly called *Microcitrus* sp. $\times$ *E. glauca*), an *E. glauca* $\times$ *Microcitrus* sp. complex hybrid, and a supposed *E. glauca* $\times$ *Citrus* $\times$ *sinensis* hybrid (eremorange), being the susceptible sweet orange cultivar 'Tobias' [*C. sinensis* (L.) Osbeck] used as control. All these accessions were propagated onto Las-susceptible 'Rangpur' lime nucellar rootstocks. Budwood used to propagate each accession came from a single donor mother tree. Plants were kept in a greenhouse with air temperature varying from 20.0 to 30.0 °C and natural sunlight condition. They were grown in 4 L polyethylene bags filled with coir, fertigated twice a week, and sprayed with insecticides and miticides monthly. Other information on the accessions, challenge-inoculation procedures and plant maintenance practices are described in detail in Alves et al. (2021b). Oceanian citrus and 'Tobias' sweet orange control plants used for experiments 1 to 5 reported here (Figure 1) were ~ 3 to 4-year-old. Las had been detected in all rootstock bark samples by qPCR with an average cycle threshold (Ct) of 30.0 ± 0.4 , and in the 'Tobias' sweet orange control scion leaves (Ct 23.8 ± 1.4), at 30 months after graft inoculation. Some composite plants from all the seven Oceanian citrus and 'Tobias' sweet orange challenge-inoculated genotypes died along the course of the experiments due to the damage caused by Las infection to their 'Rangpur' lime root system.

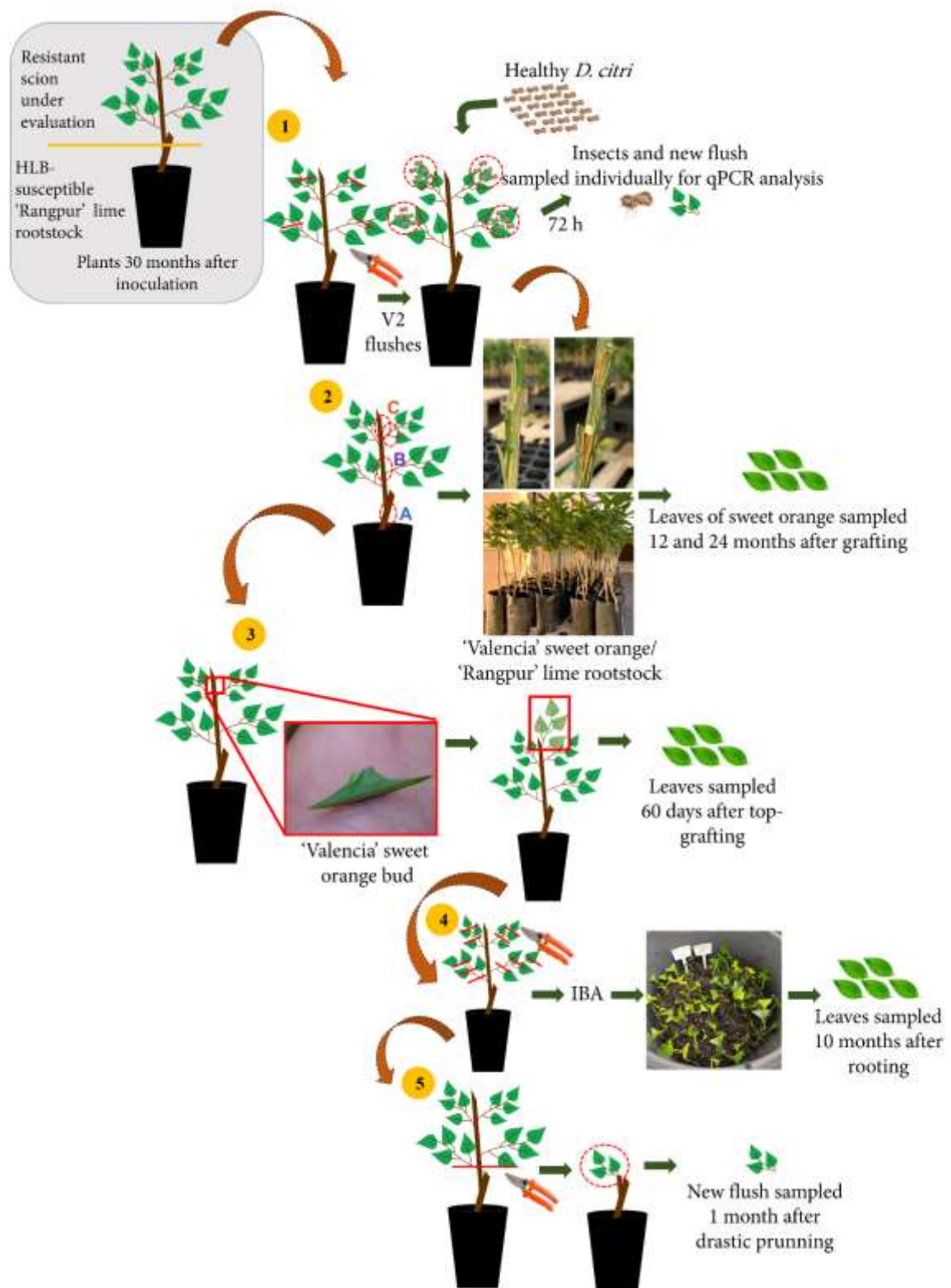


Figure 1. Schematic design of the five experiments carried out (1) Las acquisition by *Diaphorina citri* reared on Oceanian citrus genotypes; (2) Las indexing in 'Valencia' sweet orange plants grafted with (A) bark tissue from 5 cm below the graft union of

Las-infected ‘Rangpur’ lime used as rootstock, (B) bark tissue from 8 cm above the graft union and (C) budwood from the top of the Oceanian citrus genotypes; (3) Top-grafting of ‘Valencia’ sweet orange plants on scions from the Oceanian citrus genotypes; (4) Las multiplication through stem cuttings of Oceanian citrus genotypes; and (5) Forcing Las colonization from the ‘Rangpur’ lime rootstock to new sprouted shoots of Oceanian citrus genotypes after severe back-pruning.

2.5 Experiment 1: Las acquisition by *Diaphorina citri* reared on Oceanian citrus genotypes

Thirty months after Las graft challenge-inoculation and the categorization of the seven Oceanian citrus accessions as resistant, the tip of the branches from the canopy of those plants that remained alive was pruned to promote flushing. Three to four flushes per plant in the V2 phenological stage of development (Cifuentes-Arenas et al., 2018) were selected and used to confine five 20-day-old Las-free *D. citri* adults per flush for 72 h of acquisition access period (Figure 1.1). The insects were obtained from a colony that had been reared on Las-free *Murraya paniculata* (L.) Jack seedlings. After the confinement, insects and 5-10 leaves of each flush were sampled individually, DNA was extracted, and Las detection was assessed by qPCR. As control, Las-infected ‘Tobias’ sweet orange plants were used.

2.6 Experiment 2: Las infection in ‘Valencia’ sweet orange plants grafted with bark and budwood from the Oceanian citrus genotypes

174 two-year-old plants of ‘Valencia’ sweet orange on ‘Rangpur’ lime rootstock were grafted with bark patches and budwood from the seven Oceanian citrus genotypes and ‘Tobias’ sweet orange as control to assess the eventual transmission of Las from bark and budwood pieces of challenge-inoculated plants to healthy ‘Valencia’ sweet orange plants (Figure 1.2; Figure 2.A). All ‘Valencia’ sweet orange plants were grown in 4 L containers. Two patches of bark tissue (Figure 2.B) or budwood (Figure 2.C) with ca. 3 to 5 cm long by 0.8 cm wide size from 4 to 11 individual plants of each Oceanian citrus genotype were grafted at 15 cm above the scion-rootstock union of each ‘Valencia’ sweet orange tree. The bark and budwood tissues were collected from three different regions of each plant from the Oceanian citrus

genotypes evaluated: at 5 cm below the scion-rootstock union ('Rangpur' lime trunk), and 8 cm above it (Oceanian citrus genotype trunk), and at the highest shoot of the scion canopy of all available plants (budwood). As control, the same approach was used with bark and budwood tissue from Las-infected 'Tobias' sweet orange plants. A sample of 0.2 g from each bark and budwood piece to be grafted in 'Valencia' sweet orange plants, was used to assess Las by qPCR analysis. Three months after grafting, the 'Valencia' sweet orange plants were top pruned at about 1.5 cm distant from the apex to stimulate the development of new shoots and, consequently, to force Las movement from the bark and budwood tissue used as eventual inoculum source to the new tissues of 'Valencia' sweet orange (Raiol-Junior et al., 2021a). The union of the bark and budwood tissue with the 'Valencia' sweet orange stems was visually observed to evaluate the graft success (Figure 2.D). After 12 and 24 months of grafting, four to six leaves were randomly sampled in the 'Valencia' sweet orange scion of all tested plants and processed for DNA extraction and qPCR analysis for Las detection.



Figure 2. Representative aspect (A) of a 'Valencia' sweet orange plant grafted with bark or budwood from the Oceanian citrus genotypes in Experiment 2; (B) of bark tissue taken from 5 cm below the graft union of Las-infected 'Rangpur' lime used as rootstock or from 8 cm above the graft union; (C) of budwood removed from the top of the Oceanian citrus genotypes; (D) and from a failed bark graft in experiment 2.

2.7 Experiment 3: Top-grafting of ‘Valencia’ sweet orange plants on scions from the Oceanian citrus genotypes

Buds from a Las-free greenhouse-grown ‘Valencia’ sweet orange plant were collected and grafted onto the highest point of the scion top of 4-11 plants from the seven Oceanian citrus genotypes and ‘Tobias’ sweet orange, used as control (Figure 1.3). This experiment aimed to determine whether Las would be able to translocate from the infected ‘Rangpur’ lime rootstock through the Oceanian citrus genotypes, now used as interstock and colonize the top-grafted ‘Valencia’ sweet orange scions. Sixty days after the ‘Valencia’ buds had grown and developed as scions, their leaves were randomly sampled for DNA extraction and qPCR analysis.

2.8 Experiment 4: Las multiplication in cuttings of Oceanian citrus genotypes

To further assess whether the scion tissues of the Oceanian citrus genotypes were free of Las, stem cuttings were propagated for rooting (Figure 1.4). Twenty cuttings with 5-8 cm were collected from partially-lignified shoots of the canopy from the Oceanian citrus genotypes *M. australis* hybrid, *E. glauca*, eremorange, *Microcitrus* sp. hybrid, and *E. glauca* × *Microcitrus* sp. hybrid and ‘Tobias’ sweet orange as control. Two leaves were kept in each cutting top and they were cut in half to reduce transpiration. Cutting basal ends were immersed for 0.5 s in a rooting aqueous solution of synthetic auxin indole-3-butyric acid (IBA) (5,000 ppm), then they were disposed inside 12 L polyethylene plastic containers filled with decomposed pine bark substrate (Terra do Paraíso, Holambra, São Paulo, Brazil), and were manually irrigated whenever necessary. The containers were maintained inside a wet chamber for 90 days. Ten months after cutting propagation, 4 to 6 leaves of each cutting-derived plant remaining alive were sampled for Las detection through qPCR.

2.9 Experiment 5: Forcing Las colonization in shoots of Oceanian citrus genotypes

All original plants remaining alive from *M. australis* hybrid, *E. glauca*, eremorange, *Microcitrus* sp. hybrid, and *E. glauca* × *Microcitrus* sp. hybrid and ‘Tobias’ sweet orange as control were drastically back-pruned at 5 cm above the scion-rootstock union to force new flush growth and Las movement from the Las-infected ‘Rangpur’ lime rootstock to new leaves on the forced grown scion flush. Thirty days after pruning, five to ten leaves of the newly emerged flushes per plant were randomly sampled for DNA extraction and qPCR analysis (Figure 1.5).

2.10 Evaluation by qPCR

Samples were processed as described by Teixeira et al. (2005) using a TissueLyser II system (Qiagen, Valencia, CA, United States) at 45 Hz for 30 s in a 2.0-ml microtube containing 5-mm steel beads. For insect processing, one insect and a 3-mm bead were used in a 1.5 mL microtube. Total DNA were obtained from 0.5 g of leaf midribs, or 0.3 g of root and bark tissue, using a CTAB (cetyltrimethylammonium bromide) buffer (2% CTAB; 1.4 M NaCl; 2% PVP 10000; 0.5 M EDTA pH 8; 1 M Tris-HCl pH 8; 0.2% b-mercaptoethanol) as mentioned in Murray and Thompson (1980). DNA was resuspended in Milli-Q® water. DNA quality and concentration were measured using a nanodrop (Thermo Fisher Scientific, Massachusetts, EUA) (Desjardins and Conklin, 2010). For qPCR and detection of the 16S rDNA from Las, 1 µL of total DNA (100 ng/µL), TaqMan® PCR Master Mix (1x) (Invitrogen, Carlsbad, CA, United States) and Las-specific primers/probe (0.5 µM/0.2 µM) were used in a StepOnePlus thermocycler (Applied Biosystems, California, USA), as described by Li et al. (2006). As internal control for the quality of plants and psyllids DNA, the mitochondrial gene cytochrome oxidase (COX) (Li et al., 2006) and an insect wingless (*Wg*) (Manjunath et al., 2008) gene region were used, respectively. Furthermore, leaf and insect samples, negative and positive for Las, were used during DNA extractions and qPCR assays as negative and positive controls, respectively. qPCR cycle threshold (Ct) values higher than 34.0 produce variable results (Lopes et al. 2013), therefore, samples were classified as ‘positive’ with $Ct \leq 34.0$, suspected to be positive with $34.0 < Ct \text{ values} < 36.0$, and negative with $Ct > 36$ and/or when non-detected.

Average Ct and the standard deviation values of treatments were calculated using positive Ct values.

3. RESULTS

3.1 Phenotyping analysis of Oceanian citrus genotypes

The *M. warburgiana* accession used in this study showed axillary solitary spines and elongate-elliptical leaves as well as small and globular fruit with six segments (Figure 3.A), coincident with its description by Swingle and Reece (1967). The *M. papuana* accession used by us showed small and slightly elongated leaves, and yielded semi-globose fruit (Figure 3.B), which resembled a hybrid rather than the pure species. According to Winters (1976), *M. papuana* produces narrower, elongated leaves as well as long, cylindrical fruit.

The Australian round lime *M. australis* is easily distinguished from all the other *Microcitrus* species by its globose or slightly pear-shaped, rough-skinned fruit. Our *M. australis* genotype showed slightly pear-shaped fruit (Figure 3.C), and instead solitary, paired spines were predominant in its leaf axils, which are characteristic of *M. inodora* (Figure 3.D), suggesting that the *M. australis* hybrid used in this study may be an *M. australis* × *M. inodora* hybrid.

The phenotypic traits of *M. garrawayae* (Figure 3.E) and *Clymenia polyandra* (Figure 3.F) accessions from Fundecitrus also fit with the description of the species by Swingle and Reece (1967). The *M. garrawayae* accession presents small narrow, thick and leathery, lozenge-shaped to broadly lanceolate leaves and the *C. polyandra* one has spineless twigs with leaves being very peculiar, with elliptical shape, acuminate at both ends, and with a wingless petiole. In addition, *C. polyandra* produces single flowers in the axils of the leaves.

Eremocitrus genus is one of the most distinctive among near *Citrus* relatives. Morphological traits were similar in our *E. glauca* accession (Figure 3.G) and the one described by Swingle and Reece (1967). In general, the hybrids described between *Eremocitrus* and *Citrus* (Swingle and Reece, 1967) showed unifoliate leaves of intermediate size between those of the two parental species, but thinner and bifacial,

thus more similar to *Citrus* leaves, which coincided with the characteristics presented by the *E. glauca* × *C. sinensis* accession used in this study (Figure 3.H).

Regarding the other *Microcitrus* hybrids, *Microcitrus* sp. (Figure 3.I) and *E. glauca* × *Microcitrus* sp. (Figure 3.J) hybrids showed leaf, spines and fruit traits resembling the Australian finger lime *M. australasica* (Figure 3.K). However, *Microcitrus* sp. fruit is subglobose, not fusiform as that of typical finger limes, suggesting that it may be a hybrid with another *Microcitrus* species yielding globose fruit as the Australian round lime. In the case of *E. glauca* × *Microcitrus* sp. hybrid, its leaves showed a narrow, thicker, coriaceous aspect resembling those of the eremorange but fruits were long, cylindric and slender, with red color, indicating that this genotype may be a hybrid between *E. glauca* and *M. australasica* (Figure 3.I). Accumulation of anthocyanins providing red color to fruit is common in some *M. australasica* accessions.

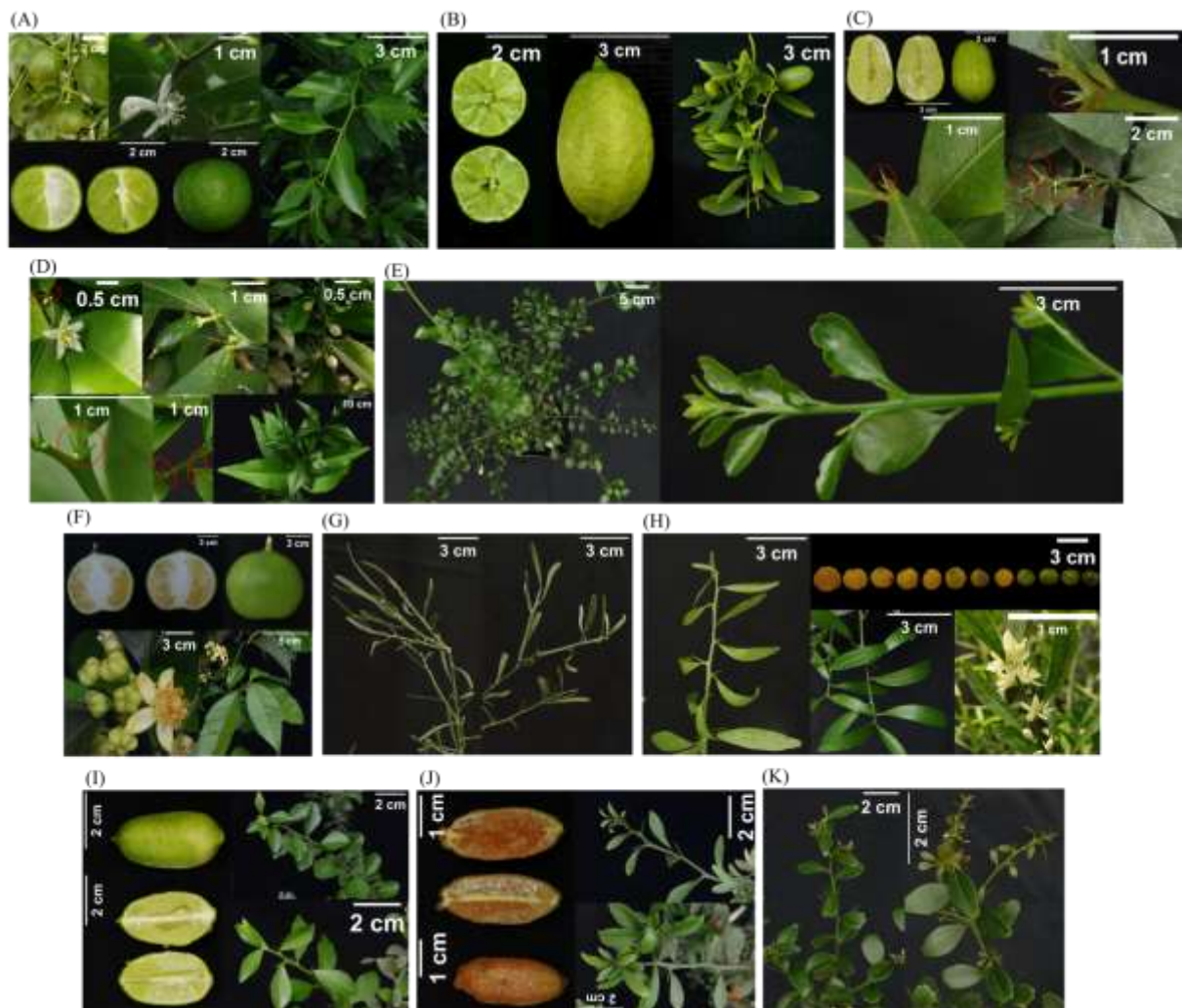


Figure 3. Morphological characterization of (A) *M. warburgiana*; (B) *M. papuana* × *M. warburgiana* hybrid; (C) *M. australis* × *M. inodora* hybrid; (D) *M. inodora*; (E) *M. garrawayae*; (F) *Clymenia polyandra*; (G) *E. glauca*; (H) *E. glauca* × *Citrus* sp. hybrid; (I) *M. australasica* × (*M. australis* × *M. australasica*) hybrid; (J) [*E. glauca* × (*M. australis* × *M. australasica*)] × *M. australasica* hybrid; and (K) *M. australasica*.

3.2 Genotyping analysis

After filtering the diallelic SNPs for less than 20% of missing data with VCF tool, we obtained a genotyping matrix of 49187 diallelic SNPs for 27 accessions for the nuclear genome and a matrix with 40 SNPs for the mitochondrial genome. Only five SNPs were obtained for the chloroplast genome and were not found useful for maternal inheritance analysis.

The analysis of heterozygosity performed for the Fundecitrus and Corsican accessions in comparison with already published accessions (Figure 4.A) allowed to identify pure species representative with most of the heterozygosity distribution curve under 10% (Figure 4.B) from admixed and interspecific hybrid accessions (Figure 4.C).

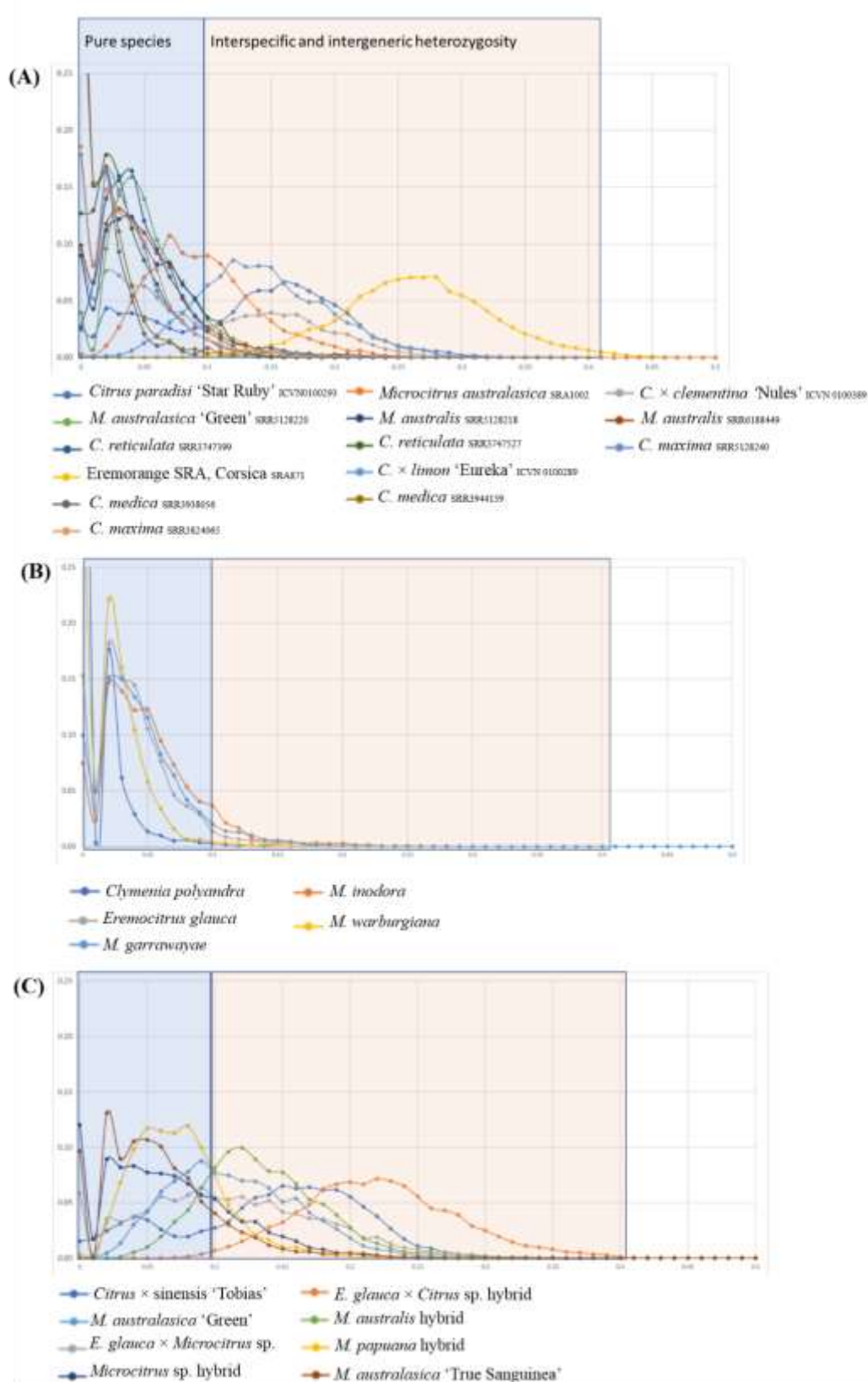


Figure 4. Distribution of heterozygosity values for the 27 accessions, established from sliding windows of 100 successive SNPs. (A): already published genome with known

phylogenomic status; (B): pure specific accessions from Fundecitrus Oceanian germplasm; (C): interspecific and admixed accessions of Fundecitrus Oceanian germplasm.

Among the Fundecitrus germplasm *M. warburgiana*, *M. garrawayae*, *M. inodora*, *E. glauca* and *C. polyandra* appears as pure representatives of their species while *M. australis*, *Eremocitrus* × *Microcitrus*, *Microcitrus* sp., *M. papuana* and the three finger limes (the 'True Sanguinea' and the 'Green' accessions from Fundecitrus and the accession from Corsica) appeared to result from interspecific admixture. *E. glauca* × *C. sinensis* from Fundecitrus displayed a very similar curve than the one of the eremorange accessions of Corsica with high value of heterozygosity resulting from the strong differentiation between *E. glauca* and the Asian *Citrus* species.

Factorial analyses of subset of the data provided first elements on the origin of the admixed accessions. A first factorial analysis focused in the Oceanian accessions (Figure 5.A). The first axis (28.9%) opposes the finger limes accessions that are close to the true *M. australasica* representative and the species from Papua-New Guinea (*M. warburgiana* and *M. papuana*). The second axis (26.0%) discriminates the *E. glauca* accessions from the *Microcitrus* ones. The *Eremocitrus* × *Microcitrus* hybrid has an intermediate position in this axis confirming the intergeneric admixture and its position on the first axis suggests a major contribution of *M. australasica*. The position of *Microcitrus* sp. accession suggests no, or very low, *E. glauca* contribution and a major *M. australasica* one. *M. australis*, *M. inodora* and *M. garrawayae* still poorly resolved in this analysis and were subjected to an independent factorial analysis (Figure 5.B). The first axis (48%) opposes *M. garrawayae* and the *M. australis* pure representative accessions while the second axis discriminates *M. inodora* from all other pure species representatives. The intermediate position of the *M. australis* accession of Fundecitrus between the two true *M. australis* accessions and *M. inodora*, in addition to its important heterozygosity, suggests that it may be an interspecific F1 hybrid of *M. australis* × *M. inodora*. The last analysis (Figure 5.C) concerned the supposed intergeneric hybrids between *Eremocitrus* and *C. sinensis*, the representative accessions of the corresponding ancestors (*E. glauca*, *C. maxima* and *C. reticulata*) as well as some admixed genotypes between these two citrus species. The first axis (61.1% of the total diversity) differentiates *E. glauca* from the *Citrus* species while the

axis 2 (26.9%) separates *C. maxima* from *C. reticulata* with clementine, sweet orange and grapefruit in intermediate positions fully in agreement with their known phylogenomic constitution (Wu et al. 2014, 2018; Ahmed et al., 2019). The supposed intergeneric *E. glauca* × *C.* × *sinensis* accession from Fundecitrus is close to the eremorange from Corsica. In axis 1 these two accessions have an intermediate position between *Eremocitrus* and *Citrus* species while their relative positions in axis 2 suggest that the eremorange from Corsica has a greater *C. maxima* contribution than the *E. glauca* × *C.* × *sinensis* from Fundecitrus. Taking into account the probable admixture or hybrid status of accessions revealed by the heterozygosity and factorial analysis, the diagnostic SNP mining for the different ancestors was performed using only pure representative for 10 considered ancestral species. A total of 20708 Diagnostic SNPs were identified and the number of DSNPs for each ancestor is given in the NJ tree of the 14 used accessions (Figure 6). The distribution over the nine chromosomes is available as Suppl. Table 1.

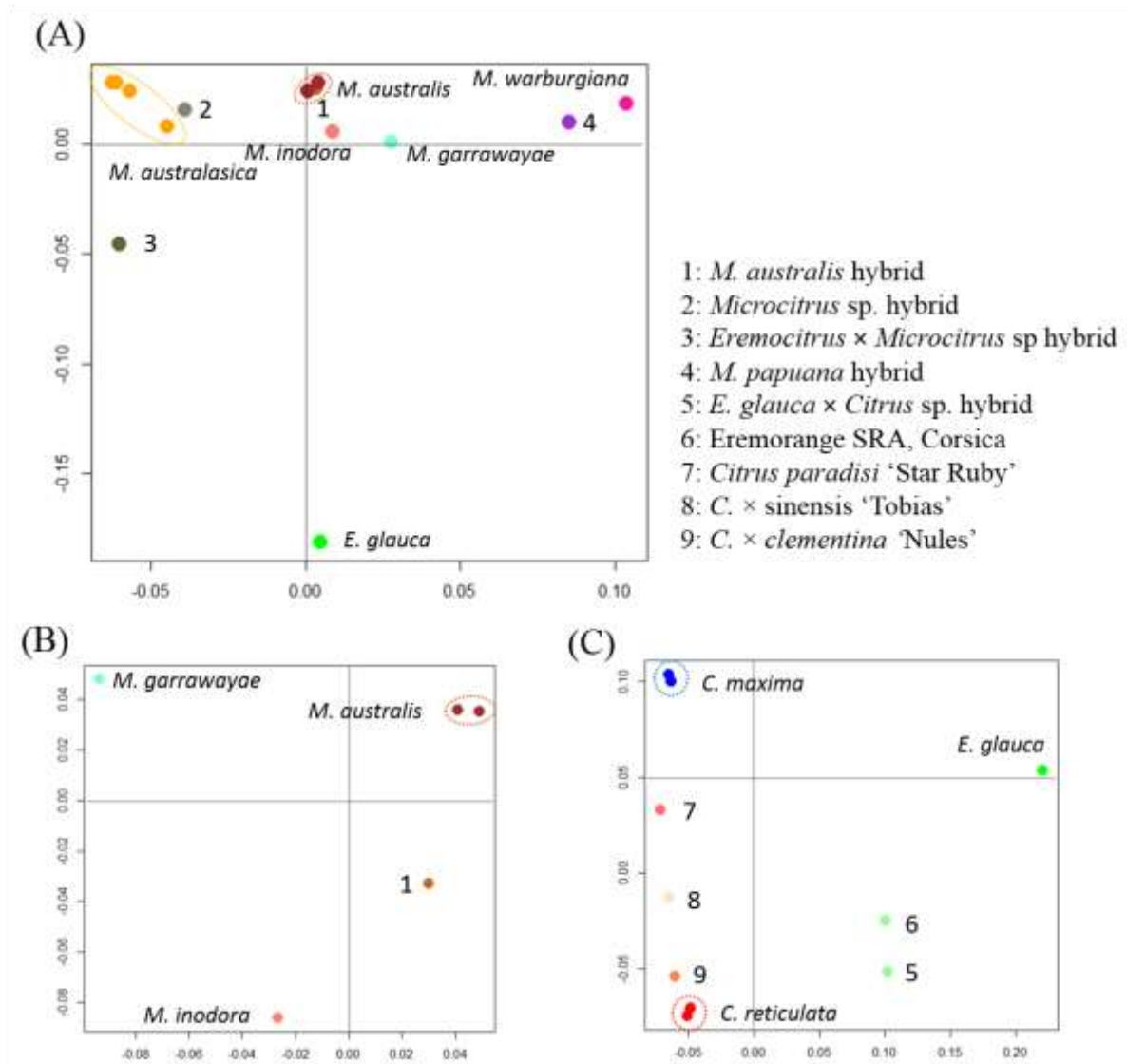


Figure 5. Factorial analysis from the genotyping of 49187 diallelic SNPs. (A): Organization of the genetic diversity of the Ocenian germplasm. (B) Focus on the relationship of *M. australis* hybrid accession with *M. australis*, *M. garrawayae* and *M. inodora*; (C): Focus on the relationship of the two supposed eremoranges with the *C. reticulata*/*C. maxima* gene pools.

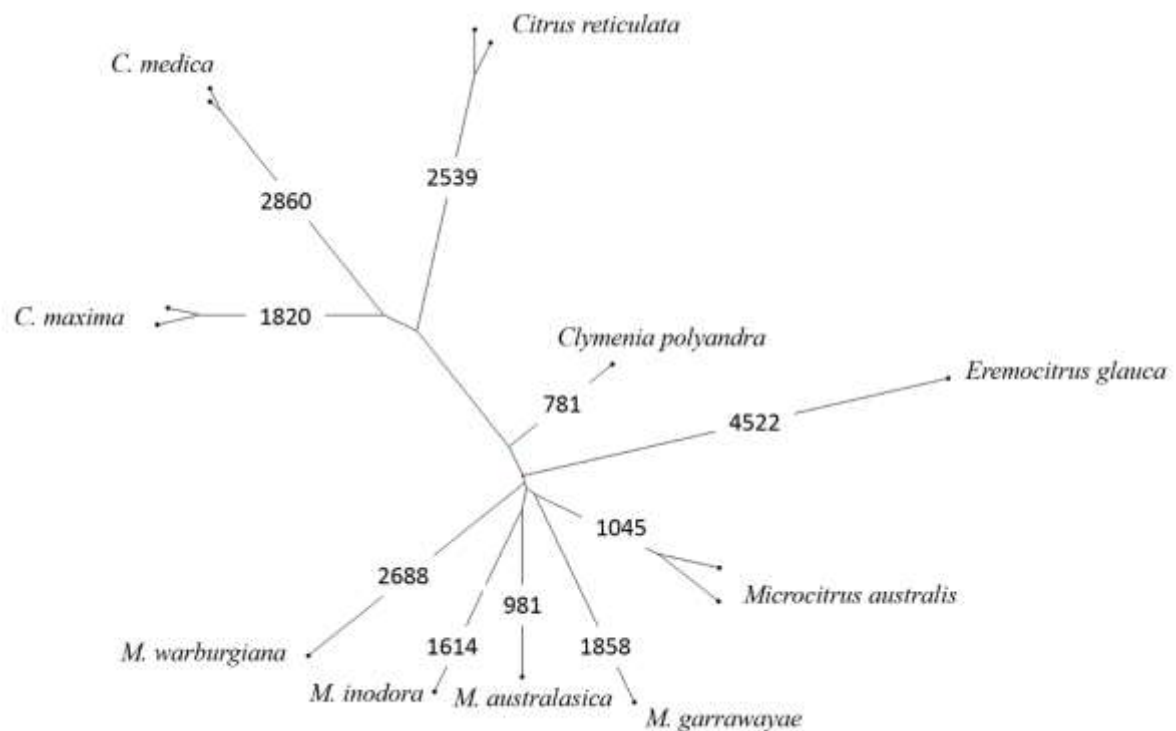


Figure 6. Organization of the nuclear diversity of 10 Asian and Oceanian species; NJ tree analysis from the genotyping of 49187 diallelic SNPs and number of DSNPs for each species.

The global contribution of the ten ancestors to the interspecific hybrids or admixed accessions was estimated from the proportion of specific alleles over the set of DSNPs of each ancestor (Table 1). Contributions below 1.5% were considered as artifactual. The contributions of *C. maxima* and *C. reticulata* to sweet orange, grapefruit and clementine are in full agreement with previous works (Wu et al. 2014, 2018; Oueslati et al. 2017) as well as the contributions of *C. medica*, *C. maxima* and *C. reticulata* to lemon (Wu et al., 2018; Ahmed et al., 2019). The two supposed *E. glauca* × *C. sinensis* hybrids displayed a contribution of 50% from *E. glauca*. Both displayed also *C. reticulata* and *C. maxima* contributions with a greater proportion of *C. reticulata* (47%) in the Fundecitrus accession than in the Corsican one (40%). All the finger limes accessions displayed introgressions of *M. australis* in a *M. australasica* background. The estimated contributions of *M. australis* and *M. inodora* to the *M. australis* accession from Fundecitrus are respectively 47 and 40%. The *Eremocitrus* × *Microcitrus* accession displayed a major contribution of *M. australasica* (69%) followed by *E.*

glauca (23%) and *M. australis* (5.9%). This analysis confirms the absence of *E. glauca* contribution for the supposed *Microcitrus* × *Eremocitrus* hybrid. The *M. papuana* accession displayed a major but incomplete contribution of *M. warburgiana* (65%) without any signal for the other ancestors considered in our study.

Table 1. Contribution of 10 ancestral species to the admixed and interspecific hybrids of Fundecitrus and Corsican Asian and Oceanian citrus germplasm.

	<i>C. maxima</i>	<i>C. reticulata</i>	<i>C. medica</i>	<i>M. garrawayae</i>	<i>E. glauca</i>	<i>M. australis</i>	<i>M. inodora</i>	<i>M. warburgiana</i>	<i>M. australasica</i>	<i>C. polyandra</i>
<i>Citrus</i> × <i>sinensis</i> 'Tobias'	41.5%	55.9%	0.7%	0.1%	0.1%	0.1%	0.0%	0.1%	0.0%	0.6%
<i>E. glauca</i> × <i>Citrus</i> sp.	4.3%	47.4%	0.2%	0.1%	50.0%	0.0%	0.1%	0.1%	0.1%	0.6%
<i>E. glauca</i> × <i>Microcitrus</i> sp.	0.2%	0.5%	0.3%	0.6%	23.2%	5.9%	1.5%	1.7%	68.6%	1.2%
<i>M. papuana</i> hybrid	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	64.7%	0.0%	0.0%
<i>M. australasica</i> 'True Sanguinea'	0.2%	0.3%	0.4%	0.5%	0.4%	21.3%	1.8%	0.6%	67.0%	0.9%
<i>M. australasica</i> 'Green'	0.3%	0.3%	0.3%	0.5%	0.3%	23.2%	1.4%	0.4%	65.7%	1.0%
<i>M. australis</i> hybrid	0.2%	0.2%	0.7%	0.5%	0.1%	47.2%	39.9%	0.8%	1.4%	0.8%
<i>Microcitrus</i> sp. hybrid	0.4%	0.7%	0.3%	0.6%	0.5%	18.1%	1.7%	0.6%	55.6%	1.3%
<i>Citrus paradisi</i> 'Star Ruby'	63.3%	36.4%	0.7%	0.3%	0.0%	0.1%	0.0%	0.1%	0.0%	0.5%
<i>M. australasica</i> SRA, Corsica	0.4%	0.3%	0.3%	0.5%	0.3%	17.6%	1.5%	0.5%	66.8%	0.5%
<i>C. × clementina</i> 'Nules'	18.1%	77.7%	0.3%	0.1%	0.0%	0.2%	0.1%	0.0%	0.0%	0.4%
Eremorange SRA, Corsica	11.9%	39.8%	0.3%	0.2%	50.1%	0.2%	0.1%	0.1%	0.1%	0.8%
<i>C. × limon</i> 'Eureka'	17.9%	33.4%	56.6%	0.1%	0.1%	0.2%	0.0%	0.0%	0.4%	0.9%

Phylogenomic karyotypes are proposed for the admixed accessions analyzed for HLB resistance. They were inferred from the curve, all along the genome, for homozygosity and heterozygosity for the specific alleles of each ancestor involved in the admixture. Figure 7 provides an example for the supposed *E. glauca* × *C. × sinensis* accession. One haplotype is fully constituted by *E. glauca* genome while the other is a mixture of *C. reticulata* and *C. maxima* with a major contribution of *C. reticulata*. This accession is clearly a direct hybrid between *E. glauca* and an Asian variety with *C. reticulata*/*C. maxima* admixture. The presence of *C. reticulata* heterozygosity signal at the end of chromosome 2, while sweet orange is homozygous for *C. maxima* in the same region discards sweet orange as the second parent. It is more probably that a cultivated mandarin or a tangor (mandarin × sweet orange hybrid) may be the second parent. The distribution of *C. maxima* genome in the clementine one (Wu et al., 2014) is compatible with the hypothesis that the second parent is a clementine. The Circos representation for *M. australis* hybrid, *Microcitrus* ×

Eremocitrus, *Eremocitrus* × *Microcitrus*, *M. papuana* hybrid, ‘Tobias’ sweet orange and the eremorange from Corsica are given in Suppl. Figure 1. The heterozygosity all along the genome for *M. australis* and *M. inodora* confirms the hypothesis that the *M. australis* accession from Fundecitrus results from a direct hybridization between *M. australis* and *M. inodora*. Among the two accessions supposed to result from admixture between *E. glauca* and *Microcitrus* species, this background is confirmed for the *Eremocitrus* × *Microcitrus* accessions. It displays homozygous regions for *M. australasica* as well as heterozygous areas of *M. australasica*/*M. australis* and *M. australasica*/*E. glauca*. It may be a hybrid between *M. australasica* and a second parent resulting from complex admixture between *E. glauca*, *M. australis* and *M. australasica*. The genome of the supposed *Microcitrus* × *Eremocitrus* hybrid is constituted in large part of *M. australasica* in homozygosity, some regions of *M. australasica*/*M. australis* heterozygosity and a small portion of *M. australis* homozygosity at the start of the chromosome 9. *M. papuana* displays significant signal for *M. warburgiana* heterozygosity in most part of the genome but also signal for *M. warburgiana* homozygosity. If it is a hybrid of *M. papuana*, as suggested by the morphology analysis, this concomitant signal for *M. warburgiana* DSNPs homozygosity and heterozygosity may be explained by the fact that *M. papuana* and *M. warburgiana* share numerous specific mutations differentiating the Papua-New Guinea species from the Australian ones. Indeed, in the absence of pure representative of *M. papuana* for the DSNPs mining, these Papua-New Guinea specific SNPs should have been assimilated as *M. warburgiana* DSNPs resulting in false homozygosity signal for *M. warburgiana* in a hybrid between two Papua-New Guinea species.

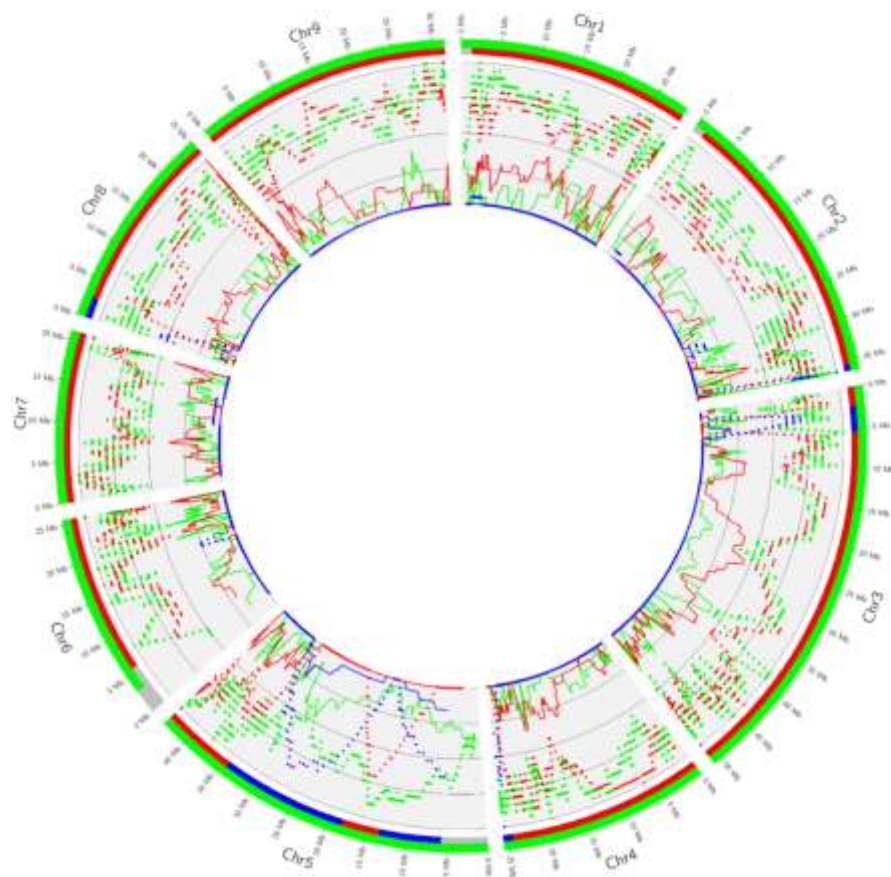


Figure 7. Distribution of heterozygosity (dashes) and homozygosity (line) of the ancestral alleles of *E. glauca* (green), *C. reticulata* (red) and *C. maxima* (blue) along the genome of the supposed *E. glauca* × *C. sinensis* hybrid and phylogenomic karyotype inference (external ribbons with the same color code). Grey corresponds to undetermined regions.

From the NJ tree based on mitochondrial data (Figure 8), it appears that: the *E. glauca* × *C. sinensis* and *Eremocitrus* × *Microcitrus* accessions inherited *E. glauca* mitochondria as well as the eremorange from Corsica. All analyzed finger limes and the *Microcitrus* hybrid accessions have *M. australasica* mitochondria while the *M. australis* from Fundecitrus has *M. australis* mitochondria. The mitochondria of the *M. papuana* accession is differentiated from the *M. warburgiana* one and may be representative of the *M. papuana* species. To summarize, genotypes used in this study, their common names and phylogenomic constitution after genotyping by sequencing (GBS) analysis are listed on Table 2.

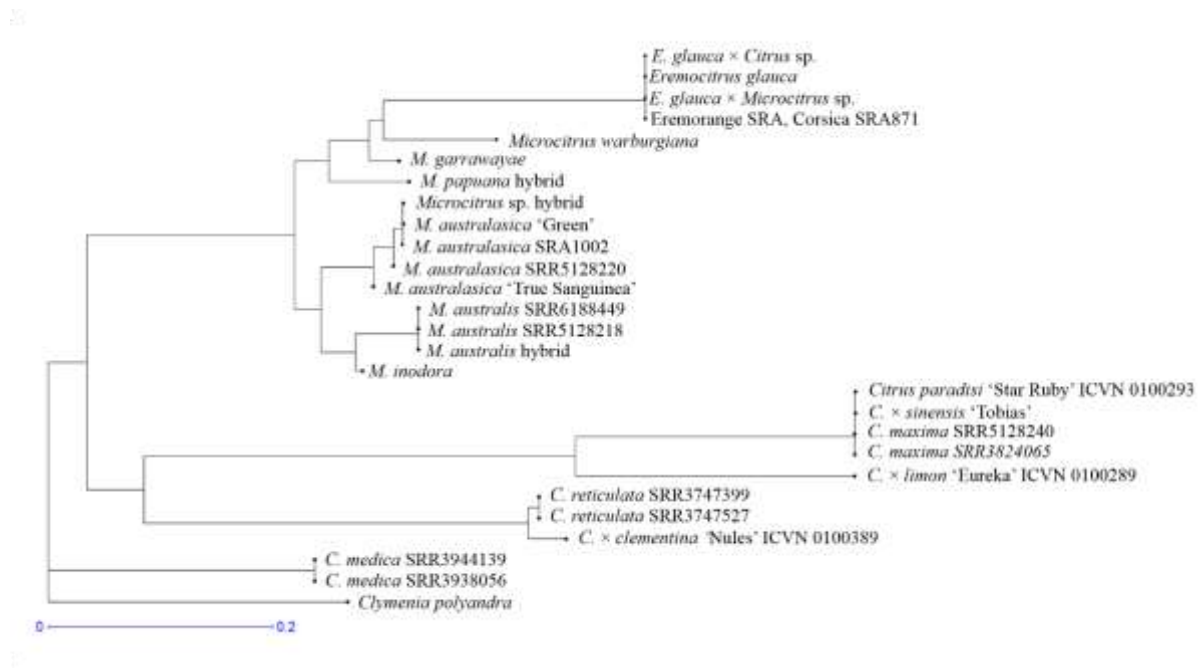


Figure 8. Assignment of the mitochondrial genome of hybrids and admixed Oceanian accessions in relation to ancestral mitochondrial ones; NJ tree based on the genotyping for 40 mitochondrial diallelic DSNPs.

Table 2. Genotypes used in this study, their common names and phylogenomic constitution after genotyping by sequencing (GBS) analysis.

Genotype ^a	Common name ^b	After GBS ^c
<i>Citrus</i> × <i>sinensis</i> (L.) Osbeck 'Tobias' (P) ^c	Tobias sweet orange	<i>C. × sinensis</i> (L.) Osbeck 'Tobias'
<i>Microcitrus warburgiana</i> (F.M. Bailey) Tanaka (M) ^d	New Guinean wild lime	<i>M. warburgiana</i> (F.M. Bailey) Tanaka
<i>M. papuana</i> Winters (M)	Brown river finger lime	<i>M. papuana</i> × <i>M. warburgiana</i> hybrid
<i>M. australis</i> (A. Cunn. Ex Mudie) Swingle (M)	Australian round lime	<i>M. australis</i> × <i>M. inodora</i> hybrid
<i>Eremocitrus glauca</i> (Lindl.) Swingle (M)	Australian desert lime	<i>Eremocitrus. glauca</i>
<i>Microcitrus</i> sp. × <i>E. glauca</i> hybrid (PM) ^e	Australian lime hybrid BGC 695 ^b	<i>M. australasica</i> × (<i>M. australis</i> × <i>M. australasica</i>) hybrid
<i>E. glauca</i> × <i>C. × sinensis</i> (P) ^f	Eremorange	<i>E. glauca</i> × <i>Citrus</i> sp. hybrid
<i>E. glauca</i> × <i>Microcitrus</i> sp. (PM)	Australian desert lime hybrid BGC 682 ^b	[<i>E. glauca</i> × (<i>M. australis</i> × <i>M. australasica</i>)] × <i>M. australasica</i> hybrid

^aThe nomenclature used follows Swingle and Reece (1967) and Bayer et al. (2009) and was also previously used in Alves et al. (2021b).

^bOriginal accession number at the Citrus Germplasm Bank (BGC) of Embrapa Cassava & Fruits in Cruz das Almas, Bahia.

^cP: Polyembryonic; ^dM: Monoembryonic; ^ePM: Possibly monoembryonic; According to Swingle and Reece (1967) and Bitters et al. (1986).

3.3 Experiment 1: Las acquisition by *Diaphorina citri* reared on Oceanian citrus genotypes

After 72 h of *D. citri* confinement, none of the new shoot flushes evaluated from plants of all Oceanian citrus genotypes resulted positive by qPCR, while in the case of 'Tobias' sweet orange 100% of the flushes were Las-positive (Table 3). Insects reared on plant flushes followed the same tendency, with none of those fed on Oceanian citrus showing Ct values for Las < 34, but 97.14% (133 of 140) of those fed on 'Tobias' sweet orange being clearly Las-positive ($Ct \leq 34$). However, a small number of insects were considered suspicious to be positive as they showed Ct values between 34 and 36, specifically 2 of 160 fed on *M. australis* hybrid, 7 of 140 on the *Microcitrus* sp. hybrid, 6 of 220 on the *E. glauca* × *Citrus* sp. hybrid and 2 of 140 on 'Tobias' sweet orange. All insects fed on *M. warburgiana*, *M. papuana* hybrid, *E. glauca* and *E. glauca* × *Microcitrus* sp. hybrid were Las-negative (Suppl. Table 2).

Table 3. Acquisition of '*Candidatus Liberibacter asiaticus*' (Las) by *Diaphorina citri* reared for 72 h on new shoot flushes from plants of seven Oceanian citrus genotypes and 'Tobias' sweet orange as control, all grafted onto Las-infected 'Rangpur' lime rootstocks. Plants had been graft-challenge inoculated with Las in both rootstock and scion 30 months earlier. Las titer in log10 of amplicon copies per gram of plant tissue was estimated based on a standard curve as described by Lopes et al. (2013).

Genotype	Number of plants used for insect acquisition	New shoot flushes		<i>D. citri</i> adults	
		Frequency of Las-positive / total flushes	Ct avg±SEM / Log±SEM ^a	Frequency of Las-positive / total insects	Ct avg±SEM / Log ±SEM ^b
<i>Citrus</i> × <i>sinensis</i> 'Tobias'	8	(28/28)	27.5±0.55 / 6.1±0.63	(133/145)	31.4±0.14 / 2.3±0.04
<i>Microcitrus warburgiana</i>	5	(0/14)	nd ^c	(0/70)	nd
<i>M. papuana</i> hybrid	4	(0/16)	nd	(0/80)	nd
<i>M. australis</i> hybrid	8	(0/32)	nd	(0/160)	nd
<i>Eremocitrus glauca</i>	6	(0/12)	nd	(0/64)	nd
<i>Microcitrus</i> sp. hybrid	7	(0/28)	nd	(0/140)	nd
<i>E. glauca</i> × <i>Citrus</i> sp. hybrid	11	(0/44)	nd	(0/220)	nd
<i>E. glauca</i> × <i>Microcitrus</i> sp. hybrid	8	(0/32)	nd	(0/160)	nd

^a Cycle threshold/log10 of amplicon copies per gram of plant tissue average and standard error from all flushes.

^b Cycle threshold/log10 of amplicon copies per gram of plant tissue average and standard error from all insects.

^c Non-detected.

3.4 Experiment 2: Las infection in 'Valencia' sweet orange plants grafted with bark and budwood from the Oceanian citrus genotypes

Las infection was confirmed in the 'Rangpur' lime rootstock bark patches from all composite plants remaining alive with scions of the seven Oceanian citrus genotypes and 'Tobias' sweet orange control, with Ct average values ranging from 28.5 ± 0.91 to 32.6 ± 0.25 (Table 4). Las was also detected in the bark patches of the scions taken at 8 cm above the scion-rootstock union, with Ct average values ranging from 26.4 ± 0.64 to 32.8 ± 0.50 , with the exception of all those coming from *E. glauca* which showed Ct values > 34 . Moreover, bark pieces at 8 cm from most *M. warburgiana* and *M. australis* hybrid scions were also Las-negative (Suppl. Table 3). In budwood collected from the apex, Las was detected in all but one (7 of 8) 'Tobias' sweet orange, and only 2 of 11 *E. glauca* × *Citrus* sp. hybrid samples with Ct average values ranging from 22.5 ± 1.09 to 24.0 ± 0.05 , while Las was hardly found in few samples of *M. australis* hybrid (2 of 8), *Microcitrus* sp. hybrid (3 of 7) and *E. glauca* × *Microcitrus* sp. hybrid (1 of 8), with Ct averages ranging from 30.7 ± 2.74 to 33.6 ± 0.00 in Las-positive samples. Las was not detected in budwood of *M. warburgiana*, *M. papuana* hybrid and *E. glauca* scions.

Table 4. 'Candidatus Liberibacter asiaticus' (Las) infection, determined by quantitative polymerase chain reaction, in bark patches and budwood collected from different regions (8 cm above the graft union and at the top, respectively) of Oceanian citrus and 'Tobias' sweet orange plants, as well as from the stem (5 cm below the graft union) of Las-infected 'Rangpur' lime used as rootstock, graft success and Las transmission from those bark patches and budwood to 'Valencia' sweet orange plants, 12 and 24 months after grafting. Las titer in log10 of amplicon copies per gram of plant tissue was estimated based on a standard curve as described by Lopes et al. (2013).

Genotype	N ^a	Average Ct±SEM ^b values of Las in bark tissue and budwood grafted onto healthy 'Valencia' sweet orange plants				Average Ct±SEM values of Las in leaves of the grafted 'Valencia' sweet orange plants					
		Rootstock	Scion			12 months after grafting			24 months after grafting		
						Rootstock	Scion		Rootstock	Scion	
			8 cm	Apex			8 cm	Apex		8 cm	Apex
<i>Citrus</i> × <i>sinensis</i> 'Tobias'	8	28.8±0.84 / 3.8±0.25 (8/8)	26.4±0.64 / 4.5±0.19 (8/8)	22.5±1.09 / 5.6±0.33 (7/8)	F ^c	21.8±1.19 / 5.9±0.36 (6/8) ^d	29.7±2.13 / 3.5±0.64 (7/8)	F	29.4±0.65/ 3.6±0.20 (6/8)	23.0±1.39 / 5.5±0.42 (7/8)	
<i>Microcitrus warburgiana</i>	5	29.9±1.1 / 3.4±0.3 (5/5)	30.4±0.00 / 3.3±0.00 (1/5)	nd ^b (0/5)	F	F	nd (0/5)	F	F	nd (0/5)	
<i>M. papuana</i> hybrid	4	28.5±0.91 / 3.8±0.27 (4/4)	29.1±1.09 / 3.7±0.33 (4/4)	nd (0/4)	F	F	nd (0/4)	F	F	nd (0/4)	
<i>M. australis</i> hybrid	8	30.1±1.20 / 3.4±0.36 (8/8)	28.5±0.00 / 3.9±0.00 (1/8)	32.5±0.67 / 2.7±0.20 (2/8)	F	F	nd (0/8)	F	F	nd (0/8)	

<i>Eremocitrus glauca</i>	7	30.5±1.13 / 3.2±0.34 (7/7)	nd (0/7)	nd (0/7)	F	F	nd (0/7)	F	F	nd (0/7)
<i>Microcitrus</i> sp. hybrid	7	29.0±1.37 / 3.7±0.41 (7/7)	27.7±1.00 / 4.1±0.30 (5/7)	30.7±2.74 / 3.2±0.81 (3/7)	F	F	nd (0/7)	F	F	nd (0/7)
<i>E. glauca</i> × <i>Citrus</i> sp. hybrid	11	28.6±0.85 / 3.8±0.25 (11/11)	29.7±0.50 / 3.5±0.15 (10/11)	24.0±0.05 / 5.2±0.01 (2/11)	F	F	nd (0/11)	F	F	nd (0/11)
<i>E. glauca</i> × <i>Microcitrus</i> sp. hybrid	8	32.6±0.25 / 2.6±0.08 (8/8)	32.8±0.50 / 2.6±0.15 (2/8)	33.6±0.00 / 2.3±0.00 (1/8)	F	F	nd (0/8)	F	F	nd (0/8)

^a Number of plants used for the Las graft challenge inoculation experiment (all with Las-positive rootstocks, Ct ≤ 34.0).

^b Non-detected.

^c F = Grafting failed.

^d Number of positive 'Valencia' sweet orange plants (Ct ≤ 34.0)/total number of indexed 'Valencia' sweet orange plants.

3.5 Experiment 3: Top-grafting of 'Valencia' sweet orange plants on scions from the Oceanian citrus genotypes

Budwood from healthy 'Valencia' sweet orange plants was grafted on the top of the original plants remaining alive from the Oceanian citrus genotypes and 'Tobias' sweet orange growing on Las-infected 'Rangpur' lime rootstock. Unfortunately, at the time this experiment was conducted, no plants of *M. warburgiana* were alive and thus this genotype was not evaluated. In addition, no 'Valencia' sweet orange budwood sprouted on *Microcitrus* sp. hybrid plants (Table 5), likely due to their highly debilitated root system. Rates of budwood sprouting varied from 1/8 for 'Tobias' sweet orange, 1/4 for *M. papuana* hybrid, 2/8 for *M. australis* hybrid, 2/6 for *E. glauca*, 4/11 for *E. glauca* × *Citrus* sp. hybrid and 3/8 for *E. glauca* × *Microcitrus* sp. hybrid. Las was detected only in 'Valencia' sweet orange samples top-grafted on the 'Tobias' sweet orange with a Ct of 26.9 (Table 5; Suppl. Table 4).

Table 5. Graft success and titer of '*Candidatus Liberibacter asiaticus*' (Las) in new shoots grown from budwood of 'Valencia' sweet orange top-grafted on Oceanian citrus genotypes and 'Tobias' sweet orange, all growing onto Las-infected 'Rangpur' lime rootstocks, as determined through detection of Las16S DNA by qPCR 60 days after bud grafting. Las titer in log10 of amplicon copies per gram of plant tissue was estimated based on a standard curve as described by Lopes et al. (2013).

Genotype	N ^a	Ct / Log ^b
<i>Citrus</i> × <i>sinensis</i> 'Tobias'	1/8	26.9 / 4.3
<i>M. papuana</i> hybrid	1/4	nd ^c
<i>M. australis</i> hybrid	2/8	nd
<i>Eremocitrus glauca</i>	2/6	nd

<i>Microcitrus</i> sp. hybrid	0/7	
<i>E. glauca</i> × <i>Citrus</i> sp. hybrid	4/11	nd
<i>E. glauca</i> × <i>Microcitrus</i> sp. hybrid	3/8	nd

^a Number of 'Valencia' sweet orange indicator sprouts over the total number of bud-grafted plants.

^b Cycle threshold / log10 of amplicon copies per gram of plant tissue.

^c Non-detected.

3.6 Experiment 4: Las multiplication in cuttings of Oceanian citrus genotypes

No plants of *M. warburgiana* or *M. papuana* hybrid were alive to attempt establishing stem cuttings for rooting and all cuttings performed from *M. australis* hybrid, *E. glauca* and *E. glauca* × *Citrus* sp. hybrid stems did not survive as the root system of the original plants was almost dead by aggressive Las infection of the 'Rangpur' lime rootstock. However, 32 and 5 cutting-derived rooted plants were obtained from *Microcitrus* sp. and *E. glauca* × *Microcitrus* sp. hybrids, respectively, which were all Las-free 10 months after propagation (Table 6; Suppl. Table 5). As controls, 33 of 39 cutting-derived plants of 'Tobias' sweet orange, had Ct average values between 21.6 ± 0.28 and 29.9 ± 1.13 .

Table 6. '*Candidatus Liberibacter asiaticus*' (Las) infection in leaves of cutting-derived plantlets obtained from Oceanian citrus genotypes and 'Tobias' sweet orange control, all grafted onto Las-infected 'Rangpur' lime rootstocks, 2 years before and ten months after cutting propagation. Las titer in log10 of amplicon copies per gram of plant tissue was estimated based on a standard curve as described by Lopes et al. (2013).

Genotype	Original Plant ^a Ct/Log at 24 MAI ^b			Cutting-derived plants	
	Plant	Scion leaves	Rootstock	N ^c	Ct avg±SEM / Log avg±SEM ^d
<i>Citrus</i> × <i>sinensis</i> 'Tobias'	1	21.2 / 6.0	30.1 / 3.4	9/10	24.8±0.34 / 5.0±0.10
	2	27.9 / 4.0	29.8 / 3.5	9/10	29.9±1.13 / 3.5±0.34
	3	22.9 / 5.5	30.1 / 3.4	6/9	28.0±2.87 / 3.6±0.67
	4	20.3 / 6.3	29.9 / 3.4	9/10	21.6±0.28 / 5.9±0.08
<i>Microcitrus australis</i> hybrid	1	nd	30.1 / 3.4		F ^e
	2	nd	30.1 / 3.4		F
	3	nd	29.9 / 3.4		F
	4	nd	30.3 / 3.3		F
<i>Eremocitrus glauca</i>	1	nd	27.9 / 4.0		F
	2	nd	30.3 / 3.3		F
	3	nd	31.7 / 2.9		F

	4	nd	30.8 / 3.2		F
<i>Microcitrus</i> sp. hybrid	1	nd	29.0 / 3.7	0/10	nd ^f
	2	nd	28.9 / 3.7	0/7	nd
	3	nd	31.7 / 2.9	0/5	nd
	4	nd	30.1 / 3.4	0/10	nd
<i>Eremocitrus glauca</i> × <i>Citrus</i> sp. hybrid	1	nd	29.0 / 3.7		F
	2	nd	27.2 / 4.2		F
	3	nd	30.6 / 3.2		F
	4	nd	29.7 / 3.5		F
<i>E. glauca</i> × <i>Microcitrus</i> sp. hybrid	1	nd	33.4 / 2.4	0/3	nd
	2	nd	33.0 / 2.5	0/2	nd
	3	nd	33.3 / 2.4		F
	4	nd	33.7 / 2.3		F

^a Plants Las-challenged by grafting from which stems were removed to attempt rooting.

^b Months after graft-challenge Las inoculation.

^c Number of rooted stem cutting per plant which were evaluated for Las infection.

^d Cycle threshold average determined through detection of the 16S DNA by qPCR ± Standard error of the mean/ log10 of amplicon copies per gram of plant tissue ± Standard error of the mean.

^e Rooting failed.

^f Non-detected.

3.7 Experiment 5: Forcing Las colonization in new shoots of Oceanian citrus genotypes

The stems of all surviving Oceanian citrus plants growing onto Las-infected 'Rangpur' lime rootstock were pruned exhaustively in attempts to force Las movement from the rootstock to the new growing tissues in the scions. New shoots grew on stems of most plants, except *E. glauca* × *Microcitrus* sp. hybrid for which only one out of eight plants sprouted. Las was detected in 6 of the 8 'Tobias' plants with an average Ct value of 26.8 ± 1.48 (Table 7; Suppl. Table 6), but new shoots from *M. australis* hybrid, *E. glauca*, *Microcitrus* sp. × *E. glauca* hybrid and *E. glauca* × *Citrus* sp. hybrid plants were all Las-negative. All original plants of *M. warburgiana* and *M. papuana* hybrid were dead before performing this experiment, as mentioned above.

Table 7. '*Candidatus* Liberibacter asiaticus' detection in new flushes from Oceanian citrus genotypes and 'Tobias' sweet orange as scions onto Las-infected 'Rangpur' lime rootstocks, one month after drastic pruning of the canopy at 5 cm above the scion-rootstock union. Las titer in log10 of amplicon copies per gram of plant tissue was estimated based on a standard curve as described by Lopes et al. (2013).

Genotypes	N ^a	New flushes after pruning	Freq. ^c
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		Ct avg±SEM ^b	
<i>Citrus × sinensis</i> 'Tobias'	6/8	26.8 ± 1.48 / 4.4±0.45	6/6
<i>M. australis</i> hybrid	8/8	nd ^d	0/8
<i>Eremocitrus glauca</i>	7/7	nd	0/7
<i>Microcitrus</i> sp. hybrid	7/7	nd	0/7
<i>E. glauca</i> × <i>Citrus</i> sp.	11/11	nd	0/11
<i>E. glauca</i> × <i>Microcitrus</i> sp. hybrid	1/8	nd	0/1

^a Number of plants that showed new shoot flushes after drastic pruning/total number of original plants that were pruned.

^b Cycle threshold average determined through detection of the 16S DNA by qPCR / log10 of amplicon copies per gram of plant tissue and standard error of the mean.

^c Number of qPCR-positive plants (Ct ≤ 34.0)/total number of plants with flushes evaluated.

^d Non-detected.

4. DISCUSSION

In view of the huge losses in citrus production and quality caused by HLB, as well as the high costs of the existing management strategies, finding a durable and sustainable solution for this disease has been the objective of different research programs worldwide. Genetic resistance to HLB has been found in several citrus relatives of the Rutaceae family, Aurantioideae subfamily, when seedling trees in the field were subjected during six years to natural disease challenge conditions in Florida, an HLB endemic region (Ramadugu et al., 2016). However, the experimental system did not reveal whether immune germplasm was actually resistance to the bacterium or to the insect vector. For example, the most HLB field-resistant species belonging to the Clauseneae tribe, such as *Clausena lansium* and *Glycosmis pentaphylla*, are resistant to *Diaphorina citri* under controlled greenhouse conditions (Felisberto et al., 2019). Conversely, other Clauseneae species such as *Bergera koenigii* and *Murraya paniculata*, are excellent *D. citri* hosts (Tomaseto et al., 2019; Eduardo et al., 2022b *in press*) but non- or transient Las hosts, respectively (Cifuentes-Arenas et al., 2019, Alves et al., 2021a). Among genera from the true citrus species, partial resistance to *D. citri* has been found in *Poncirus* (Westbrook et al., 2011; Richardson and Hall, 2013; Hall et al., 2015; Felisberto et al., 2019) and in Oceanian *Microcitrus* and *Eremocitrus* species (Eduardo et al., 2022a *in press*). Therefore, to assess resistance within *Citrus* and *Citrus* relatives to either the bacterium, the insect vector or both, experiments performed under controlled environmental conditions and using challenge procedures rendering unequivocal results are advisable.

In a previous study, after propagation of over 20 citrus relatives within the Citrinae subtribe, Citreae tribe, onto the Las-susceptible 'Rangpur' lime rootstock and graft-inoculation with Las-infected budwood on both the scion and rootstock in controlled greenhouse conditions, we selected seven Oceanian citrus genotypes as full-resistant to Las, that is, Las was never detected in their scion leaves over a period of 24 months after inoculation but it did in all rootstocks tested (Alves et al., 2021b). However, as Las was identified through qPCR in the stem tissue at 5 cm above the scion-rootstock union in most plants of the resistant genotypes, and also in stem bark tissue at 30 cm height in a few plants of two of the genotypes under evaluation, we wondered whether these genotypes were actually partial-resistant to Las or transient hosts. Moreover, we performed a phenotypic description and a fine phylogenomic characterization of the seven Oceanian citrus accessions.

The phenotypic analysis of the Fundecitrus accessions considered as representative of *M. papuana* and *M. australis* revealed that they should be rather ascribed as interspecific hybrids. Moreover, the characteristics of the supposed interspecific or intergeneric hybrids did not allow concluding on their origin accurately. Therefore, we performed, by GBS, a fine genetic characterization of their nuclear and mitochondrial genomes in comparison with those of pure representatives of ten ancestral species of Asian and Oceanian citrus. The identification of 20708 DSNPs allowed us to efficiently infer ancestral karyotypes of the Fundecitrus germplasm, including full-resistant accessions. It extended to Oceanian species the TraceAncestor approach on GBS data proposed by Ahmed et al. (2019) for Asian citrus. The DSNPs set should be improved by analyzing several true representatives of the different species and particularly representatives of *M. papuana* that we missed in the present study. In agreement with the phenotypic observations, this analysis confirmed that the *M. inodora*, *M. garrawayae*, *M. warburgiana*, *E. glauca* and *C. polyandra* accessions of Fundecitrus germplasm were pure representative of these species. According to nuclear and cytoplasmic genome data, the *M. australis* accession should be a *M. australis* × *M. inodora* hybrid while the *M. papuana* accession is a probable admixture between *M. papuana* and *M. warburgiana* with *M. papuana* mitochondria. It would be necessary to include a true *M. papuana* representative in the phylogenomic analysis to conclude this unquestionably. The supposed *E. glauca* × *C. sinensis* appeared to

result from an intergeneric cross between *E. glauca* as female parent and an admixed *C. reticulata*/*C. maxima* Asian variety as male parent. However, the phylogenomic profiles discarded sweet orange as male parent and suggested that the second parent was a clementine. This accession is different from the eremorange from Corsica whose phylogenomic profile is fully compatible with an *E. glauca* × *C. sinensis* origin (Wu et al., 2018). The *Eremocitrus* × *Microcitrus* accession is a complex hybrid involving, in order of importance, *M. australasica*, *E. glauca*, and *M. australis*. According to the nuclear and mitochondrial data, it should have resulted from [*E. glauca* × (*M. australis* × *M. australasica*)] × *M. australasica* hybridizations. The *Microcitrus* hybrid may have resulted from a kind of backcross *M. australasica* × (*M. australis* × *M. australasica*) with however a small *M. australis* introgression in the *M. australasica* recurrent parent. It is interesting to notice that the three Australian finger limes from *Fundecitrus* and Corsica are not pure *M. australasica* representatives but all them display *M. australis* introgressions. As a whole, these results highlight the important inclination of the Oceanian species to produce interspecific and even intergeneric hybrids, likely favored by the absence of facultative apomixis in these species. Consequently, this results sometimes in confusing classifications of these accessions in germplasm collections, particularly if the accessions were introduced by seeds. It also stresses the importance of clonal propagation of this germplasm for any analysis of genetic resistance to Las using Oceanian citrus species.

Next, to better characterize the resistance phenotype of the seven full-resistant Oceanian citrus species and hybrids, new experiments with the same plants were designed. We first considered whether *D. citri* adults were able to acquire Las when fed on Las challenge-inoculated Oceanian citrus scions still surviving on Las-infected ‘Rangpur’ lime rootstocks. Adult *D. citri* can acquire Las within 5-7 h feeding on infected plants (Xu et al., 1988). In our study, after 72 h of confinement on new flushes of the Las-susceptible ‘Tobias’ sweet orange in an environment proper for Las acquisition (Alves et al., 2021a), 97% of the insects acquired the bacteria. Conversely, Las was not detected in any insect fed on the shoot flushes of the seven Oceanian citrus genotypes, which, as expected, were also negative. These results suggested that Las detected in the Oceanian citrus stems did not reach the scions canopy or did at titers insufficient for insect acquisition and then transmission. However, Las was detected in

budwood of the canopy of at least some plants from the *M. australis* hybrid and the other three hybrids with *M. australasica* or sweet orange background in concentrations theoretically sufficient to be acquired by *D. citri* and transmitted to other host plants (Lopes and Cifuentes-Arenas, 2021).

Then, we attempted Las transmission by grafting bark pieces and budwood from the Oceanian citrus scions onto 'Valencia' sweet orange healthy plants. Although bark grafting failed for all Oceanian species and hybrids, budwood grafting was highly successful in all cases, revealing that Las was efficiently transmitted from Las-positive 'Tobias' to 'Valencia' sweet orange but, if present, it was not transmitted in any case from the Oceanian citrus genotypes to 'Valencia' sweet orange receptor plants. Efficiency of Las transmission by grafting infective budwood is usually high. Notwithstanding, its frequency depends on the bacterial titer in the plant tissue used for grafting, the kind, number, and size of the infected materials used for inoculation, the season of the year and the environmental conditions (Lopes et al., 2009). Frequency of successful Las transmission in graft-inoculated, greenhouse-grown *C. × sinensis* plants of 'Hamlin', 'Natal', 'Pera', and 'Valencia' sweet orange cultivars was reported to span from 54 to 88% (Lopes et al., 2009; Albrecht et al., 2014). The failure to transmit Las from apical budwood of the Oceanian citrus genotypes suggests that Las detected by qPCR on them was at low titer, with few or no cells alive, and that viable cells were below a minimum threshold number insufficient to be transmitted by grafting.

Afterwards, we tried to force Las infection by either top-grafting healthy 'Valencia' sweet orange onto the Oceanian citrus scions or by establishing Oceanian citrus scion stem cuttings in pots for rooting. Once either 'Valencia' sweet orange or Oceanian citrus cuttings started to grow, they were evaluated for Las detection. However, few plants survived after attempting top-grafting or set up cuttings, mainly because the 'Rangpur' lime rootstock was so much compromised by Las infection that Oceanian citrus scions were already dying when these experiments were carried out, about 36 to 48 months after challenge inoculation. Even with this, 'Valencia' sweet orange plants were Las-negative on *M. papuana* hybrid, *M. australis* hybrid, *E. glauca*, *Microcitrus* sp. hybrid, *E. glauca* × *Citrus* sp. hybrid and *E. glauca* × *Microcitrus* sp. hybrid remaining alive while only 'Tobias' sweet orange interstock was able to infect

the top-grafted ‘Valencia’ sweet orange. The graft compatibility of those Las-resistant Oceanian citrus genotypes with a citrus elite variety such as ‘Valencia’ opens the possibility for their use as putatively HLB-resistant rootstocks/interstocks for other citrus cultivars. In citriculture, there are several examples of successful disease control by using resistant/tolerant rootstocks, such as in the cases of citrus tristeza (Garnsey et al., 1987; Yoshida, 1996; Yokomi et al., 2017), citrus sudden death (Roman et al., 2004; Yamamoto et al., 2011) and *Phytophthora* spp. gummosis (Matheron et al. 1998; Wutscher, 1979), though for all these scion-rootstock combinations, scion cultivars used should be also disease resistant or tolerant as a premise. For HLB, the situation would be different because there are no citrus cultivars resistant to Las and most important varieties are quite sensitive to the bacterium. However, the use of Las-resistant rootstocks may alleviate symptoms and perhaps delay tree decline. Specific experiments should be planned to test this.

On the other hand, rooted cuttings were successfully established only for *Microcitrus* sp. and *E. glauca* × *Microcitrus* sp. hybrids as well as for ‘Tobias’ sweet orange, being 84% of the latter Las-positive while none of the Oceanian citrus hybrid cuttings were Las-infected. The lack of infection in 16% of ‘Tobias’ sweet orange cuttings may be explained by the uneven distribution of Las within the infected cuttings and composite trees (Tatineni et al., 2008; Li et al., 2009; Raiol-Junior et al., 2021a). Lack of Las-infection in rooted cuttings of the Oceanian citrus hybrids remaining alive may be indicative of the inability of the bacterium to accumulate in the phloem of these genotypes and suggested indirectly that erratic detection of Las in Oceanian citrus may result from bacterial movement from the infected rootstock favored under specific physiological conditions.

Aiming to press the canopy of Oceanian citrus to become invaded by viable bacteria from the rootstock, we pruned severely the Oceanian citrus genotypes close to the bud union with the rootstock to promote vigorous scion sprouting and sap movement up with the new flush. Most of the pruned plants produced new growth successfully. Whereas new flushes from ‘Tobias’ sweet orange were all Las-positive with high bacterial titers, those from *M. australis* hybrid, *E. glauca* and three other Oceanian citrus hybrids were all Las-negative further indicating that the bacterium was unable to infect consistently Oceanian citrus species and hybrids previously

considered by us as full-resistant to Las. Under greenhouse conditions, Las moves 2.9 - 3.9 cm day⁻¹ in a citrus host (Raiol-Junior et al., 2021b), preferably towards new flushes and newly developed roots, by following the source:sink driven sap flow (Raiol-Junior et al., 2021a). The presence of Las only in 'Tobias' sweet orange shoot flushes after 30 days of sprouting confirmed the viability and the fast movement of Las within the phloem of susceptible plants. On the other hand, the absence of Las in new flushes of *M. australis* hybrid, *E. glauca*, *Microcitrus* sp. hybrid, *E. glauca* × *Citrus* sp. hybrid and *E. glauca* × *Microcitrus* sp. hybrid, proved the inability of Las to colonize new tissues of these resistant genotypes.

Overall, these experiments together confirmed that the Oceanian true species, *M. warburgiana* and *E. glauca* together with the *M. papuana* × *M. warburgiana* hybrid were full-resistant to Las, as their canopies resulted Las-negative in all our thorough tests, *D. citri* was unable to acquire Las from their young leaves and attempts to force Las flow from the 'Rangpur' lime rootstocks to them as scions or interstocks were unsuccessful. Based on Swingle and Reece (1967) all known species of *Microcitrus* and *Eremocitrus* emerged from a common ancestor likely resembling *M. warburgiana*, the Papua/New Guinea species, from which different lines of evolution produced the quite unique, pronounced xerophytic *E. glauca* and other *Microcitrus* species adapted to different regions of Australia. In the context of resistance to Las, it should be noticed that the two *Microcitrus* species from Papua/New Guinea (*M. warburgiana* and *M. papuana*) as well as *E. glauca* are close phylogenetically according to their mitochondrial genomes and this correlates with these three Oceanian species or hybrids being the most consistently full-resistant to Las, which suggests that full-resistance to Las within Oceanian citrus may have arisen within these few related species.

The response of the other four Oceanian citrus hybrids to Las-aggressive challenge inoculation conceived to compel infection or at least Las translocation from the infected rootstock to the Oceanian hybrids scions was trickier to interpret. Las-positive samples were detected not only close to the rootstock-scion bud unions but also at their canopies, at titers high enough to indicate bacterial infection. As these four genotypes are hybrids between full-resistant and either partial-resistance or susceptible parents, it could be claimed that they may have inherited partial-resistant

actually. However, *D. citri* hardly acquired the bacterium from their young leaves and their budwood was unable to transmit Las to 'Valencia' sweet orange healthy plants used for biological indexing. This together with the unfeasibility to detect Las in rooted cuttings and in new flushes after hard pruning from some of them suggested that most of the bacterium found in Oceanian citrus hybrid tissues was unviable, likely multiplied at the 'Rangpur' lime root systems and moved to sink scion leaves through the phloem vessels under proper physiological conditions and after several years of vascular connections between the heavily Las-infected rootstocks and the Oceanian hybrid scions. Being phloem vessels from Oceanian citrus hybrids incompetent as culture media to support Las survival and multiplication, bacterial cells flowing from the rootstock would progressively decline and die, though unviable bacteria could be detectable by qPCR during months (Josephson et al., 1993). Assessing cell viability of bacterial pathogens in plant cells is important to understand their interactions and disease progress or resistance responses. There are several methods to study bacterial cell viability, including plating assays, serological techniques, *in vivo* imaging markers and flow cytometry, among others (Chitarra and van den Bulk, 2003) but they are mostly applicable to culturable bacteria. Although it has been recently proposed a method to discriminate damaged and viable cells from unculturable bacteria in infected plants based on PCR and plant cell dye staining (Sicard et al., 2020), it is not applicable to phloem vessels-restricted Las. Therefore, distinguishing live and dead bacteria is still unresolved for Las. qPCR provides great sensitivity for Las detection but does not allow to get conclusions on bacterial viability. Another characteristic of qPCR detection for Las is that, in our conditions, Ct values higher than 34 produce variable, inconsistent results (Lopes et al. 2013). Moreover, Ct values between 34 and 36 may result from amplification of non-specific genetic material of other microorganisms also living in the phloem (Bao et al., 2019) as high sequence homology between 16S rDNA from Las and citrus-associated bacteria has already been described (Lin et al., 2008). In our study, only Ct values lower than 34 were considered Las-positive to avoid over-interpretation of Las infection in specific samples on plants that resulted Las-negative repeatedly over years.

Consistent and durable full-resistance in the seven Oceanian citrus genotypes together with proper characterization of their genomic backgrounds and parentages

are essential to be able of properly mapping their genomic regions involved in resistance to Las as well as to use them in breeding programs aimed to generate new citrus-like cultivars yielding immunity or high resistance to HLB. The Las-resistance mechanism in Oceanian citrus species and the nature of Las cells detected in budwood from the Oceanian citrus hybrid genotypes worth further research.

5. AUTHOR CONTRIBUTIONS

MNA, LP, EAG, NAW, SAL and LLR-J conceptualized and designed the work. MNA, LLR-J and EVC collected the data. MM generated GBS data and PO performed genetic and phylogenomic analysis. MNA, LLR-J, EVC, NAW, EAG, PO, SAL, JAF and LP contributed to data analysis and interpretation, drafting and critical revision of the article. All authors contributed to the article and approved the submitted version.

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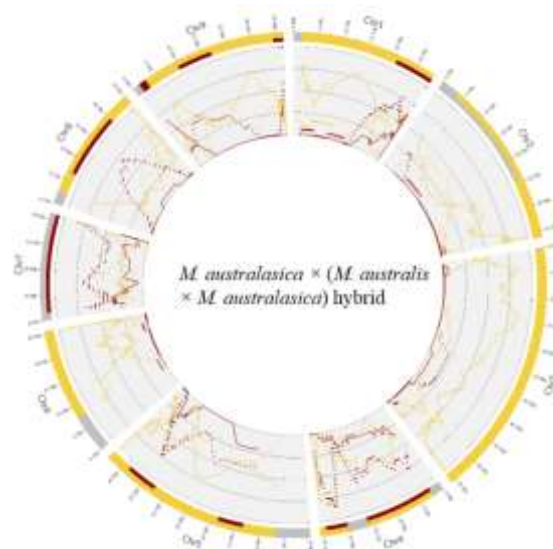
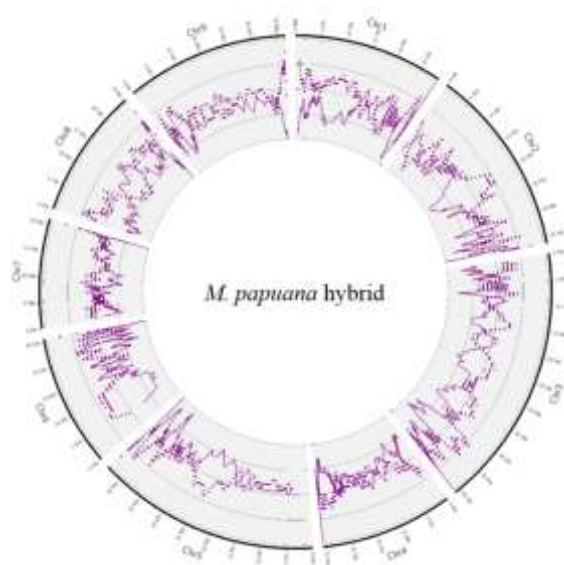
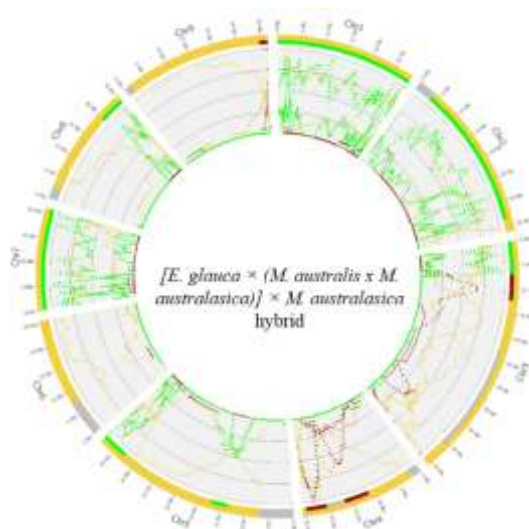
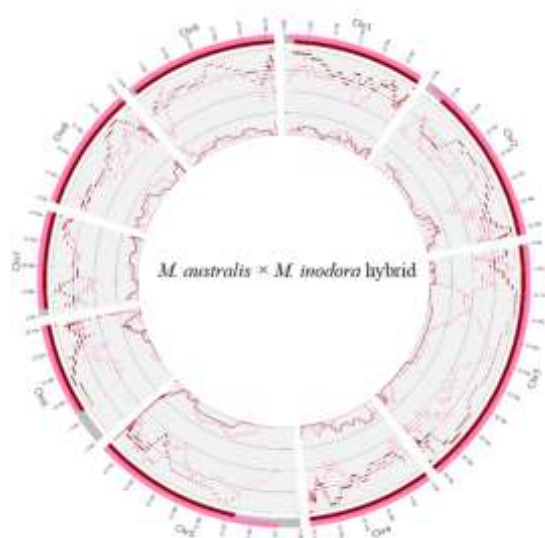
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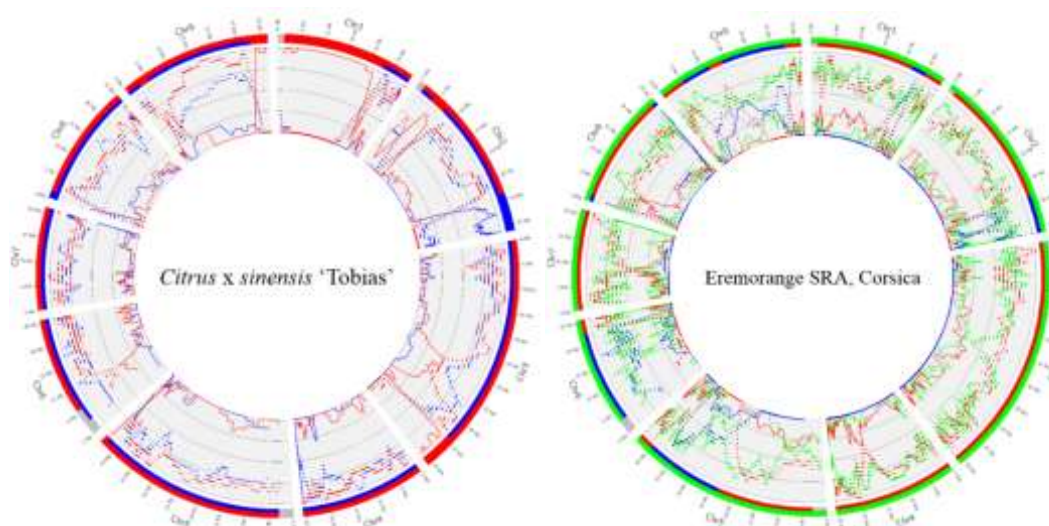
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9. SUPPLEMENTARY MATERIALS

Supplementary Figure 1 Distribution of heterozygosity (dashes) and homozygosity (line) of the ancestral alleles along the genome of the interspecific hybrids and admixed Oceanian accessions and phylogenomic karyotype inference (external ribbons). *E. glauca*: green; *M. australis*: brown, *M. australasica*: yellow; *M. inodora*: pink; *C. reticulata*: red; *C. maxima*: blue; undetermined: grey.





Supplementary Table 1 Diagnostic SNPs of the Oceanian genotypes and Asian reference species analyzed through genotyping by sequencing

Genotype/ Cromossome	Chr1	Chr2	Chr3	Chr4	Chr5	Chr6	Chr7	Chr8	Chr9	Total
<i>M. inodora</i>	163	219	282	194	176	149	128	153	150	1614
<i>M. garrawayae</i>	272	226	328	229	178	106	94	194	231	1858
<i>E. glauca</i>	588	584	779	460	464	443	437	370	397	4522
<i>M. australis</i>	140	123	183	119	113	125	76	84	82	1045
<i>C. maxima</i>	197	253	333	207	199	195	178	120	138	1820
<i>M. warburgiana</i>	305	360	493	287	272	303	222	206	240	2688
<i>C. reticulata</i>	298	341	457	279	245	217	237	235	230	2539
<i>C. medica</i>	266	402	567	362	275	204	198	271	315	2860
<i>M. australasica</i>	112	116	198	110	119	97	71	69	89	981
<i>Clymenia polyandra</i>	95	103	120	79	86	70	80	69	79	781
Total	2436	2727	3740	2326	2127	1909	1721	1771	1951	20708

Supplementary Table 2 Individual Ct values of acquisition of '*Candidatus Liberibacter asiaticus*' (Las) by *Diaphorina citri* reared for 72 h on new shoot flushes from 4 to 11 plants of seven CLas-challenge inoculated Oceanian citrus genotypes and 'Tobias' sweet orange as control, all grafted onto Las-infected 'Rangpur' lime rootstocks. Plants had been graft-challenge inoculated with Las in both rootstock and scion 30 months earlier.

[illegible]

8	nd	nd	nd	ne		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	ne	ne	ne	ne	ne
9	d	d	d	d		d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d
<i>M. papuana</i> hybrid																								
1	d	d	d	d		d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d
2	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
3	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
4	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
5	d	d	d	d		d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d
6	d	d	d	d		d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d
7	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
8	d	d	d	d		d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d
<i>M. australis</i> hybrid																								
1	d	d	d	d		d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d
2	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
3	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	35.7	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
4	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
5	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
6	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	35.1	nd	nd	nd	nd	nd	nd	nd
7	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
8	d	d	d	d		d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d	d
9	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
10	nd	nd	nd	nd		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
<i>E. glauca</i> (Lindl.) Swingle																								
1	nd	nd	nd	ne		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	ne	ne	ne	ne	ne
2	nd	nd	nd	ne		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	ne	ne	ne	ne	ne
3	nd	nd	nd	ne		nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	ne	ne	ne	ne	ne

1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
3	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
4	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
5	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
6	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
7	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
8	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

^aPlant propagation evaluated (with Las-positive rootstocks, Ct ≤ 34.0).

^bCycle threshold determined through detection of the 16S DNA from Las by qPCR.

^cNot-evaluated.

^dPlant died before starting this experiment and .

^eNon-detected.

2	25.3	nd	nd	F	F	nd	F	F	nd
3	29.4	28.5	35.6	F	F	nd	F	F	nd
4	29.4	nd	nd	F	F	nd	F	F	nd
5	33.9	35.0	33.1	F	F	nd	F	F	nd
6	33.2	nd	nd	F	F	nd	F	F	nd
7	25.8	nd	nd	F	F	nd	F	F	nd
8	d	d	d	d	d	d	d	d	d
9	30.0	35.7	31.8	F	F	nd	F	F	nd
10	33.8	nd	nd	F	F	nd	F	F	nd
<i>E. glauca</i> (Lindl.) Swingle									
1	31.8	nd	nd	F	F	nd	F	F	nd
2	27.4	nd	nd	F	F	nd	F	F	nd
3	27.1	nd	nd	F	F	nd	F	F	nd
4	27.6	35.3	nd	F	F	nd	F	F	nd
5	33.1	35.8	nd	F	F	nd	F	F	nd
6	32.9	nd	nd	F	F	nd	F	F	nd
7	33.7	nd	nd	F	F	nd	F	F	nd
<i>Microcitrus</i> sp. hybrid									
1	25.6	26.9	24.4	F	F	nd	F	F	nd
2	25.2	25.7	nd	F	F	nd	F	F	nd
3	33.2	35.2	36.0	F	F	nd	F	F	nd
4	29.5	27.7	33.8	F	F	nd	F	F	nd
5	29.9	31.4	35.0	F	F	nd	F	F	nd
6	34.0	nd	nd	F	F	nd	F	F	nd
7	26.0	26.6	33.9	F	F	nd	F	F	nd
<i>E. glauca</i> x <i>Citrus</i> sp. hybrid									
1	26.0	28.8	nd	F	F	nd	F	F	nd
2	30.7	28.6	23.9	F	F	nd	F	F	nd
3	33.5	30.4	nd	F	F	nd	F	F	nd
4	26.6	30.3	nd	F	F	nd	F	F	nd
5	25.9	30.2	nd	F	F	nd	F	F	nd
6	33.6	nd	nd	F	F	nd	F	F	nd
7	28.3	28.7	35.5	F	F	nd	F	F	nd
8	d	d	d	d	d	d	d	d	d
9	28.5	27.4	nd	F	F	nd	F	F	nd
10	26.7	28.2	nd	F	F	nd	F	F	nd
11	28.1	32.2	24.0	F	F	nd	F	F	nd
12	26.7	31.8	35.0	F	F	nd	F	F	nd
<i>E. glauca</i> x <i>Microcitrus</i> sp. hybrid									
1	32.1	34.4	33.6	F	F	nd	F	F	nd
2	32.9	35.4	nd	F	F	nd	F	F	nd
3	31.9	35.5	nd	F	F	nd	F	F	nd

4	32.2	nd	nd	F	F	nd	F	F	nd
5	32.0	35.7	nd	F	F	nd	F	F	nd
6	33.1	32.3	nd	F	F	nd	F	F	nd
7	32.7	33.3	nd	F	F	nd	F	F	nd
8	34.0	nd	nd	F	F	nd	F	F	nd

^a Months after inoculation

^b Grafting for indexing failed.

^c Plant died and evaluation was not possible to be done.

^d Non-detected.

Supplementary Table 4 Graft success and Ct values of '*Candidatus* Liberibacter asiaticus' (Las) to new shoots grown from budwood of 'Valencia' sweet orange top-grafted on Oceanian citrus genotypes and 'Tobias' sweet orange, all growing onto Las-infected 'Rangpur' lime rootstocks, as determined through detection of CLas16S DNA by qPCR 60 days after bud grafting.

Plant ^a	'Valencia' sweet orange leaves top-grafted
<i>C. × sinensis</i> (L.) Osbeck 'Tobias'	
1	F ^b
2	F
3	F
4	F
5	d ^c
6	26.9
7	F
8	F
9	F
<i>M. warburgiana</i> (F.M. Bailey) Tanaka	
1	F
2	d
3	F
4	d
5	F
6	d
7	F
8	F
9	d
<i>M. papuana</i> hybrid	
1	d
2	F
3	nd ^d
4	F
5	d

6	d
7	F
8	d
<i>M. australis</i> hybrid	
1	d
2	F
3	F
4	nd
5	F
6	F
7	nd
8	d
9	F
10	F
<i>E. glauca</i> (Lindl.) Swingle	
1	F
2	F
3	nd
4	F
5	F
6	F
7	nd
<i>Microcitrus</i> sp. hybrid	
1	F
2	F
3	F
4	F
5	F
6	F
7	F
<i>E. glauca</i> x <i>Citrus</i> sp. hybrid	
1	nd
2	nd
3	F
4	F
5	F
6	F
7	nd
8	d
9	F
10	nd
11	F

12	F
<i>E. glauca</i> x <i>Microcitrus</i> sp. hybrid	
1	F
2	nd
3	F
4	F
5	F
6	F
7	nd
8	nd

^a Plant evaluated (with Las-positive rootstocks, Ct ≤ 34.0).

^b Top-grafting failed.

^c Plant died and evaluation was not possible to be done.

^d Non-detected.

Supplementary Table 5 '*Candidatus* Liberibacter asiaticus' (Las) Ct values in scion leaves and rootstock 24 months after graft-inoculation and in leaves of cutting-derived plantlets obtained from Oceanian citrus genotypes and 'Tobias' sweet orange control, all grafted onto Las-infected 'Rangpur' lime rootstocks, ten months after cutting propagation.

[illegible]

<i>Microcitrus</i> sp. hybrid	1	nd	29.0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
	2	nd	28.9	nd	nd	nd	nd	nd	nd	nd			
	3	nd	31.7	nd	nd	nd	nd	nd					
	4	nd	30.1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
<i>Eremocitrus</i> <i>glauca</i> × <i>Citrus</i> sp. hybrid	1	nd		F									
	2	nd	29.0										
	3	nd	27.2	F									
	4	nd	30.6	F									
<i>E. glauca</i> × <i>Microcitrus</i> sp.	1	nd	29.7	F									
	2	nd	33.4	nd	nd	nd							
	3	nd	33.0	nd	nd								
	4	nd	33.3	F									
	4	nd	33.7	F									

^a Plant Las-challenged by grafting from which stems were removed to attempt rooting

^b Months after inoculation by grafting

^c Number of rooted stem cutting per plant with were evaluated for Las infection

^d Non-detected

^e Rooting failed

*All plants were dead at the moment of the experiment

Supplementary Table 6 '*Candidatus* Liberibacter asiaticus' detection in new flushes from Oceanian citrus genotypes and 'Tobias' sweet orange as scions onto Las-infected 'Rangpur' lime rootstocks, one month after drastic pruning of the canopy at 5 cm above the scion-rootstock union.

Plant	Oceanian citrus genotypes	
	New Flush after pruning	
<i>C. × sinensis</i> (L.) Osbeck ‘Tobias’		
1		32.0
2		29.0
3		F ^a
4		F
5		d
6		28.6
7		24.9
8		23.0
9		23.2
<i>M. warburgiana</i> (F.M. Bailey) Tanaka		
1		F
2		d ^b
3		F
4		d
5		F

6	d
7	F
8	F
9	d
<i>M. papuana</i> hybrid	
1	d
2	nd ^c
3	nd
4	nd
5	d
6	d
7	nd
8	d
<i>M. australis</i> hybrid	
1	d
2	nd
3	nd
4	nd
5	nd
6	nd
7	nd
8	d
9	nd
10	nd
<i>E. glauca</i> (Lindl.) Swingle	
1	nd
2	nd
3	nd
4	nd
5	nd
6	nd
7	nd
<i>Microcitrus</i> sp. hybrid	
1	nd
2	nd
3	nd
4	nd
5	nd
6	nd
7	nd
<i>E. glauca</i> x <i>Citrus</i> sp. hybrid	
1	nd

2	nd
3	nd
4	nd
5	nd
6	nd
7	nd
8	d
9	nd
10	nd
11	nd
12	nd
<i>E. glauca</i> x <i>Microcitrus</i> sp. hybrid	
1	F
2	F
3	F
4	F
5	F
6	F
7	nd
8	F

^a Failed: no new flush obtained after pruning

^b Plant died and evaluation was not possible to be done

^c Non-detected.

CHAPTER 3 – Early population dynamics of ‘*Candidatus Liberibacter asiaticus*’ in susceptible and resistant genotypes after inoculation with infected *Diaphorina citri* feeding on young shoots^A

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Mônica Neli Alves^{1,2}, Juan Camilo Cifuentes-Arenas², Laudecir Lemos Raiol-Junior³, Jesus Aparecido Ferro¹ and Leandro Peña^{2,4*}

¹Faculdade de Ciências Agrárias e Veterinárias (FCAV), Universidade Estadual Paulista (UNESP), Jaboticabal, Brazil,

²Fundo de Defesa da Citricultura (Fundecitrus), Araraquara, Brazil,

³Empresa Brasileira de Pesquisa Agropecuária (Embrapa), Cruz das Almas, Brazil,

⁴Instituto de Biología Molecular y Celular de Plantas (IBMCP), Consejo Superior de Investigaciones Científicas (CSIC), Universidad Politécnica de Valencia (UPV), Valencia, Spain

ABSTRACT

Huanglongbing is a highly destructive citrus disease associated with '*Candidatus Liberibacter asiaticus*' (Las), a phloem-limited and non-culturable bacterium, naturally transmitted by the psyllid *Diaphorina citri*. Although diverse approaches have been used to understand the molecular mechanisms involved in the pathogen–host interaction, such approaches have focused on already infected and/or symptomatic plants, missing early events in the initial days post-inoculation. This study aimed to identify the time course of Las multiplication and whole-plant colonization immediately following inoculation by infected psyllids feeding for 2 days. Thus, the experimental approach was to track Las titers after psyllid inoculation in new shoots (NS) of *Citrus × sinensis* (susceptible), *Murraya paniculata* (partially resistant), and *Berberis koenigii* (fully resistant). Soon after psyllid removal, Las titers dropped until the 10–12th days in all three species. Following this, Las titers increased exponentially only in *C. × sinensis* and *M. paniculata*, indicating active bacterial multiplication. In *C. × sinensis*, Las reached a stationary phase at ~ 5 log Las cells/g of tissue from the 40th day onward, while in *M. paniculata*, Las increased at a lower rate of up to ~ 3 log Las cells/g of tissue between the 40th and 60th days, decreasing gradually thereafter and becoming undetectable from the 160th day onward. In *B. koenigii*, Las titers decreased from the start and remained undetectable. In *Citrus × sinensis*, an average of 2.6 log of Las cells/g of tissue was necessary for Las to move out of 50% of the NS in 23.6 days and to colonize the rest of the plant, causing a successful infection. Conversely, the probability of Las moving out of the NS remained below 50% in *M. paniculata* and zero in *B. koenigii*. To our knowledge, this is the first study on Las dynamics and whole-plant colonization during the earliest stages of infection. Identification of critical time-points for either successful multiplication or Las resistance may help to elucidate initial events of Las–host interactions that may be missed due to longer sampling intervals and at later stages of infection.

Keywords: HLB, Las multiplication, *Citrus* spp., *Murraya paniculata*, *Bergera koenigii*, bacterial growth, qPCR

1. INTRODUCTION

Huanglongbing (HLB) is the most severe citrus disease worldwide (Bové, 2006). In Asia and America, the unculturable α -proteobacteria, phloem-limited '*Candidatus Liberibacter asiaticus*' (Las) is associated with HLB (Gottwald, 2010). It is transmitted in the field by the Asian citrus psyllid *Diaphorina citri* (Kuwayama) (Sternorrhyncha: Liviidae) (Capoor et al., 1967). As there is no known cure for this disease, HLB control in Brazil is based on the use of healthy plants from certified nurseries, elimination of symptomatic plants in the groves, and inspection and rigorous control of the insect vector (Bassanezi et al., 2020). However, these strategies may be economically and environmentally unsustainable and inefficient for the management of HLB, whose incidence continues to increase, and it is currently estimated to be present in more than 20% of symptomatic trees in the citrus belt of São Paulo and Minas Gerais (Fundecitrus, 2020). In Florida, United States, the citrus industry relies on the production of more than 80% of the orchards that are Las-infected (Singerman and Useche, 2016). Leaves from affected trees show typical HLB symptoms such as asymmetrical chlorosis called blotchy mottle, enlarged veins, and intense canopy defoliation (Bové, 2006). The evolution of symptoms in Las-positive plants is rapid once they appear, and as the disease severity increases, the yield and fruit quality decrease (Gottwald, 2010). HLB-affected fruits are smaller, lighter, and highly acidic and have a reduced Brix ratio (Bassanezi et al., 2009).

Diaphorina citri acquires Las when they feed on the phloem sap from infected trees by inserting their stylets into the sieve elements. Las infects psyllids in a propagative and circulative manner (Inoue et al., 2009; Ammar et al., 2016; Canale et al., 2017). After a minimum latent period of 2–3 weeks, infected psyllids can transmit the bacterium to healthy plants by releasing it directly into the phloem when feeding (Canale et al., 2017). Electrical penetration graph (EPG) experiments have shown that 8 h is usually sufficient for the psyllid to come in contact with the phloem (Bonani et al., 2010; Wu et al., 2016; Carmo-Souza et al., 2020), but the efficiency of Las transmission is strongly dependent on the developmental stage of the plant tissue in which the insect

settles as well as the environmental conditions (Raiol-Junior et al., 2017; Lopes and Cifuentes-Arenas, 2021; Pandey et al., 2021). The nature of insect saliva effectors capable of counteracting host defenses, similar to those shown for other phloem-feeding insects (Hogenhout and Bos, 2011), is largely unknown for *D. citri*. Since Las is released into the phloem, it is notable that symptoms appear in infected plants after the bacterium has already reached high titers, in a time period ranging from 4 to 8 months after inoculation under experimental conditions (Lopes et al., 2009) and to more than a year under field conditions (Gottwald, 2010). However, little is known about citrus host–Las interactions before the onset of symptoms, especially during the initial days and weeks after psyllid inoculation. Pandey and Wang (2019) detected Las as early as 2 days after *D. citri* fed on new shoots (NS), but the subsequent population growth was not monitored. It has been reported that Las moves through the phloem pores in an elongated form and adheres to the plasma membrane in sieve plates through unknown extracellular dark materials (Achor et al., 2020). Las preferentially invades new tissues during development (Raiol-Junior et al., 2021a) and irregularly colonizes different parts of the plant (Tatineni et al., 2008; Li et al., 2009), which makes the selection of the tissue used for study and/or diagnostics very important (Tatineni et al., 2008; Johnson et al., 2014; Raiol-Junior et al., 2021a). Moreover, losses in the root system caused by Las occur before the appearance of symptoms in the canopy (Johnson et al., 2014). Therefore, the first steps of the vector–plant–pathogen interaction are challenging to study. In addition, most previous studies were conducted using only susceptible *Citrus* species.

Although susceptibility to Las varies among *Citrus* species and varieties (Folimonova et al., 2009), full resistance has already been described in species that are sexually and/or graft-compatible with *Citrus* (Alves et al., 2021) as well as in species that are phylogenetically more distant within the family Rutaceae, subfamily Aurantioideae (Ramadugu et al., 2016; Beloti et al., 2018; Cifuentes-Arenas et al., 2019). *Murraya paniculata* (L.) Jack and *Bergera koenigii* L. plants, known as orange jasmine and curry leaf, respectively, are commonly used as ornamental plants in tropical and subtropical regions of the world (Swingle and Reece, 1967). Both these species are good hosts for *D. citri* (Damsteegt et al., 2010; Teck et al., 2011; Westbrook et al., 2011), being much more attractive to psyllid females as compared to sweet

orange under laboratory and field conditions (Beloti et al., 2017; Tomaseto et al., 2019). However, low suitability and immunity to Las have been reported in *M. paniculata* and *B. koenigii*, respectively (Damsteegt et al., 2010; Beloti et al., 2018; Cifuentes-Arenas et al., 2019). The role of *D. citri* as a Las vector and the first stages of Las infection in the phloem of the two plant species remain unexplored.

We studied the time course of Las multiplication in the susceptible host *Citrus × sinensis* ‘Valencia’, in the transient host *M. paniculata*, and in the fully resistant *B. koenigii* to better understand the initial bacterial dynamics between the delivery of Las into the phloem by adults psyllids, bacterial multiplication at the first stages post-inoculation, and the time taken for Las to get out from the NS and spread through the rest of the plant. The use of a well-controlled challenge-inoculation system using infective psyllids allowed us to replicate experiments with consistent results, leading to the establishment of the earliest events of Las infection and whole-plant colonization. Understanding Las progression and defense responses triggered by resistant species may help to develop bacterial control strategies.

2. MATERIALS AND METHODS

2.1 Plant Material and Growth Conditions

One-year-old plantlets of *Citrus × sinensis* (L.) Osbeck ‘Valencia’ sweet orange grafted on ‘Rangpur’ lime (*Citrus × limonia* Osbeck) rootstock and 1-year-old seedlings of *M. paniculata* (orange jasmine) and *B. koenigii* (curry leaf) were used. All plants were grown in 300-ml conical tubes (6.5 × 5.9 cm, upper × lower diameter; 16 cm, height), filled with coconut fiber, and irrigated twice a week. Prior to the experimental use, the *C. × sinensis* plantlets were 20 to 30 cm above the substrate level, and *M. paniculata* and *B. koenigii* were 20–40 cm above the substrate level. Rearing of Las-infected *Diaphorina citri* and studies on the time course of Las multiplication were carried out inside a controlled environmental room (CER) using shelves where the light source was 20 to 30 cm above the plants. The daily temperature varied from 24 °C to 27 °C, relative humidity ranged between 55% and 78%, and photoperiod was set to 12 h of light at 300 mmol m⁻² s⁻¹ of photosynthetically active radiation and 12 h of darkness. At the end of the longest sampling time (see below) for up to 12 months,

plants were transferred from CER to a greenhouse in which the mean daily air temperature varied between 18.5 °C and 34.4 °C, and illumination was natural.

2.2 Rearing of Las-Infective Psyllids and Inoculation Procedure

A methodology adapted from Lopes and Cifuentes-Arenas (2021) was used for insect rearing and inoculation purposes. Las-positive, fully symptomatic, and 2-year-old plants of *C. × sinensis* grafted on ‘Swingle’ citrumelo (*Citrus × paradise* Macfad. × *Poncirus trifoliata* L. Raf.) growing in 4.7-l pots were used for rearing Las-infective *D. citri* insects. The Ct value of Las in these source plants was 20.7 ± 0.33 (equivalent to ca. 6.2 ± 0.1 log of Las cells/g of tissue). Las infection in *C. × sinensis* plants was confirmed by qPCR (Li et al., 2006). Plants were lightly pruned to stimulate the growth of NS, and when they reached the V2 stage of development (Cifuentes-Arenas et al., 2018), four Las-negative 10- to 20-day-old psyllids were confined per shoot for oviposition for 7 days. The Las-negative mixed sex psyllids were obtained from a colony kept at Fundecitrus, reared on healthy *M. paniculata*, adapting the methodology proposed by Skelley and Hoy (2004). The adult psyllids were then removed from the plants, and the plants remained inside the CER to allow for the eggs to hatch and nymphs to develop into first-generation adults (F1). Once F1 adults began to emerge, a random sample of three insects per source plant was taken at 5 days post-emergence to confirm Las presence by qPCR, and the rest were maintained in the same plants up to 10–15 days before being used (Figure 1).

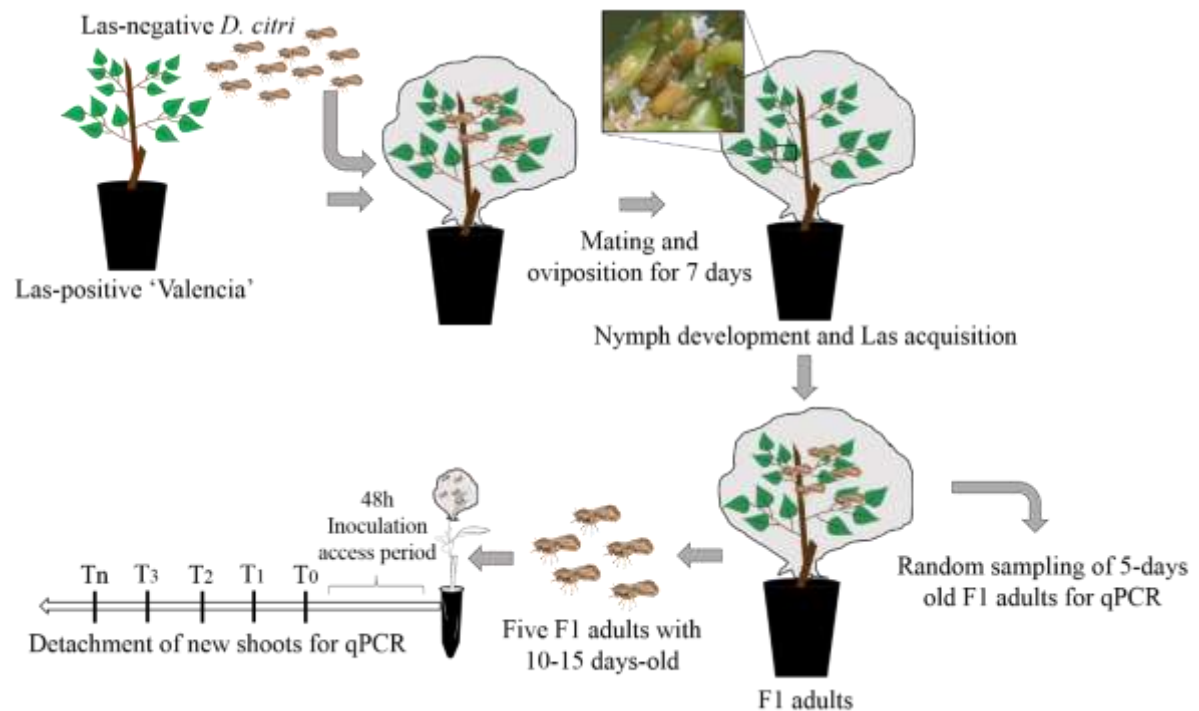


Figure 1. Experimental procedures for '*Candidatus Liberibacter asiaticus*' transmission by *Diaphorina citri*.

The *C. × sinensis* plantlets and the *M. paniculata* and *B. koenigii* seedlings were pruned at ~15 cm above the substrate level to force NS growth until they reached stage V3 of development 18 to 23 days post-pruning (Cifuentes-Arenas et al., 2018). In *M. paniculata* and *B. koenigii*, V3 shoots were characterized by the presence of one partially unfurled and one or more tender expanding leaflets (Figure 2).

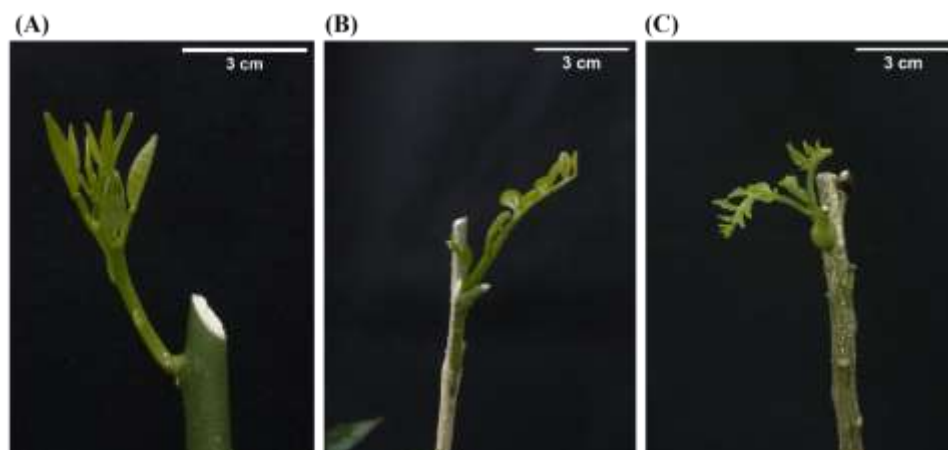


Figure 2. Aspect of new shoots in (A) *Citrus × sinensis*, (B) *Murraya paniculata*, and (C) *Bergera koenigii*. Such shoot stages were used for '*Candidatus Liberibacter asiaticus*' challenge *Diaphorina citri*-mediated inoculation experiments.

Five 10–15-day-old Las-exposed adults were confined to a single V3 NS per plant for 48 h of inoculation access period (IAP). The adults were then removed and stored at -20 °C. Subsequently, random-stratified samplings were performed, where one of the five adult insects from each of three randomly selected plants per NS detaching time (see below) for qPCR analysis was selected. The plants were then sprayed with insecticide (Abamectin EC 7.2 g of active ingredient per 1 l of water) to eliminate any psyllid eggs or nymphs present. This experiment was repeated twice with 70, 100, and 41 individuals of *C. × sinensis*, *M. paniculata*, and *B. koenigii*, respectively, in the first replication, and 95, 106, and 70 individuals in the second replication. As a control group, 8–10 plants of each species in both experimental replicates were exposed to Las-negative adults of similar age and conditions as described above.

2.3 Time Course of Las Multiplication and Whole Plant Colonization

To determine the population dynamics of Las multiplication after psyllid feeding, 5–10 plants per genotype were randomly selected at each time point, and the NS on which the Las-positive adults fed was detached from time 0 (day of psyllid removal) and at variable times post-IAP. Each plant was used at a single time point. For *C. × sinensis*, sampling was performed at 0, 5, 10, 15, 25, 40, 55, and 70 days and 0, 5, 10, 15, 18, 20, 30, 40, 65, and 95 days post-IAP in the first and second experiment replicates, respectively. For *M. paniculata*, sampling was performed at 0, 5, 10, 15, 25, 40, 55, 70, 95, 110, and 180 days and 0, 5, 10, 15, 20, 25, 30, 40, 65, 95, and 170 days post-IAP in the first and second experiment replicates, respectively. For *B. koenigii*, sampling was performed at 0, 5, 10, 15, 30, 60, and 95 days and 0, 5, 10, 15, 20, 34, and 55 days post-IAP in the first and second experiment replicates, respectively. NS was analyzed to verify the presence of Las by qPCR. In the first sampling intervals, the whole NS on which the Las-positive adults fed was used for processing, but as they grew, only a proportion of the tissue was necessary; it was ensured that tissue pieces were randomly sampled from all parts (tip to base) of the NS on which the Las-positive adults fed. At the end of the longest sampling time (180 days), the plants were transferred from the CER to a greenhouse to complete up to 12 months post-IAP. Then, these plants were sampled again, selecting four to five leaves

randomly distributed in the whole canopy. The entire leaves were processed to detect Las presence using qPCR; thus, the successful establishment of the infection was verified.

2.4 DNA Extraction and qPCR

Tissue samples (0.3 g) were processed as described by Teixeira et al. (2005) using a TissueLyser II system (Qiagen, Valencia, CA, United States) at 45 Hz for 30 s in a 2.0-ml microtube containing 5-mm steel beads. For psyllid DNA extraction, one insect and a 3-mm steel bead were used in a 1.5-ml microtube. Total DNA was extracted using the cetyltrimethylammonium bromide (CTAB) extraction buffer as mentioned in Murray and Thompson (1980), with adaptations. Briefly, for *C. × sinensis*, *M. paniculata*, and *D. citri* DNA extraction, 1.5 ml of CTAB buffer was used (2% CTAB; 1.4 M NaCl; 2% PVP 10000; 0.5 M EDTA pH 8; 1 M Tris–HCl pH 8; 0.2% b-mercaptoethanol), incubated at 65 °C for 30 min, and then centrifuged for 10 min at 12000 × g. The supernatant was transferred to a new 1.5-ml microtube, followed by extraction with chloroform/isoamyl alcohol (24:1). For *B. koenigii*, to avoid oxidation of phenolic compounds after sampling, 0.3 g of the tissue was immediately cooled with liquid nitrogen and stored at -80 °C until DNA extraction. The tissue trituration was done with 350 ml of a modified CTAB buffer (2% CTAB; 1.4 M NaCl; 2% PVP 10000; 0.5 M EDTA pH 8; 1 M Tris–HCl pH 8; 5% b-mercaptoethanol) and then 1.15 ml of this buffer was added, incubated at 60 °C for 20 min, and centrifuged for 10 min at 12000 × g. The supernatant was transferred to a new 1.5-ml microtube, followed by two extractions with phenol/chloroform/isoamyl alcohol (25:24:1) and one with chloroform/isoamyl alcohol (24:1). In both extractions, DNA precipitation was performed with 0.6 V isopropanol, washed twice with 70% ethanol, and resuspended in 50 µl of Milli-QR water. DNA quality was checked using a NanoDrop (Thermo Fisher Scientific, Massachusetts, EUA) (Desjardins and Conklin, 2010).

For real-time polymerase chain reactions (qPCR) and the identification of the 16S rRNA of Las, 1.0 µl of total DNA (100 ng/µl), TaqManR PCR Master Mix (1x) (Invitrogen, Carlsbad, CA, United States), and HLBas primer/probe (0.5 mM/0.2 mM) were used in a StepOnePlus thermocycler (Applied Biosystems, California, USA) as described by Li et al. (2006). To estimate Las multiplication in time-course experiments,

qPCR analysis was performed in triplicate, and for Las colonization and Las detection in the whole plant, qPCR analyses were performed only once. The mitochondrial gene cytochrome oxidase (COX) and an insect wingless (*Wg*) gene region were used in all samples as internal controls for the quality of plants and psyllid DNA, respectively (Li et al., 2006; Manjunath et al., 2008). In addition, negative and positive citrus leaf and psyllid samples were included during DNA extraction and qPCR assays. Las was quantified based on the linear relationship between Ct and the 16S rRNA log, according to Lopes et al. (2013). As Ct values higher than 34.0 (below 0.9 log Las cells/g of tissue) produce variable results (Lopes et al., 2013), samples were considered Las-positive when the qPCR cycle threshold (Ct) was lower than 34.0, suspected to be positive when Ct values were between 34.0 and 36.0, and negative when Ct > 36 and/or when undetermined.

2.5 Data Analysis

Data on adult survival at the time of psyllid removal were analyzed using a chi-square test by adjusting a binomial generalized linear model (GLM). To determine if there were significant differences in Las titers between the groups of psyllids used to inoculate the three plant species (in each repetition of the experiment and between time points within each species), a two-way ANOVA with interactions was first performed considering the species and the repetition of the experiment as a factor with three and two levels, respectively. Subsequently, the possible differences between the time points within each species were analyzed by performing a one-way ANOVA, considering each shoot detachment time point as a treatment. These analyses were performed by adjusting a Gaussian GLM. Similarly, to compare the initial bacterial load in NS between species at the time of psyllid removal, a two-way ANOVA (plant species × experiment repetition) was carried out. When significant differences were found, the means were separated using Tukey's post hoc test.

To analyze the time course of Las population multiplication in NS after psyllid removal, data on Las titer were submitted to nonlinear regressions to compare several growth models. The best model was selected based on the overall model significance and R². For this, data consisted of log values estimated over the Ct values (including those above 34) according to Lopes et al. (2013). An arbitrary value of 39.9999 was

assigned to the qPCR results that gave “undetermined” outputs. Data on new shoot growth were subjected to nonlinear regression to adjust the Gompertz growth model (Tjørve and Tjørve, 2017). To analyze the probability of successful infection after 48 h of psyllid IAP and subsequent detachment of NS at different time intervals, data on Las-positive leaf samples ($Ct < 34.0$) taken 1 year after the start of the experiments were subjected to a logistic regression by adjusting a binomial GLM (Peng et al., 2002).

3. RESULTS

Adult survival after 48 h of IAP was not significantly different between plant species [$\chi^2_{(2,2403)} = 466.81$; $p > 0.05$]. On an average, psyllid survival after the confinement period was 99.1%, 99.6%, and 96.6% in the first experiment and 95.8, 97.4, and 99.4% in the second experiment replicates on *C. × sinensis* ‘Valencia’, *M. paniculata*, and *B. koenigii*, respectively (Supplementary Table 1). Las titers in psyllids did not differ between plant species on which they were confined [$F_{(2,158)} = 1.4863$; $p > 0.05$] or between replicates [$F_{(1,158)} = 0.1535$; $p > 0.05$], and there was no significant interaction [$F_{(2,158)} = 2.6445$; $p > 0.05$] between these two factors (Supplementary Table 2). At different time points after NS detachment, there was no difference in Las titers in psyllids used in *C. × sinensis* [$F_{(12,53)} = 0.9967$; $p > 0.05$], *M. paniculata* [$F_{(14,64)} = 1.7563$; $p > 0.05$], and *B. koenigii* [$F_{(10,38)} = 1.7061$; $p > 0.05$]. The averages of Las titers varied from 5.33 to 5.98 log (min. = 1.94; max. = 6.94; Figure 3). The amount of initial bacterial load injected by the adult psyllids after 48 h of IAP was not influenced by the plant species [$F_{(2,50)} = 0.003$; $p > 0.05$] or by the repetition of the experiment [$F_{(1,50)} = 1.542$; $p > 0.05$]. The interaction between the two factors was not significant [$F_{(2,50)} = 3.220$; $p > 0.05$]. Details on Ct and log values of each plant and time of evaluation are shown in Supplementary Tables 3, 4.

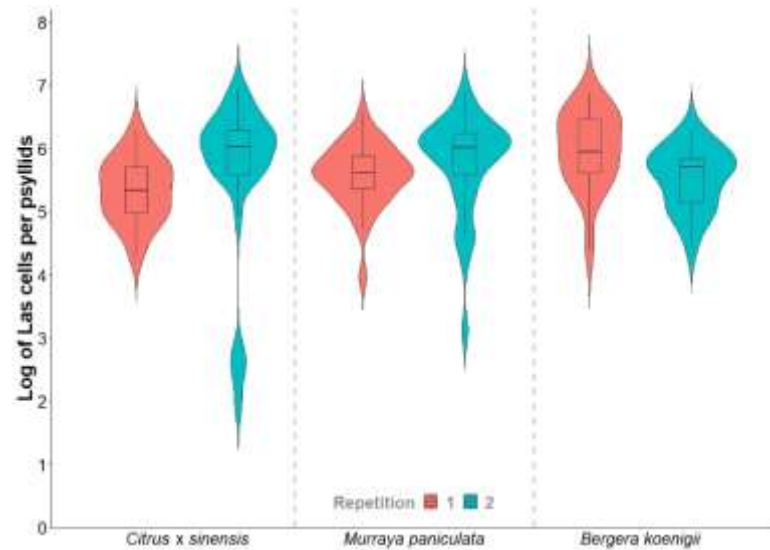


Figure 3. Violin plots of the '*Candidatus Liberibacter asiaticus*' titer in a subsample of 15- to 20-day-old *Diaphorina citri* adults reared on Las-positive *Citrus × sinensis* plants and confined during 48 h of inoculation access period (IAP) in new shoots of healthy *Citrus × sinensis*, *M. paniculata*, and *B. koenigii*.

The data on Las titers in *C. × sinensis* and *M. paniculata* adjusted well to the Lorentzian growth model (Table 1 and Supplementary Figure 1). This model describes two contrasting situations for both species in both replicates (Figure 4). At time 0 (the day of psyllid removal after 48 h of IAP), 10/10 and 7/10 *C. × sinensis* plants had Ct values lower than 34.0, in the first and second replicates, respectively. The average Las titer in NS at time 0 was 2.91 ± 0.16 log cell/g of tissue. After this, there was an initial decrease in Las titers soon after psyllid removal until the 10–12th days, up to ~ 2.0 log cell/g of tissue. Subsequently, Las multiplied exponentially, reaching $\sim 90\%$ of its final population after ~ 40 days (Figure 4). A stationary phase was then observed, and the bacterial titer remained stable (~ 4.5 – 5 log cell/g of tissue) until the last evaluation (70 and 95 days post-IAP in experiment replicates 1 and 2, respectively) with 80%–100% Las-positive plants.

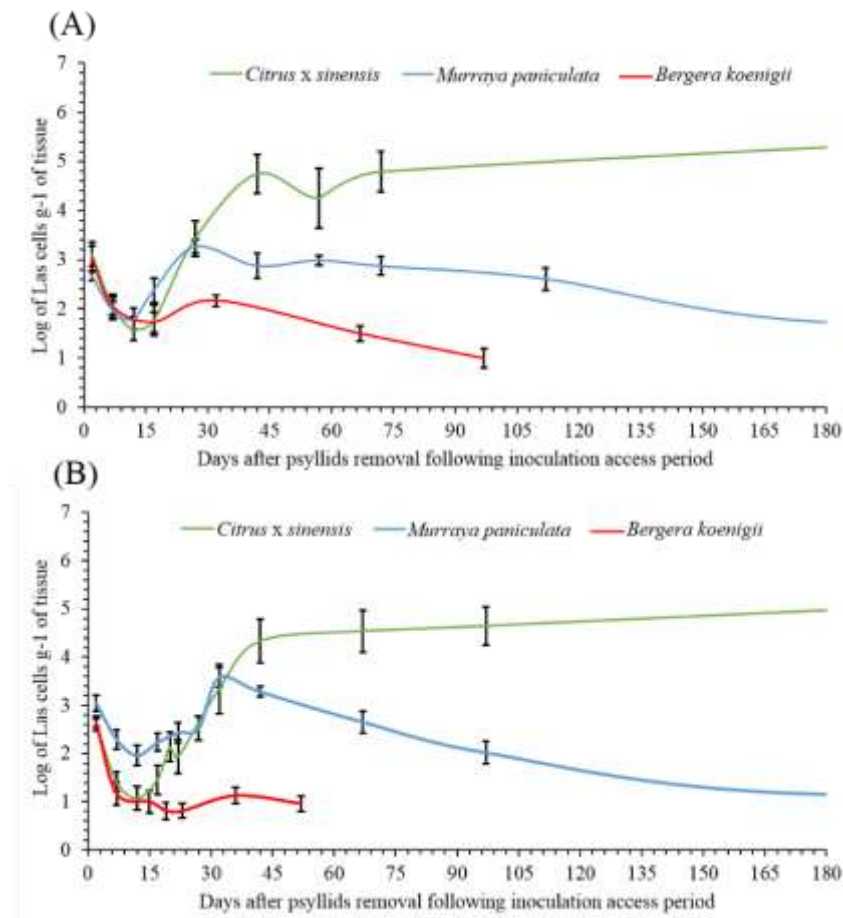


Figure 4. Evolution of the population dynamics of ‘*Candidatus Liberibacter asiaticus*’ over time in new shoots of *Citrus × sinensis*, *Murraya paniculata*, and *Bergera koenigii*, following a 48-h period of Las-exposed *Diaphorina citri* confinement in experiment replicates 1 (A) and 2 (B).

Table 1. Nonlinear regression models describing the time course of ‘*Candidatus Liberibacter asiaticus*’ population dynamics in new shoots after 48 h of Las-exposed *Diaphorina citri* confinement at 25°C ± 2°C.

	Model parameters				Model statistics		
	$\beta_0 \pm \text{SE}$	$\beta_1 \pm \text{SE}$	$\beta_2 \pm \text{SE}$	$\beta_3 \pm \text{SE}$	$F(df)$	$P(>F)$	R^2
Replicate 1							
<i>C. × sinensis</i> ^a	5.356 ± 0.333	13.343 ± 1.730	29.682 ± 7.507	-176.41 ± 47.940	24.85 _(3, 9)	0.002	0.8994
<i>M. paniculata</i> ^a	1.389 ± 0.418	64.819 ± 12.341	130.6 ± 57.449	339.349 ± 201.587	4.92 _(3, 11)	0.0381	0.5403
<i>B. koenigii</i> ^b	3.424 ± 0.445	-0.211 ± 0.051	--	--	105.73 _(2, 6)	<0.001	0.7627
Replicate 2							
<i>C. × sinensis</i> ^a	5.172 ± 0.220	12.798 ± 1.044	32.917 ± 5.326	-206.907 ± 34.916	69.42 _(3, 11)	<0.001	0.9535
<i>M. paniculata</i> ^a	1.118 ± 0.388	45.641 ± 6.797	84.272 ± 32.31	277.056 ± 125.897	6.66 _(3, 12)	0.0126	0.6207
<i>B. koenigii</i> ^b	3.285 ± 0.488	-0.405 ± 0.073	--	--	76.80 _(2, 7)	<0.001	0.8018

^a The final model selected was $Las = \beta_0 + \frac{2\beta_3}{\pi} \frac{\beta_2}{4(x-\beta_1)^2 + (\beta_2)^2}$, where β_0 is the baseline offset, β_1 is the center of the peak, β_2 is the full width of the peak at half height, and β_3 is the total area of the curve from the base line.

^b The final model selected was $Las = \beta_0 x^{\beta_1}$, where β_0 is the coefficient and β_1 is power.

Moreover, 9/10 and 10/10 *M. paniculata* plants were Las-positive at time 0 in the first and second experiment replicates, with an average titer of 2.90 ± 0.10 log cell/g of tissue. The initial decrease in Las titer up to the 10–12th days was less severe than that in *C. × sinensis*, with an average titer of ~ 2.5 log cell/g of tissue. The subsequent exponential increase was also less pronounced, and Las reached a maximum titer between the 40th and 60th days, with a population similar to that of the day of psyllid removal (~ 3 log cell/g of tissue). After reaching this maximum, Las titer decreased slowly until it reached undetectable levels from the ~ 180 th and 170th days onward in the first and second experiment replicates, respectively (Figure 4).

In *B. koenigii*, the best model describing Las population progress was the negative exponential model (Table 1 and Supplementary Figure 1). At time 0, 6/8 and 6/10 *B. koenigii* plants showed Ct values lower than 34.0, in the first and second replicates, respectively. The average of the Las titer in NS at time 0 was 2.93 ± 0.15 log cells/g of tissue, but after this, a fast reduction in Las titer following psyllid removal was observed, reaching undetectable levels from the 30th and 10th days onward in the first and second replicates, respectively (Figure 4).

For the three species, the Gompertz model described sufficiently well the growth of NS (Table 2), with very active growth from 15 to approximately 45 days after pruning. The maximum rate of NS growth was found in *C. × sinensis* at days 25–30 in both experimental replicates, followed by *B. koenigii* at days 33–35 also in both experimental replicates. *M. paniculata* presented the lowest growth rate, reaching the maximum rate at days 20 and 23 in replicates 1 and 2, respectively (Figure 5 and Supplementary Table 5).

TABLE 2 | Parameters of the Gompertz growth model of new shoots of *Citrus × sinensis*, *Murraya paniculata*, and *Bergera koenigii*.

	Model parameters			Model statistics		
	$\beta_0 \pm \text{SE}$	$\beta_1 \pm \text{SE}$	$\beta_2 \pm \text{SE}$	$F_{(df)}$	$P(>F)$	R^2
Replicate 1						
<i>C. × sinensis</i> ^a	$15,586 \pm 0,872$	$25,027 \pm 0,99$	$0,255 \pm 0,082$	$157.18_{(3, 77)}$	<0.001	0.5711
<i>M. paniculata</i> ^a	$6,52 \pm 0,214$	$20,53 \pm 2,068$	$0,123 \pm 0,037$	$483.95_{(3, 107)}$	<0.001	0.6226
<i>B. koenigii</i> ^b	$17,103 \pm 0,814$	$33,345 \pm 1,268$	$0,153 \pm 0,034$	$221.61_{(3, 41)}$	<0.001	0.8922
Replicate 2						
<i>C. × sinensis</i> ^a	$10,579 \pm 0,447$	$24,697 \pm 0,922$	$0,249 \pm 0,074$	$271.25_{(3, 102)}$	<0.001	0.5606

<i>M. paniculata</i> ^a	4,139 ± 0,221	23,431 ± 2,57	0,062 ± 0,015	316.01 _(3, 113)	<0.001	0.5615
<i>B. koenigii</i> ^b	22,132 ± 1,599	32,672 ± 1,434	0,11 ± 0,024	179.62 _(2, 77)	<0.001	0.6964

^a The final model selected was $\text{Shoot size} = \beta_0 * e^{(-e^{(-\beta_2 * (\text{time} - \beta_1)})}$, where β_0 is maximum shoot size, β_1 is the center of the curve, and β_2 is the growth rate.

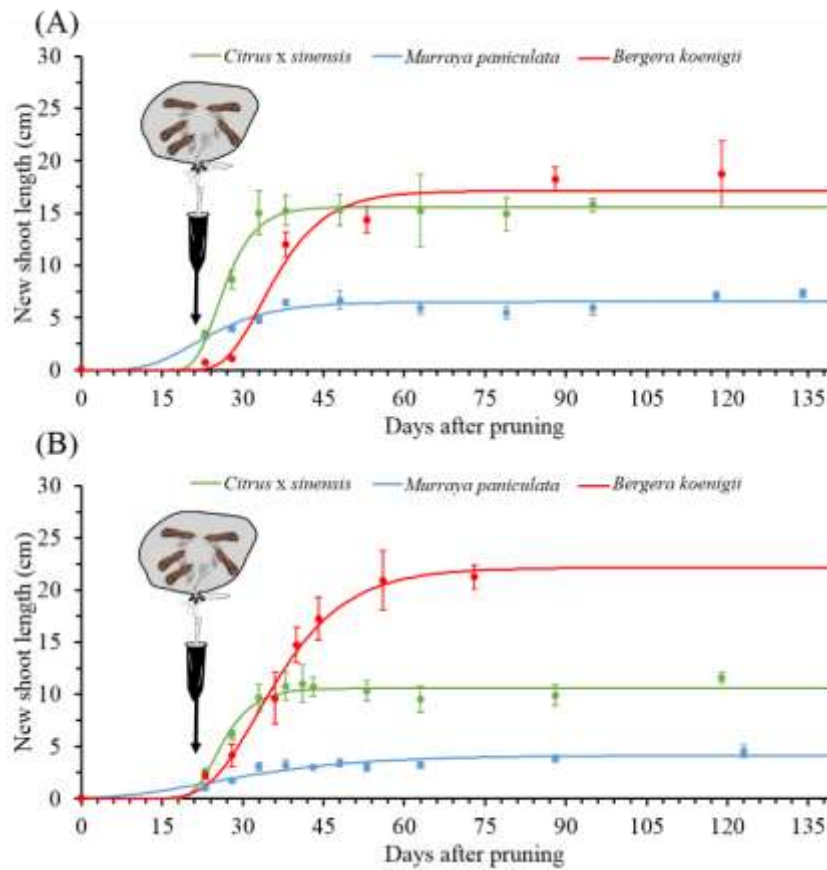


Figure 5. The Gompertz models for new shoot growth of *Citrus × sinensis*, *M. paniculata*, and *B. koenigii* in an acclimatized room at 25°C (black arrows indicate the day of psyllid confinement for a 48-h inoculation access period (IAP), (see section “Materials and Methods” for details), in experiment replicates 1 (A) and 2 (B).

In *C. × sinensis*, the time after 48 h of IAP in which NS was detached significantly affected the probability of a successful infection in the whole plant, evaluated by qPCR 1 year after the initial insect confinement (Table 3), indicating that Las was restricted to the NS during the first weeks post-inoculation. In *C. × sinensis*, the mean time for the NS to begin to serve as source of Las for the rest of the plant and thus to establish a successful infection was 21.8 days (~15 to 30 days) in the first experiment replicate and 25.3 (~18 to 33 days) in the second replicate. For *M. paniculata*, the results indicated that Las was almost unable to achieve whole-plant stable infections in the host (Figure 6), as only 18 out of 100 and 10 out of 106

seedlings in the first and second experiment replicates, respectively, were infected out of the NS at 12 months after psyllid inoculation, and such Las-positive plants were distributed randomly along the time-points evaluated in both replicates (Supplementary Table 4). In *B. koenigii*, Las could not establish successful infections regardless of the NS detachment time.

Table 3. Logistic models for the probability of a successful infection after 48 h of Las-positive inoculation by *Diaphorina citri* adults onto new shoots.

Specie	Model parameters ^a		Model statistics		
	$\beta_0 \pm \text{SE}$	$\beta_1 \pm \text{SE}$	$X^2_{(\text{df})}$	$P(>X^2)$	pseudo-R ²
Replicate 1					
<i>C. × sinensis</i>	-1.917 ± 0.494	0.088 ± 0.022	67.25 _(1, 68)	5.40x10 ⁻⁸	0.3054
<i>M. paniculata</i>	-1.920 ± 0.391	0.008 ± 0.005	92.01 _(1, 98)	0.1322	0.0240
Replicate 2					
<i>C. × sinensis</i>	-2.305 ± 0.503	0.091 ± 0.021	88.06 _(1, 88)	4.21x10 ⁻⁹	0.2816
<i>M. paniculata</i>	-2.110 ± 0.438	0.004 ± 0.008	65.97 _(1, 104)	0.6058	0.0040

^a SE: standard error of the parameter; bold values are significant ($p < 0.05$)

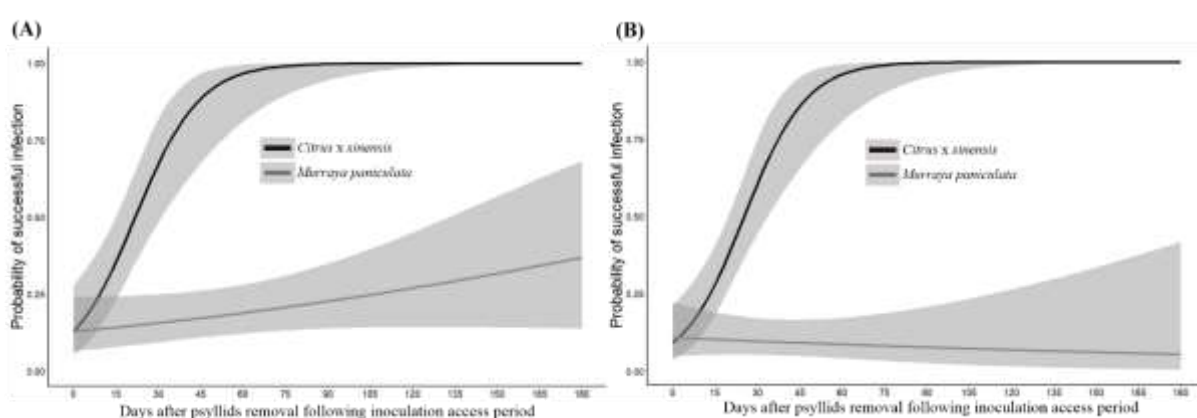


Figure 6. Probability of occurrence of a successful infection of the whole plant of *Citrus × sinensis* and *M. paniculata* after an initial 48 h of Las-exposed psyllid confinement and subsequent removal of new shoots at different time intervals in experiment replicates 1 (A) and 2 (B).

4. DISCUSSION

In this study, we investigated the earliest steps of *D. citri*–Las–host interactions, which resulted in the establishment of either bacterial infection in the susceptible *C. × sinensis* ‘Valencia’ host or in resistance to Las in *M. paniculata* and *B. koenigii*. These two latter species were considered among the most suitable *D. citri* hosts among the

Rutaceae, because volatile and visual cues made them more attractive than *Citrus* (Beloti et al., 2017; Tomaseto et al., 2019). The number of adult psyllids settled on the seedlings, number of eggs laid, and nymphs developed were also higher in *M. paniculata* and *B. koenigii* than in *Citrus* in host choice assays (Teck et al., 2011). However, we did not find differences among these three host plants in the ability of infected psyllids to inoculate Las into them, because Las showed similar titers at time 0 in NS of the three plant species, after the infected insects fed on them for 48 h. Although it has been reported that Las infection negatively affects *D. citri* performance (Ghanim et al., 2016) and induces important transcriptome reprogramming in the insect midgut (Yu et al., 2020), most infected *D. citri* individuals used in our experiments remained alive after 48 h of IAP and were efficient in transmitting Las to the three hosts. Morphological, developmental, and chemical differences among the three Rutaceae species (Beloti et al., 2018; Cifuentes-Arenas et al., 2019; Tomaseto et al., 2019) did not influence the ability of psyllids to overcome physical barriers and access sieve elements through their stylets to release the bacterium at relatively high titers. However, it is possible that Las acquisition by the psyllids may differ between these three host plants, whether due to the above differences or for other reasons including different Las titers in these hosts.

Once released into the phloem, bacterial titers decreased during the first 10–12 days after inoculation, reaching very low concentrations in the three species investigated. Such bacterial titer decline may be attributed, at least in part, to a dilution effect due to active NS growth a few days after pruning at the V2–V3 developmental stages in *C. × sinensis*, according to Cifuentes-Arenas et al. (2018). However, there was a clear lag between Las titer fall and NS enlargement, which occurred from 15 to 45 days after pruning. Moreover, Las decline was similar in the three species and quite comparable in the two replicates, while NS development followed slightly differing patterns in the two replicates, especially for *C. × sinensis* and *B. koenigii* (Figures 4, 5). Las has a reduced genome size and lacks genes involved in type 3 and most part of type 2 and 4 secretion systems, as well as those required for life in vitro (Duan et al., 2009). Indeed, it is only able to metabolize some sugars, which makes it dependent on phloem sap as a carbon source (Fagen et al., 2014), as it occurs in many other phloem-restricted bacteria (Huang et al., 2020). It is possible that Las needs some time

to adapt to the new phloem environment to sequester metabolites from the host required to live and multiply. On the other hand, such initial bacterial titer decline does not occur when *Liberibacter crescens* strain BT-1, the only cultured wild-type strain of the *Liberibacter* genus, is grown *in vitro* under optimal nutritional and environmental conditions (Sena-Vélez et al., 2019). This suggests that the phloem does not provide the necessary metabolites to Las so fast and/or they need to be transformed for use by bacterial cells, as it occurs with sugars for spiroplasmas (André et al., 2005). Killiny (2016) proposed that the *M. paniculata* and *B. koenigii* phloem sap has poor nutrient qualities, which may be associated with the limited or lack of Las multiplication in these two species observed in later time-course phases, but not during the first 12 days after inoculation, because the bacterial titer decrease was similar in *C. × sinensis*, an excellent Las host.

Alternatively, bacterial titer decrease may be caused as a consequence of defense responses induced by plant cells to either psyllid damage through herbivore-associated molecular patterns or to Las through microbe-associated molecular patterns (Huang et al., 2020). Although it is unclear whether the latter occurs in phloem sieve elements (Jiang et al., 2019), it has been recently found that Las can attach and move into nucleated cells associated with the phloem, at least in seed coat phloem tissue (Achor et al., 2020), which would likely activate immune cell responses. Moreover, such a drastic and consistent decline in bacterial titers in different plant species at a few days post-inoculation may explain, at least in part, the relatively low efficiency of Las inoculation and the frequent failures in attempts to inoculate Las into citrus plants through psyllids under controlled conditions (Pelz-Selinski et al., 2010; Ammar et al., 2016; Canale et al., 2017). Therefore, Las should be able to overcome such a lag, probably caused by the presence of physical and chemical obstacles, to remain alive and proceed with the infection process. It is known that the Las genome encodes a functional salicylic acid hydroxylase capable of degrading salicylic acid to neutralize plant defenses (Li et al., 2017). Moreover, the Las prophage gene SC2-gp095 encodes a ROS-scavenging peroxidase, which may assist Las in evading plant defense responses (Jain et al., 2015).

After the decrease phase, the Las concentration increased exponentially until it reached a titer of approximately 5 log Las cells/g of NS tissue at 45 days, which was

maintained stationary in the susceptible *C. × sinensis* host. Other studies conducted with already Las-infected plants, inoculated by grafting, obtained similar results in terms of stationary phase titers in susceptible citrus genotypes (Hilf and Luo, 2018; Raiol-Junior et al., 2021a). However, in our study, there was a lag of approximately 15–30 days for Las to reach a plateau of 5 log Las cells/g of tissue compared to the previous studies, which may be due to differences in the initial inoculum titers (budwoods vs. psyllids), a dose–effect already reported (Raiol-Junior et al., 2017), which seems to lose relevance once Las is established (Cifuentes-Arenas et al., 2019). An average of 23.6 (16.5–31.5) days of post-IAP was required for Las to move out of 50% of the NS and colonize the rest of the plant, causing a successful infection, that is, for the initially infected shoot to serve as a source of Las for the rest of the *C. × sinensis* plant. This occurred several months before Las-secreted proteins may be suitable for interaction with host cell enzymes to induce disease symptoms (Clark et al., 2018; Pang et al., 2020), including conspicuous callose deposition on sieve plates and consequent leaf mottling in infected *C. × sinensis* plants (Kim et al., 2009; Achor et al., 2010). Other phloem responses arising just after Las phloem translocation from infected tissues, but much earlier than the expression of symptoms, remain poorly understood.

In *M. paniculata*, Las population dynamics were similar to those of *C. × sinensis* up to the 30th day. Las reached a semi-stationary phase for about 15–30 days, and then bacterial titers declined progressively to become undetectable at 180 days onward. Moreover, Las translocation from the NS to the rest of the plant was rare and uneven. In previous controlled experiments with *M. paniculata*, the earliest samplings were taken from the second to fourth months post-IAP (Damsteegt et al., 2010; Cifuentes-Arenas et al., 2019). However, the continuous decrease in Las titers to undetectable levels confirms previous consideration of *M. paniculata* as a transient host for Las (Damsteegt et al., 2010; Cifuentes-Arenas et al., 2019). Even at low titers, the transitory presence of Las in *M. paniculata* (Damsteegt et al., 2010; Walter et al., 2012; Cifuentes-Arenas et al., 2019) suggests that this species may be a suitable host for Las spread. However, Las transmission from *M. paniculata* to *C. × sinensis* by *D. citri* was observed at very low frequencies (less than 1%) in controlled experiments and only when environmental and plant developmental conditions were highly

favorable for Las infection (Cifuentes-Arenas et al., 2019). Indeed, both the transient nature of Las infection and its low titers when the bacterium remains detectable in this host explain the 43% reduction in HLB incidence in a sweet orange orchard when *M. paniculata* was used as a trap crop fence to efficiently attract and control the *D. citri* population and its spread (Tomaseto et al., 2019).

In contrast, Las could not be established in NS of *B. koenigii*, as shown previously by Beloti et al. (2018). However, we were able to detect Las multiplication in *B. koenigii* NS in one of the experiment replicates, indicating that under specific, highly suitable conditions, the bacterium could attain low but clearly detectable concentrations in the inoculated shoots, although only occasionally. Moreover, the probability of Las moving out of the NS was null in the *B. koenigii*. Whether *M. paniculata* and *B. koenigii* phloem sap are unsuitable for Las survival (Killiny, 2016) and/or their cells activate efficient defense responses to hold bacterial infections remains to be further investigated. Nevertheless, *B. koenigii* is more efficient than *M. paniculata* in limiting Las. Considering the different responses of Las infection between *Murraya paniculata* and *Bergera koenigii*, their close phylogenetic relationships, and the wide diversity of *Murraya* and *Bergera* species found in Asia (Nguyen et al., 2019), the interactions of the two genera with Las is worth investigating, as they seem to represent hosts at the frontier between resistance and susceptibility to the bacterium.

This is the first study to investigate the early population dynamics of Las in three species with contrasting susceptibility/resistance responses to this bacterium, by simulating the natural Las inoculation system using infective *D. citri* and evaluating the presence of Las in NS by qPCR at different short time intervals post-IAP. Using this controlled and reproducible system, we were able to expose all the plants to similar initial inoculum pressure, as they received the same initial bacterial load injected into the phloem by psyllids and the environmental conditions were adequate to maximize the chances of successful infection occurrence (Raiol-Junior et al., 2021b). Our results allowed us to identify the key time points of Las growth in plant genotypes with different responses to Las infection. This information could be essential to investigating whether or when plant cell defense responses are activated after wounding and bacterial release into the phloem by *D. citri*, as well as how Las is able to evade such responses

in *C. × sinensis* but not in *B. koenigii* and only partially in *M. paniculata*. It may also help to identify suitable targets to be knocked out or overexpressed biotechnologically with the aim of creating HLB-resistant varieties.

5. DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

6. AUTHOR CONTRIBUTIONS

MA, LP, and JF conceptualized the work. MA, LP, JF, and J-CA designed the work. MA, J-CA, and LR-J collected the data. J-CA performed the statistical analysis. MA, J-CA, LR-J, JF, and LP contributed to interpretation, drafting the article, and critical revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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10. SUPPLEMENTARY MATERIAL

Supplementary Table 1. *Diaphorina citri* adult survival after 48 h inoculation access period (IAP) on *Citrus × sinensis*, *Murraya paniculata* and *Bergera koenigii* in an acclimatized room at 25 °C, in experiment replicates 1 and 2. In the column “Psyllid survival”, “1” means alive and “0” dead.

Experiment replicate 1				Experiment replicate 2			
Days after psyllids confinement	Plant number	Psyllid number	Psyllid survival	Days after psyllids confinement	Plant number	Psyllid number	Psyllid survival
<i>Citrus × sinensis</i>				<i>Citrus × sinensis</i>			
0	117	1	1	0	122	1	1
0	117	2	1	0	122	2	1
0	117	3	1	0	122	3	1
0	117	4	1	0	122	4	1
0	117	5	1	0	122	5	1
0	124	1	1	0	127	1	1
0	124	2	1	0	127	2	1
0	124	3	1	0	127	3	1
0	124	4	1	0	127	4	1
0	124	5	1	0	127	5	1
0	130	1	1	0	136	1	1
0	130	2	1	0	136	2	1
0	130	3	1	0	136	3	1
0	130	4	1	0	136	4	1
0	130	5	1	0	136	5	1
0	134	1	1	0	139	1	1
0	134	2	1	0	139	2	1
0	134	3	1	0	139	3	1
0	134	4	1	0	139	4	1
0	134	5	1	0	139	5	1
0	139	1	1	0	147	1	1
0	139	2	1	0	147	2	1
0	139	3	1	0	147	3	1
0	139	4	1	0	147	4	1
0	139	5	1	0	147	5	1
0	145	1	1	0	150	1	1
0	145	2	1	0	150	2	1
0	145	3	1	0	150	3	1
0	145	4	1	0	150	4	1
0	145	5	1	0	150	5	1
0	152	1	1	0	171	1	1
0	152	2	1	0	171	2	1
0	152	3	1	0	171	3	1

0	152	4	1	0	171	4	1
0	152	5	1	0	171	5	1
0	156	1	1	0	183	1	1
0	156	2	1	0	183	2	1
0	156	3	1	0	183	3	1
0	156	4	1	0	183	4	1
0	156	5	0	0	183	5	1
0	162	1	1	0	187	1	1
0	162	2	1	0	187	2	1
0	162	3	1	0	187	3	1
0	162	4	1	0	187	4	1
0	162	5	1	0	187	5	1
0	168	1	1	0	196	1	1
0	168	2	1	0	196	2	1
0	168	3	1	0	196	3	1
0	168	4	1	0	196	4	1
0	168	5	1	0	196	5	1
5	101	1	1	5	117	1	1
5	101	2	1	5	117	2	1
5	101	3	1	5	117	3	1
5	101	4	1	5	117	4	1
5	101	5	1	5	117	5	1
5	107	1	1	5	131	1	1
5	107	2	1	5	131	2	1
5	107	3	1	5	131	3	1
5	107	4	1	5	131	4	1
5	107	5	1	5	131	5	1
5	112	1	1	5	138	1	1
5	112	2	1	5	138	2	1
5	112	3	1	5	138	3	1
5	112	4	1	5	138	4	1
5	112	5	1	5	138	5	1
5	122	1	1	5	141	1	1
5	122	2	1	5	141	2	1
5	122	3	1	5	141	3	1
5	122	4	1	5	141	4	1
5	122	5	1	5	141	5	1
5	127	1	1	5	145	1	1
5	127	2	1	5	145	2	1
5	127	3	1	5	145	3	1
5	127	4	1	5	145	4	1
5	127	5	1	5	145	5	1
5	138	1	1	5	153	1	1
5	138	2	1	5	153	2	1

5	138	3	1	5	153	3	1
5	138	4	1	5	153	4	1
5	138	5	1	5	153	5	1
5	150	1	1	5	156	1	1
5	150	2	1	5	156	2	1
5	150	3	1	5	156	3	1
5	150	4	1	5	156	4	1
5	150	5	1	5	156	5	1
5	159	1	1	5	166	1	1
5	159	2	1	5	166	2	1
5	159	3	1	5	166	3	1
5	159	4	1	5	166	4	1
5	159	5	1	5	166	5	0
5	164	1	1	5	199	1	1
5	164	2	1	5	199	2	1
5	164	3	1	5	199	3	1
5	164	4	1	5	199	4	1
5	164	5	1	5	199	5	0
5	169	1	1	5	209	1	1
5	169	2	1	5	209	2	1
5	169	3	1	5	209	3	1
5	169	4	1	5	209	4	1
5	169	5	1	5	209	5	0
10	102	1	1	10	120	1	1
10	102	2	1	10	120	2	1
10	102	3	1	10	120	3	1
10	102	4	1	10	120	4	1
10	102	5	1	10	120	5	1
10	110	1	1	10	125	1	1
10	110	2	1	10	125	2	1
10	110	3	1	10	125	3	1
10	110	4	1	10	125	4	1
10	110	5	1	10	125	5	0
10	116	1	1	10	133	1	1
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				5	65	5	1
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				5	69	2	1
				5	69	3	1
				5	69	4	1
				5	69	5	1
				5	70	1	1
				5	70	2	1
				5	70	3	1
				5	70	4	1
				5	70	5	1
				5	82	1	1

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13	45	4	1
13	45	5	1
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13	47	3	1
13	47	4	1
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17	35	3	1
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17	59	5	1
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17	74	3	1
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17	81	4	1
17	81	5	1
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17	92	2	1
17	92	3	1
17	92	4	1
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21	15	2	1
21	15	3	1
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21	15	5	1
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21	27	3	1
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21	31	2	1
21	31	3	1
21	31	4	1
21	31	5	1
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21	39	3	1
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34	16	3	1
34	16	4	1
34	16	5	1
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34	26	3	1

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34	37	4	1
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34	54	2	1
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34	54	4	1
34	54	5	1
34	63	1	1
34	63	2	1
34	63	3	1
34	63	4	1
34	63	5	0
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34	68	2	1
34	68	3	1
34	68	4	1
34	68	5	1
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34	76	2	1
34	76	3	1
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34	99	5	1
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50	4	3	1
50	4	4	1
50	4	5	1
50	11	1	1
50	11	2	1

50	11	3	1
50	11	4	1
50	11	5	1
50	20	1	1
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50	20	3	1
50	20	4	1
50	20	5	1
50	30	1	1
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50	75	4	1
50	75	5	1
50	90	1	1
50	90	2	1
50	90	3	1
50	90	4	1
50	90	5	1
50	96	1	1
50	96	2	1
50	96	3	1
50	96	4	1
50	96	5	1

Supplementary Table 2. Data on Ct and log values of '*Candidatus Liberibacter asiaticus*' from the random stratified sampling that consisted of one of the 5 adult insects from each of three randomly selected plants per new shoots detaching time. Insects were reared on Las-positive plants and confined on the new shoots of *Citrus × sinensis*, *Murraya paniculata* and *Bergera koenigii* during 48 h of inoculation access period (IAP). Ct and log values from experiment replicates 1 and 2 were determined through detection of the 16S rDNA by qPCR.

Experiment replicate 1				Experiment replicate 2			
Days after psyllid confinement	Plant number	Ct ^a	Log ^b	Days after psyllid confinement	Plant number	Ct ^a	Log ^b
<i>Citrus × sinensis</i>				<i>Citrus × sinensis</i>			
0	117	21,5	5,13	0	122	18,0	6,15
0	139	22,2	4,93	0	171	20,5	5,44
0	168	21,9	5,02	0	196	19,9	5,59
5	101	19,8	5,64	5	117	18,3	6,06
5	138	22,8	4,77	5	156	16,1	6,70
5	169	17,6	6,29	5	209	27,0	3,56
10	102	19,9	5,60	10	120	15,3	6,94
10	116	18,9	5,90	10	160	20,0	5,59
10	165	19,4	5,76	10	205	20,2	5,52
15	105	20,7	5,37	15	186	22,3	4,91
15	121	22,6	4,82	15	201	19,9	5,59
15	163	24,5	4,28	15	206	26,7	3,64
25	108	21,0	5,30	18	137	17,2	6,40
25	133	22,5	4,86	18	151	19,7	5,68
25	167	23,8	4,49	18	207	17,8	6,21
40	103	19,2	5,81	20	140	17,8	6,23
40	125	21,2	5,23	20	175	18,4	6,05
40	166	20,4	5,47	20	194	18,0	6,16
55	114	19,2	5,81	30	118	15,6	6,86
55	158	22,0	5,01	30	159	18,6	5,99
55	161	21,4	5,17	30	211	18,9	5,89
70	104	20,0	5,58	40	121	17,5	6,30
70	111	18,2	6,11	40	157	16,6	6,57
70	170	19,6	5,69	40	208	18,1	6,14
<i>Murraya paniculata</i>				65	123	17,8	6,23
0	3	19,1	5,85	65	167	19,4	5,77
0	53	18,7	5,96	65	210	17,5	6,30
0	98	18,0	6,15	95	116	16,7	6,55

5	6	20,3	5,49	95	162	19,0	5,87
5	65	17,7	6,25	95	188	19,6	5,70
5	96	21,7	5,09	<i>Muraya paniculata</i>			
10	5	18,7	5,96	0	2	17,6	6,27
10	23	19,4	5,76	0	47	17,4	6,33
10	49	16,6	6,56	0	79	19,0	5,86
15	37	20,6	5,40	5	5	17,1	6,43
15	69	18,0	6,16	5	52	18,9	5,90
15	86	20,1	5,56	5	98	23,2	4,65
25	2	21,4	5,16	10	9	17,9	6,20
25	61	20,8	5,36	10	59	17,3	6,37
25	97	18,9	5,89	10	100	19,5	5,73
40	4	18,5	6,00	15	21	18,1	6,13
40	60	20,0	5,58	15	95	17,8	6,22
40	93	22,9	4,73	15	114	16,2	6,67
55	1	20,1	5,55	20	3	20,0	5,58
55	8	21,4	5,16	20	15	23,4	4,58
55	87	19,2	5,81	20	84	24,7	4,22
70	27	19,4	5,75	25	24	16,3	6,64
70	39	20,0	5,59	25	54	18,5	6,03
70	100	21,5	5,15	25	115	18,7	5,97
95	10	22,5	4,85	30	6	17,7	6,23
95	15	19,8	5,62	30	48	17,4	6,33
95	82	19,9	5,61	30	72	19,4	5,77
110	7	19,8	5,64	40	4	15,4	6,92
110	24	21,5	5,13	40	55	18,1	6,12
110	99	17,8	6,21	40	87	21,6	5,11
180	22	20,7	5,39	65	10	18,5	6,01
180	90	19,6	5,69	65	85	21,0	5,27
180	92	21,1	5,26	65	113	23,2	4,64
<i>Bergera koenigii</i>				95	1	21,0	5,30
0	215	17,7	6,25	95	57	18,3	6,08
0	245	21,2	5,23	95	110	18,5	6,02
0	251	20,5	5,42	170	27	19,1	5,85
5	212	18,9	5,91	170	91	18,7	5,97
5	238	15,6	6,86	170	108	20,0	5,58
5	242	18,8	5,94	<i>Bergera koenigii</i>			
15	214	16,6	6,57	0	42	18,1	6,12
15	234	18,8	5,92	0	86	24,0	4,42
15	248	18,8	5,94	0	98	22,6	4,83
30	213	16,3	6,66	5	22	17,5	6,31
30	235	16,9	6,47	5	43	20,1	5,54

30	249	19,7	5,67	5	51	19,5	5,73
60	216	17,2	6,39	13	44	22,0	5,00
60	241	20,3	5,50	13	55	19,5	5,74
60	246	24,0	4,41	13	89	21,5	5,15
95	202	17,0	6,46	17	7	19,5	5,71
95	237	20,0	5,58	17	29	18,5	6,03
95	250	16,7	6,54	17	74	20,8	5,35
				21	1	21,7	5,10
				21	27	19,5	5,74
				21	72	21,1	5,25
				34	26	19,5	5,73
				34	54	22,5	4,86
				34	76	19,8	5,64
				50	11	18,5	6,01
				50	30	19,1	5,83
				50	75	18,7	5,97

^aCt: Cycle threshold.

^bLog: Las titer in log10 of amplicon copies per gram of insect tissue estimated based on a standard curve as described by Lopes et al., 2013.

Supplementary Table 3. Data on Ct and log values of '*Candidatus Liberibacter asiaticus*' over time in each new shoots of *Citrus x sinensis*, *Murraya paniculata* and *Bergera koenigii* following a 48 h period of *Diaphorina citri* confinement in experiment replicates 1 and 2 as determined through detection of the 16S rDNA by qPCR.

Experiment replicate 1				Experiment replicate 2			
Days after psyllid confinement	Plant number	Ct ^a	Log ^b	Days after psyllid confinement	Plant number	Ct ^a	Log ^b
<i>Citrus x sinensis</i>				<i>Citrus x sinensis</i>			
0	117	32,1	2,75	0	122	30,1	3,37
0	117	30,3	3,31	0	122	30,1	3,35
0	117	31,5	2,93	0	122	30,1	3,35
0	124	33,1	2,47	0	127	29,4	3,58
0	124	31,0	3,09	0	127	29,6	3,52
0	124	32,9	2,54	0	127	29,4	3,57
0	130	32,3	2,70	0	136	34,5	2,04
0	130	28,9	3,72	0	136	34,4	2,09
0	130	30,8	3,15	0	136	34,4	2,08
0	134	33,3	2,39	0	139	33,0	2,49
0	134	30,6	3,22	0	139	33,2	2,43

0	134	31,9	2,82	0	139	34,3	2,10
0	139	33,3	2,40	0	147	31,9	2,83
0	139	31,4	2,98	0	147	32,8	2,54
0	139	32,3	2,71	0	147	31,9	2,84
0	145	35,0	1,88	0	150	33,9	2,23
0	145	32,6	2,61	0	150	33,0	2,50
0	145	33,8	2,27	0	150	33,4	2,38
0	152	33,0	2,49	0	171	33,9	2,21
0	152	30,8	3,16	0	171	33,1	2,45
0	152	32,1	2,75	0	171	33,7	2,28
0	156	25,0	4,90	0	183	35,0	1,88
0	156	23,1	5,47	0	183	34,6	2,02
0	156	23,8	5,25	0	183	33,9	2,24
0	162	30,5	3,24	0	187	33,4	2,38
0	162	28,6	3,81	0	187	nd	nd
0	162	29,6	3,52	0	187	35,8	1,65
0	168	32,9	2,53	0	196	32,1	2,76
0	168	31,3	3,00	0	196	32,4	2,68
0	168	31,9	2,84	0	196	31,9	2,83
5	101	nd ^c	nd	5	117	34,3	2,10
5	101	34,9	1,94	5	117	nd	nd
5	101	nd	nd	5	117	36,0	1,58
5	107	nd	nd	5	131	nd	nd
5	107	35,0	1,90	5	131	nd	nd
5	107	34,1	2,18	5	131	nd	nd
5	112	nd	nd	5	138	nd	0,00
5	112	34,1	2,16	5	138	nd	nd
5	112	34,3	2,12	5	138	nd	nd
5	122	34,7	1,99	5	141	32,1	2,77
5	122	32,0	2,81	5	141	32,4	2,69
5	122	32,6	2,62	5	141	33,2	2,44
5	127	35,8	1,66	5	145	34,2	2,13
5	127	32,8	2,55	5	145	34,8	1,97
5	127	33,2	2,45	5	145	36,0	1,60
5	138	nd	nd	5	153	34,6	2,01
5	138	34,6	2,02	5	153	35,2	1,85
5	138	34,0	2,20	5	153	nd	nd
5	150	nd	nd	5	156	nd	nd
5	150	nd	nd	5	156	35,1	1,85
5	150	nd	nd	5	156	34,5	2,04
5	159	35,5	1,74	5	166	nd	nd
5	159	33,2	2,44	5	166	nd	nd
5	159	32,8	2,55	5	166	nd	nd
5	164	35,8	1,65	5	199	nd	nd

5	164	35,2	1,82	5	199	nd	nd
5	164	nd	nd	5	199	nd	nd
5	169	34,2	2,13	5	209	nd	nd
5	169	31,5	2,94	5	209	nd	nd
5	169	33,0	2,49	5	209	nd	nd
10	102	nd	nd	10	120	nd	nd
10	102	nd	nd	10	120	nd	nd
10	102	nd	nd	10	120	nd	nd
10	110	nd	nd	10	125	35,7	1,70
10	110	35,2	1,85	10	125	35,2	1,85
10	110	nd	nd	10	125	nd	nd
10	116	nd	nd	10	133	nd	nd
10	116	nd	nd	10	133	nd	nd
10	116	nd	nd	10	133	nd	nd
10	126	35,4	1,78	10	134	36,0	1,59
10	126	33,0	2,51	10	134	35,7	1,68
10	126	33,4	2,38	10	134	nd	nd
10	129	nd	nd	10	143	nd	nd
10	129	35,1	1,87	10	143	nd	nd
10	129	nd	nd	10	143	nd	nd
10	136	nd	nd	10	160	nd	nd
10	136	32,6	2,60	10	160	nd	nd
10	136	33,3	2,40	10	160	nd	nd
10	140	34,9	1,91	10	168	nd	nd
10	140	33,0	2,50	10	168	nd	nd
10	140	33,7	2,30	10	168	nd	nd
10	144	nd	nd	10	172	nd	nd
10	144	nd	nd	10	172	nd	nd
10	144	nd	nd	10	172	nd	nd
10	148	35,8	1,65	10	193	33,3	2,39
10	148	36,0	1,61	10	193	32,6	2,61
10	148	nd	nd	10	193	33,2	2,43
10	165	nd	nd	10	205	nd	nd
10	165	nd	nd	10	205	nd	nd
10	165	nd	nd	10	205	nd	nd
15	105	nd	nd	15	126	nd	nd
15	105	35,5	1,76	15	126	32,4	2,69
15	105	34,9	1,93	15	126	31,9	2,81
15	115	nd	nd	15	128	nd	nd
15	115	35,4	1,78	15	128	nd	nd
15	115	34,9	1,94	15	128	nd	nd
15	120	nd	nd	15	129	32,0	2,79
15	120	nd	nd	15	129	30,3	3,30
15	120	nd	nd	15	129	30,6	3,23

15	121	nd	nd	15	174	nd	nd
15	121	nd	nd	15	174	35,3	1,82
15	121	nd	nd	15	174	nd	nd
15	128	nd	nd	15	176	nd	nd
15	128	nd	nd	15	176	nd	nd
15	128	nd	nd	15	176	nd	nd
15	137	34,1	2,16	15	180	nd	nd
15	137	32,0	2,80	15	180	35,6	1,71
15	137	33,2	2,45	15	180	nd	nd
15	142	29,0	3,68	15	186	nd	nd
15	142	27,0	4,29	15	186	nd	nd
15	142	27,5	4,13	15	186	nd	nd
15	147	nd	nd	15	190	nd	nd
15	147	34,2	2,13	15	190	34,2	2,14
15	147	nd	nd	15	190	nd	nd
15	153	nd	nd	15	201	34,6	2,01
15	153	nd	nd	15	201	34,8	1,97
15	153	nd	nd	15	201	35,4	1,78
15	163	nd	nd	15	206	nd	nd
15	163	35,2	1,84	15	206	nd	nd
15	163	35,9	1,62	15	206	nd	nd
25	108	25,8	4,65	18	137	nd	nd
25	108	24,0	5,20	18	137	33,9	2,23
25	108	24,9	4,91	18	137	35,3	1,80
25	119	28,6	3,81	18	151	35,5	1,75
25	119	26,6	4,42	18	151	34,4	2,09
25	119	27,7	4,09	18	151	34,3	2,11
25	123	30,8	3,16	18	161	32,2	2,72
25	123	31,2	3,02	18	161	30,3	3,31
25	123	32,2	2,72	18	161	30,9	3,12
25	132	29,6	3,53	18	173	nd	nd
25	132	27,7	4,08	18	173	nd	nd
25	132	28,0	4,01	18	173	nd	nd
25	133	28,1	3,98	18	179	30,3	3,29
25	133	26,6	4,41	18	179	28,1	3,96
25	133	26,7	4,39	18	179	28,9	3,74
25	141	nd	nd	18	184	nd	nd
25	141	36,0	1,60	18	184	nd	nd
25	141	nd	nd	18	184	nd	nd
25	143	30,8	3,16	18	195	nd	nd
25	143	28,9	3,72	18	195	nd	nd
25	143	29,1	3,65	18	195	nd	nd
25	154	29,3	3,60	18	197	31,2	3,04
25	154	27,9	4,02	18	197	33,1	2,45

25	154	27,8	4,06	18	197	33,9	2,21
25	157	30,1	3,38	18	203	nd	nd
25	157	28,6	3,83	18	203	nd	nd
25	157	28,4	3,87	18	203	nd	nd
25	167	nd	nd	18	207	32,9	2,53
25	167	35,3	1,80	18	207	30,8	3,15
25	167	35,4	1,78	18	207	31,5	2,94
40	103	23,9	5,22	20	119	33,1	2,46
40	103	23,0	5,48	20	119	31,8	2,85
40	103	23,1	5,47	20	119	32,0	2,80
40	113	nd	nd	20	132	nd	nd
40	113	nd	nd	20	132	nd	nd
40	113	nd	nd	20	132	nd	nd
40	118	23,3	5,41	20	140	30,8	3,16
40	118	22,2	5,75	20	140	29,1	3,67
40	118	22,1	5,75	20	140	29,3	3,60
40	125	23,0	5,50	20	148	31,0	3,09
40	125	24,5	5,04	20	148	29,6	3,51
40	125	24,6	5,01	20	148	29,7	3,49
40	131	24,8	4,95	20	155	nd	nd
40	131	23,8	5,27	20	155	nd	nd
40	131	23,8	5,24	20	155	35,2	1,84
40	135	26,6	4,41	20	164	nd	nd
40	135	25,5	4,74	20	164	nd	nd
40	135	25,5	4,75	20	164	nd	nd
40	146	24,0	5,19	20	175	nd	nd
40	146	24,2	5,13	20	175	nd	nd
40	146	24,1	5,16	20	175	nd	nd
40	151	23,3	5,41	20	181	nd	nd
40	151	23,3	5,40	20	181	nd	nd
40	151	23,4	5,37	20	181	nd	nd
40	160	26,5	4,46	20	189	31,6	2,91
40	160	25,5	4,74	20	189	30,7	3,18
40	160	25,5	4,73	20	189	30,8	3,17
40	166	25,3	4,80	20	194	nd	nd
40	166	24,5	5,05	20	194	nd	nd
40	166	24,4	5,08	20	194	nd	nd
55	114	34,8	1,95	30	118	26,9	4,32
55	114	34,6	2,03	30	118	25,9	4,63
55	114	35,0	1,88	30	118	26,0	4,61
55	149	25,3	4,80	30	130	25,6	4,72
55	149	25,5	4,73	30	130	25,9	4,62
55	149	25,5	4,74	30	130	28,9	3,72
55	155	23,5	5,34	30	144	25,6	4,73

55	155	22,7	5,58	30	144	25,1	4,87
55	155	22,7	5,59	30	144	25,1	4,87
55	158	24,5	5,04	30	152	27,7	4,07
55	158	25,0	4,89	30	152	24,8	4,97
55	158	26,0	4,59	30	152	24,8	4,96
55	161	23,4	5,37	30	159	28,4	3,88
55	161	29,0	3,69	30	159	27,5	4,14
55	161	29,5	3,54	30	159	27,7	4,07
70	104	24,0	5,20	30	169	nd	nd
70	104	23,0	5,49	30	169	nd	nd
70	104	22,0	5,79	30	169	nd	nd
70	106	24,8	4,94	30	185	28,6	3,81
70	106	26,0	4,59	30	185	27,8	4,05
70	106	26,2	4,53	30	185	28,1	3,98
70	109	23,6	5,30	30	192	nd	nd
70	109	22,8	5,55	30	192	28,3	3,90
70	109	22,6	5,60	30	192	28,6	3,82
70	111	25,4	4,78	30	204	nd	nd
70	111	24,1	5,15	30	204	nd	nd
70	111	24,4	5,08	30	204	nd	nd
70	170	29,7	3,49	30	211	34,8	1,95
70	170	31,0	3,09	30	211	nd	nd
70	170	30,5	3,25	30	211	nd	nd
<i>Muraya paniculata</i>				40	121	23,7	5,28
0	3	34,5	2,06	40	121	25,4	4,77
0	3	34,5	2,06	40	121	25,4	4,78
0	3	35,1	1,86	40	135	20,8	6,15
0	20	32,9	2,52	40	135	22,8	5,54
0	20	32,0	2,79	40	135	23,1	5,46
0	20	32,0	2,78	40	142	23,3	5,39
0	26	34,6	2,01	40	142	25,3	4,80
0	26	32,0	2,79	40	142	25,6	4,72
0	26	33,5	2,36	40	149	22,8	5,55
0	40	33,9	2,22	40	149	24,4	5,07
0	40	31,8	2,87	40	149	24,6	5,01
0	40	32,5	2,64	40	157	25,1	4,86
0	53	33,4	2,37	40	157	26,5	4,46
0	53	30,9	3,12	40	157	26,5	4,46
0	53	31,9	2,83	40	165	26,8	4,35
0	62	33,4	2,37	40	165	28,9	3,72
0	62	31,1	3,07	40	165	29,0	3,71
0	62	32,2	2,73	40	177	35,3	1,82
0	68	32,8	2,54	40	177	nd	nd
0	68	30,9	3,13	40	177	nd	nd

0	68	31,9	2,83	40	182	25,6	4,71
0	83	31,8	2,85	40	182	25,4	4,77
0	83	30,5	3,24	40	182	23,3	5,40
0	83	30,7	3,19	40	191	23,9	5,24
0	89	33,4	2,38	40	191	23,1	5,46
0	89	32,0	2,80	40	191	23,5	5,34
0	89	32,2	2,73	40	208	33,8	2,27
0	98	32,7	2,58	40	208	36,0	1,59
0	98	31,3	2,99	40	208	nd	nd
0	98	32,1	2,77	65	123	21,9	5,82
5	6	nd	nd	65	123	21,5	5,95
5	6	34,5	2,06	65	123	21,7	5,89
5	6	nd	nd	65	124	23,9	5,22
5	12	nd	nd	65	124	23,6	5,33
5	12	nd	nd	65	124	23,6	5,31
5	12	nd	nd	65	146	23,9	5,22
5	18	33,9	2,22	65	146	23,8	5,25
5	18	31,2	3,03	65	146	23,7	5,28
5	18	32,5	2,66	65	158	26,2	4,52
5	31	nd	nd	65	158	26,4	4,47
5	31	32,3	2,71	65	158	26,1	4,56
5	31	34,6	2,00	65	163	32,9	2,52
5	46	nd	nd	65	163	33,5	2,33
5	46	35,1	1,85	65	163	33,2	2,45
5	46	35,8	1,66	65	167	24,1	5,16
5	56	nd	nd	65	167	23,4	5,37
5	56	35,9	1,63	65	167	23,5	5,34
5	56	35,9	1,64	65	178	35,9	1,62
5	65	35,4	1,76	65	178	nd	nd
5	65	35,3	1,81	65	178	nd	nd
5	65	35,2	1,83	65	198	23,9	5,22
5	71	35,0	1,91	65	198	24,5	5,04
5	71	32,7	2,60	65	198	24,1	5,16
5	71	34,0	2,19	65	200	23,9	5,22
5	75	35,5	1,76	65	200	23,4	5,36
5	75	34,7	1,98	65	200	24,9	4,92
5	75	34,8	1,96	65	210	25,9	4,62
5	96	34,4	2,07	65	210	25,0	4,89
5	96	32,9	2,52	65	210	24,9	4,93
5	96	34,2	2,13	95	116	21,6	5,90
10	5	34,8	1,94	95	116	22,1	5,77
10	5	32,6	2,61	95	116	21,9	5,82
10	5	33,0	2,49	95	154	26,8	4,35
10	9	35,6	1,72	95	154	26,8	4,35

10	9	32,6	2,61	95	154	26,9	4,32
10	9	34,3	2,12	95	162	25,4	4,78
10	14	35,2	1,82	95	162	28,1	3,97
10	14	33,7	2,30	95	162	28,0	4,00
10	14	35,0	1,91	95	170	28,3	3,92
10	16	nd	nd	95	170	27,1	4,26
10	16	nd	nd	95	170	26,8	4,35
10	16	nd	nd	95	188	nd	nd
10	23	nd	nd	95	188	nd	nd
10	23	nd	nd	95	188	nd	0,00
10	23	nd	nd	<i>Murraya paniculata</i>			
10	29	nd	nd	0	2	30,1	3,38
10	29	nd	nd	0	2	32,3	2,70
10	29	34,6	2,03	0	2	31,8	2,85
10	33	nd	0,00	0	11	30,4	3,27
10	33	nd	nd	0	11	31,8	2,86
10	33	nd	nd	0	11	31,8	2,85
10	41	nd	nd	0	18	28,2	3,93
10	41	32,4	2,68	0	18	29,0	3,71
10	41	32,9	2,52	0	18	29,3	3,60
10	55	nd	nd	0	22	29,0	3,69
10	55	nd	nd	0	22	29,9	3,43
10	55	34,2	2,13	0	22	30,1	3,35
10	49	nd	nd	0	31	32,4	2,69
10	49	nd	nd	0	31	31,8	2,85
10	49	34,7	1,99	0	31	32,3	2,71
15	37	35,7	1,69	0	34	33,2	2,43
15	37	32,5	2,65	0	34	33,9	2,21
15	37	33,1	2,48	0	34	32,8	2,55
15	38	33,1	2,48	0	47	33,1	2,45
15	38	31,3	3,00	0	47	33,3	2,39
15	38	31,9	2,82	0	47	32,9	2,51
15	44	nd	nd	0	68	32,9	2,52
15	44	nd	nd	0	68	33,0	2,48
15	44	nd	nd	0	68	33,4	2,38
15	51	34,7	1,98	0	74	29,3	3,60
15	51	32,3	2,69	0	74	29,1	3,66
15	51	34,0	2,20	0	74	29,0	3,69
15	58	35,1	1,86	0	79	29,6	3,50
15	58	31,5	2,93	0	79	29,4	3,58
15	58	32,0	2,80	0	79	29,5	3,55
15	64	nd	nd	5	5	33,3	2,40
15	64	nd	nd	5	5	32,7	2,58
15	64	35,1	1,86	5	5	33,8	2,27

15	69	33,9	2,23	5	13	35,4	1,77
15	69	32,1	2,77	5	13	nd	nd
15	69	33,1	2,46	5	13	nd	nd
15	74	nd	0,00	5	20	28,8	3,76
15	74	nd	nd	5	20	29,3	3,60
15	74	nd	0,00	5	20	30,5	3,26
15	79	31,7	2,89	5	25	31,9	2,82
15	79	29,5	3,54	5	25	32,0	2,79
15	79	29,8	3,46	5	25	32,6	2,61
15	86	31,7	2,89	5	28	32,3	2,71
15	86	29,1	3,67	5	28	32,3	2,69
15	86	29,9	3,41	5	28	33,7	2,30
25	2	33,5	2,35	5	36	32,2	2,75
25	2	31,4	2,98	5	36	32,3	2,71
25	2	31,8	2,87	5	36	33,7	2,28
25	11	30,0	3,39	5	40	33,8	2,25
25	11	27,4	4,18	5	40	33,5	2,36
25	11	28,5	3,84	5	40	34,9	1,93
25	19	30,9	3,12	5	52	35,6	1,70
25	19	29,0	3,69	5	52	nd	nd
25	19	29,9	3,42	5	52	35,4	1,79
25	36	31,9	2,84	5	88	nd	nd
25	36	29,8	3,45	5	88	34,7	2,00
25	36	30,5	3,25	5	88	35,1	1,86
25	54	30,4	3,28	5	98	nd	nd
25	54	28,2	3,93	5	98	35,7	1,68
25	54	29,3	3,62	5	98	nd	nd
25	61	31,7	2,88	10	9	33,2	2,43
25	61	28,7	3,78	10	9	32,6	2,62
25	61	29,1	3,65	10	9	32,4	2,66
25	77	33,8	2,25	10	16	nd	nd
25	77	32,8	2,57	10	16	35,4	1,79
25	77	33,6	2,30	10	16	35,4	1,79
25	84	32,0	2,79	10	19	33,7	2,28
25	84	30,0	3,40	10	19	32,9	2,53
25	84	30,4	3,29	10	19	35,0	1,91
25	94	30,1	3,35	10	30	nd	nd
25	94	28,2	3,92	10	30	nd	nd
25	94	28,7	3,78	10	30	nd	nd
25	97	31,8	2,84	10	43	35,7	1,68
25	97	29,3	3,60	10	43	35,3	1,82
25	97	29,9	3,43	10	43	34,2	2,13
40	4	30,8	3,16	10	59	33,5	2,34
40	4	29,1	3,65	10	59	33,0	2,50

40	4	29,5	3,54	10	59	33,2	2,44
40	25	nd	nd	10	76	34,5	2,05
40	25	nd	nd	10	76	35,4	1,79
40	25	nd	nd	10	76	36,0	1,60
40	32	32,4	2,68	10	82	35,8	1,65
40	32	32,6	2,62	10	82	nd	nd
40	32	32,1	2,78	10	82	35,4	1,76
40	35	30,0	3,38	10	93	34,1	2,15
40	35	29,3	3,59	10	93	33,4	2,37
40	35	29,3	3,60	10	93	33,4	2,38
40	43	31,8	2,86	10	100	34,8	1,96
40	43	31,0	3,09	10	100	33,1	2,47
40	43	30,9	3,12	10	100	34,4	2,08
40	48	nd	nd	15	21	34,2	2,15
40	48	nd	nd	15	21	35,0	1,89
40	48	35,1	1,86	15	21	35,3	1,82
40	60	30,2	3,35	15	56	nd	nd
40	60	29,5	3,56	15	56	nd	nd
40	60	29,5	3,55	15	56	nd	nd
40	67	30,4	3,29	15	58	32,9	2,54
40	67	29,7	3,48	15	58	32,7	2,58
40	67	29,5	3,53	15	58	32,9	2,53
40	73	33,2	2,44	15	65	31,9	2,81
40	73	33,0	2,49	15	65	32,4	2,68
40	73	32,9	2,52	15	65	32,3	2,71
40	93	29,4	3,58	15	94	nd	nd
40	93	29,2	3,64	15	94	33,4	2,36
40	93	29,3	3,60	15	94	33,6	2,32
55	1	32,5	2,63	15	95	34,0	2,20
55	1	31,6	2,93	15	95	32,9	2,53
55	1	32,0	2,79	15	95	32,9	2,52
55	8	31,9	2,83	15	99	31,2	3,03
55	8	31,7	2,90	15	99	30,8	3,14
55	8	31,1	3,06	15	99	31,1	3,07
55	13	31,9	2,81	15	109	35,5	1,74
55	13	32,0	2,80	15	109	35,6	1,73
55	13	31,4	2,98	15	109	nd	nd
55	17	30,3	3,31	15	111	33,4	2,39
55	17	29,8	3,46	15	111	31,2	3,03
55	17	29,8	3,44	15	111	31,8	2,85
55	28	31,9	2,83	15	114	nd	0,00
55	28	30,4	3,27	15	114	35,1	1,85
55	28	30,6	3,22	15	114	35,7	1,69
55	42	32,8	2,57	20	3	nd	nd

55	42	31,8	2,84	20	3	32,6	2,63
55	42	31,9	2,84	20	3	33,6	2,31
55	45	30,0	3,38	20	15	32,8	2,57
55	45	29,2	3,62	20	15	31,5	2,93
55	45	29,5	3,54	20	15	31,7	2,89
55	50	33,2	2,45	20	32	31,4	2,97
55	50	32,3	2,70	20	32	30,7	3,18
55	50	32,8	2,56	20	32	31,0	3,10
55	76	34,0	2,19	20	45	34,9	1,92
55	76	32,6	2,61	20	45	34,5	2,04
55	76	31,7	2,87	20	45	33,6	2,31
55	87	31,3	3,00	20	49	35,5	1,74
55	87	30,3	3,31	20	49	32,3	2,70
55	87	30,0	3,38	20	49	32,5	2,64
70	27	30,7	3,18	20	63	30,1	3,36
70	27	29,4	3,57	20	63	29,9	3,42
70	27	29,2	3,65	20	63	30,0	3,40
70	39	31,1	3,07	20	78	33,5	2,35
70	39	29,4	3,58	20	78	32,2	2,73
70	39	29,4	3,56	20	78	32,3	2,70
70	52	31,4	2,98	20	84	34,3	2,11
70	52	30,6	3,23	20	84	33,9	2,22
70	52	30,6	3,20	20	84	34,6	2,01
70	57	32,0	2,80	20	97	nd	nd
70	57	31,2	3,03	20	97	nd	nd
70	57	31,5	2,94	20	97	36,0	1,61
70	63	30,1	3,38	20	104	nd	nd
70	63	30,3	3,30	20	104	35,7	1,68
70	63	30,3	3,31	20	104	nd	nd
70	66	31,7	2,89	25	24	32,2	2,75
70	66	30,6	3,22	25	24	30,6	3,23
70	66	30,6	3,20	25	24	30,9	3,13
70	72	33,1	2,46	25	33	nd	nd
70	72	31,9	2,81	25	33	nd	nd
70	72	32,0	2,80	25	33	nd	nd
70	85	nd	nd	25	41	29,6	3,53
70	85	nd	nd	25	41	28,3	3,91
70	85	nd	nd	25	41	28,2	3,93
70	91	34,9	1,92	25	53	32,9	2,52
70	91	33,5	2,35	25	53	35,7	1,69
70	91	32,9	2,54	25	53	nd	nd
70	100	33,3	2,41	25	54	33,5	2,34
70	100	31,5	2,94	25	54	34,6	2,00
70	100	31,4	2,97	25	54	33,7	2,28

95	10	30,8	3,15	25	73	32,6	2,62
95	10	29,5	3,55	25	73	32,7	2,60
95	10	29,3	3,61	25	73	35,2	1,84
95	15	35,3	1,82	25	101	32,2	2,74
95	15	32,9	2,52	25	101	35,4	1,76
95	15	33,0	2,51	25	101	33,9	2,22
95	21	31,5	2,95	25	105	nd	nd
95	21	30,4	3,28	25	105	nd	nd
95	21	30,6	3,21	25	105	nd	nd
95	47	nd	nd	25	112	nd	nd
95	47	33,9	2,24	25	112	31,5	2,95
95	47	33,1	2,45	25	112	31,4	2,98
95	59	nd	nd	25	115	33,5	2,33
95	59	35,4	1,77	25	115	29,7	3,48
95	59	nd	nd	25	115	29,8	3,45
95	70	32,5	2,65	30	6	29,2	3,64
95	70	31,1	3,07	30	6	27,3	4,22
95	70	30,8	3,16	30	6	27,4	4,17
95	82	36,0	1,60	30	8	26,0	4,59
95	82	34,3	2,10	30	8	26,0	4,60
95	82	33,2	2,43	30	8	26,2	4,54
110	7	33,2	2,44	30	12	29,3	3,61
110	7	34,5	2,04	30	12	30,1	3,36
110	7	33,4	2,38	30	12	30,4	3,27
110	24	32,2	2,75	30	23	29,1	3,67
110	24	30,7	3,18	30	23	27,9	4,02
110	24	30,3	3,32	30	23	28,1	3,97
110	78	32,6	2,61	30	35	30,0	3,39
110	78	31,5	2,94	30	35	27,0	4,29
110	78	31,2	3,03	30	35	27,2	4,22
110	81	31,9	2,83	30	48	33,1	2,48
110	81	30,5	3,26	30	48	nd	nd
110	81	30,4	3,28	30	48	nd	nd
110	88	nd	nd	30	60	31,6	2,93
110	88	nd	nd	30	60	33,2	2,44
110	88	35,3	1,80	30	60	32,9	2,54
110	95	nd	nd	30	64	30,7	3,18
110	95	35,1	1,88	30	64	29,9	3,44
110	95	33,6	2,32	30	64	29,9	3,42
110	99	31,4	2,96	30	69	29,9	3,42
110	99	30,4	3,28	30	69	28,2	3,95
110	99	31,6	2,92	30	69	28,3	3,90
180	22	nd	nd	30	72	26,3	4,52
180	22	nd	nd	30	72	29,9	3,42

180	22	nd	nd	30	72	29,8	3,44
180	30	34,9	1,93	40	4	30,2	3,34
180	30	35,0	1,90	40	4	30,5	3,24
180	30	36,0	1,60	40	4	31,0	3,09
180	34	nd	nd	40	26	32,2	2,73
180	34	35,2	1,84	40	26	34,5	2,04
180	34	nd	nd	40	26	34,3	2,11
180	80	33,2	2,43	40	38	27,9	4,01
180	80	32,1	2,77	40	38	29,4	3,58
180	80	33,3	2,41	40	38	29,4	3,58
180	90	32,7	2,58	40	44	31,4	2,96
180	90	32,6	2,62	40	44	31,2	3,04
180	90	33,4	2,38	40	44	31,5	2,94
180	92	nd	nd	40	55	30,9	3,13
180	92	nd	nd	40	55	31,0	3,10
180	92	35,9	1,63	40	55	31,6	2,93
<i>Bergera koenigii</i>				40	62	29,9	3,43
0	215	25,8	4,66	40	62	31,1	3,08
0	215	28,0	4,00	40	62	31,3	3,00
0	215	31,8	2,85	40	70	29,3	3,61
0	223	34,5	2,04	40	70	30,0	3,39
0	223	34,8	1,96	40	70	30,5	3,24
0	223	34,7	1,99	40	75	29,7	3,49
0	228	26,2	4,55	40	75	31,1	3,06
0	228	28,1	3,98	40	75	31,2	3,04
0	228	33,6	2,30	40	83	27,1	4,27
0	233	30,6	3,23	40	83	28,8	3,75
0	233	30,2	3,33	40	83	28,8	3,76
0	233	30,7	3,18	40	87	28,8	3,75
0	236	27,9	4,03	40	87	30,7	3,19
0	236	28,4	3,87	40	87	30,4	3,26
0	236	29,0	3,69	65	10	nd	nd
0	245	32,6	2,61	65	10	nd	nd
0	245	31,8	2,86	65	10	nd	nd
0	245	32,4	2,68	65	37	31,3	3,01
0	247	33,5	2,34	65	37	32,0	2,80
0	247	35,0	1,91	65	37	32,0	2,78
0	247	35,2	1,83	65	46	29,3	3,61
0	251	32,0	2,79	65	46	31,1	3,07
0	251	32,7	2,60	65	46	31,0	3,11
0	251	33,0	2,50	65	61	29,1	3,65
5	212	33,5	2,35	65	61	30,7	3,19
5	212	35,6	1,72	65	61	31,5	2,94
5	212	nd	nd	65	77	29,3	3,60

5	217	31,0	3,11	65	77	32,0	2,80
5	217	33,5	2,34	65	77	33,1	2,48
5	217	33,9	2,23	65	85	31,7	2,88
5	219	36,0	1,60	65	85	34,0	2,19
5	219	nd	nd	65	85	35,1	1,87
5	219	nd	nd	65	90	33,0	2,51
5	221	nd	nd	65	90	32,2	2,72
5	221	nd	nd	65	90	33,0	2,51
5	221	nd	nd	65	96	34,5	2,04
5	230	29,9	3,41	65	96	nd	nd
5	230	29,7	3,48	65	96	nd	nd
5	230	30,5	3,23	65	106	29,1	3,68
5	231	35,5	1,74	65	106	30,5	3,24
5	231	34,2	2,15	65	106	30,5	3,24
5	231	nd	nd	65	113	34,0	2,21
5	238	35,7	1,68	65	113	32,0	2,78
5	238	nd	nd	65	113	32,6	2,62
5	238	nd	nd	95	1	nd	nd
5	242	35,5	1,74	95	1	35,3	1,81
5	242	34,4	2,08	95	1	nd	nd
5	242	34,7	2,00	95	17	34,4	2,07
15	214	32,5	2,66	95	17	34,0	2,20
15	214	31,7	2,87	95	17	34,3	2,10
15	214	32,1	2,76	95	29	nd	nd
15	220	nd	nd	95	29	31,0	3,11
15	220	nd	nd	95	29	30,8	3,17
15	220	nd	nd	95	42	nd	nd
15	222	35,6	1,72	95	42	nd	nd
15	222	34,3	2,11	95	42	nd	nd
15	222	35,6	1,71	95	57	nd	nd
15	225	nd	nd	95	57	34,9	1,93
15	225	nd	nd	95	57	34,0	2,19
15	225	nd	nd	95	80	35,2	1,84
15	227	nd	nd	95	80	35,0	1,90
15	227	34,6	2,01	95	80	33,6	2,32
15	227	nd	nd	95	89	31,9	2,81
15	234	nd	nd	95	89	31,3	3,00
15	234	nd	nd	95	89	31,5	2,95
15	234	nd	nd	95	103	nd	nd
15	240	35,2	1,83	95	103	nd	nd
15	240	33,4	2,38	95	103	nd	nd
15	240	34,4	2,07	95	110	nd	nd
15	248	nd	nd	95	110	nd	nd
15	248	nd	nd	95	110	nd	nd

15	248	nd	nd	170	27	nd	nd
30	213	nd	nd	170	27	nd	nd
30	213	35,1	1,87	170	27	nd	nd
30	213	35,8	1,67	170	51	nd	nd
30	218	33,3	2,41	170	51	nd	nd
30	218	nd	nd	170	51	nd	nd
30	218	34,9	1,92	170	66	34,1	2,16
30	226	34,0	2,20	170	66	34,7	1,99
30	226	33,3	2,42	170	66	34,7	1,99
30	226	nd	nd	170	81	nd	nd
30	232	33,1	2,46	170	81	nd	nd
30	232	34,8	1,95	170	81	nd	nd
30	232	33,9	2,23	170	91	nd	nd
30	235	33,1	2,46	170	91	nd	nd
30	235	34,6	2,01	170	91	35,8	1,66
30	235	34,8	1,95	170	102	nd	nd
30	239	35,5	1,75	170	102	nd	nd
30	239	33,7	2,30	170	102	36,0	1,59
30	239	33,6	2,31	170	108	nd	nd
30	243	34,8	1,96	170	108	nd	nd
30	243	nd	nd	170	108	nd	nd
30	243	36,0	1,60	<i>Bergera koenigii</i>			
30	249	nd	nd	0	10	32,4	2,67
30	249	33,9	2,23	0	10	32,5	2,66
30	249	35,4	1,78	0	10	33,5	2,35
60	216	nd	nd	0	12	33,2	2,44
60	216	nd	nd	0	12	32,9	2,53
60	216	nd	nd	0	12	31,9	2,83
60	229	nd	nd	0	17	nd	nd
60	229	nd	nd	0	17	nd	nd
60	229	nd	nd	0	17	nd	nd
60	241	nd	nd	0	25	31,3	3,01
60	241	35,7	1,67	0	25	33,4	2,38
60	241	nd	nd	0	25	32,6	2,61
60	246	nd	nd	0	42	nd	nd
60	246	35,0	1,91	0	42	nd	nd
60	246	nd	nd	0	42	nd	nd
95	202	nd	nd	0	62	nd	nd
95	202	nd	nd	0	62	nd	nd
95	202	nd	nd	0	62	nd	nd
95	224	nd	nd	0	78	32,2	2,74
95	224	nd	nd	0	78	33,9	2,23
95	224	nd	nd	0	78	33,4	2,39
95	237	nd	nd	0	86	32,7	2,58

95	237	nd	nd	0	86	33,0	2,50
95	237	nd	nd	0	86	33,1	2,47
95	244	nd	nd	0	97	31,4	2,98
95	244	nd	nd	0	97	30,9	3,13
95	244	nd	nd	0	97	31,4	2,96
95	250	nd	nd	0	98	nd	nd
95	250	nd	nd	0	98	nd	nd
95	250	nd	nd	0	98	nd	nd
				5	22	nd	nd
				5	22	35,7	1,69
				5	22	nd	nd
				5	24	nd	nd
				5	24	nd	nd
				5	24	nd	nd
				5	43	34,6	2,03
				5	43	35,2	1,84
				5	43	35,6	1,71
				5	51	34,6	2,02
				5	51	nd	nd
				5	51	nd	nd
				5	65	33,7	2,27
				5	65	34,9	1,93
				5	65	nd	0,00
				5	69	nd	nd
				5	69	nd	nd
				5	69	nd	nd
				5	70	nd	nd
				5	70	nd	nd
				5	70	nd	nd
				5	82	nd	nd
				5	82	nd	nd
				5	82	nd	nd
				5	83	nd	nd
				5	83	nd	nd
				5	83	nd	nd
				5	88	35,1	1,87
				5	88	35,1	1,86
				5	88	nd	nd
				13	13	35,7	1,69
				13	13	35,2	1,82
				13	13	nd	nd
				13	36	nd	nd
				13	36	nd	nd
				13	36	nd	nd

13	41	nd	nd
13	41	nd	nd
13	41	nd	nd
13	44	nd	nd
13	44	nd	nd
13	44	nd	nd
13	45	nd	nd
13	45	nd	nd
13	45	nd	nd
13	47	33,1	2,47
13	47	32,6	2,61
13	47	33,0	2,49
13	52	nd	nd
13	52	nd	nd
13	52	nd	nd
13	55	nd	nd
13	55	nd	nd
13	55	nd	nd
13	87	nd	nd
13	87	nd	nd
13	87	nd	nd
13	89	nd	nd
13	89	nd	nd
13	89	nd	nd
17	3	nd	nd
17	3	nd	nd
17	3	nd	nd
17	7	nd	nd
17	7	nd	nd
17	7	nd	nd
17	19	nd	nd
17	19	nd	nd
17	19	nd	nd
17	29	34,6	2,00
17	29	nd	nd
17	29	nd	nd
17	35	nd	nd
17	35	nd	nd
17	35	nd	nd
17	59	nd	nd
17	59	nd	nd
17	59	nd	nd
17	74	nd	nd
17	74	34,9	1,94

17	74	nd	nd
17	81	nd	nd
17	81	nd	nd
17	81	nd	nd
17	92	nd	nd
17	92	nd	nd
17	92	nd	nd
17	95	nd	nd
17	95	35,9	1,63
17	95	nd	nd
21	1	nd	nd
21	1	nd	nd
21	1	nd	nd
21	5	nd	nd
21	5	35,7	1,69
21	5	36,0	1,61
21	15	nd	nd
21	15	nd	nd
21	15	nd	nd
21	27	nd	nd
21	27	nd	nd
21	27	nd	nd
21	31	nd	nd
21	31	nd	nd
21	31	nd	nd
21	39	nd	nd
21	39	nd	nd
21	39	nd	nd
21	48	nd	nd
21	48	nd	nd
21	48	nd	nd
21	57	nd	nd
21	57	nd	nd
21	57	nd	nd
21	72	nd	nd
21	72	nd	nd
21	72	nd	nd
21	80	nd	nd
21	80	nd	nd
21	80	nd	nd
34	6	nd	nd
34	6	35,2	1,82
34	6	nd	nd
34	16	nd	nd

34	16	nd	nd
34	16	nd	nd
34	26	nd	nd
34	26	nd	nd
34	26	nd	nd
34	37	nd	nd
34	37	nd	nd
34	37	nd	nd
34	54	nd	nd
34	54	nd	nd
34	54	nd	nd
34	63	nd	nd
34	63	nd	nd
34	63	nd	nd
34	68	35,1	1,87
34	68	nd	nd
34	68	nd	nd
34	76	35,0	1,90
34	76	nd	nd
34	76	nd	nd
34	85	nd	nd
34	85	nd	nd
34	85	nd	nd
34	99	nd	nd
34	99	35,9	1,64
34	99	nd	nd
50	4	nd	nd
50	4	nd	nd
50	4	nd	nd
50	11	nd	nd
50	11	nd	nd
50	11	nd	nd
50	20	nd	nd
50	20	nd	nd
50	20	nd	nd
50	30	nd	nd
50	30	nd	nd
50	30	nd	nd
50	40	nd	nd
50	40	nd	nd
50	40	nd	nd
50	58	nd	nd
50	58	nd	nd
50	58	nd	nd

50	66	nd	nd
50	66	nd	nd
50	66	nd	nd
50	75	nd	nd
50	75	36,0	1,59
50	75	nd	nd
50	90	nd	nd
50	90	nd	nd
50	90	35,1	1,86
50	96	nd	nd
50	96	nd	nd
50	96	nd	nd

^aCt: Cycle threshold.

^bLog: Las titer in log₁₀ of amplicon copies per gram of plant tissue estimated based on a standard curve as described by Lopes et al., 2013.

^cnd: non-detected.

Supplementary Table 4. Data on Ct and log values of '*Candidatus Liberibacter asiaticus*' in *Citrus x sinensis*, *Murraya paniculata* and *Bergera koenigii* after one year of the 48 h *Diaphorina citri* confinement in experiment replicates 1 and 2, as determined through detection of the 16S rDNA by qPCR.

Experiment replicate 1				Experiment replicate 2			
Days after psyllid confinement	Plant number	Ct ^a	Log ^b	Days after psyllid confinement	Plant number	Ct ^a	Log ^b
<i>Citrus x sinensis</i>				<i>Citrus x sinensis</i>			
0	117	nd ^c	nd	0	122	nd	nd
0	124	nd	nd	0	127	nd	nd
0	130	35,7	1,70	0	136	nd	nd
0	134	nd	nd	0	139	nd	nd
0	139	nd	nd	0	147	34,7	1,98
0	145	34,3	2,12	0	150	nd	nd
0	152	34,9	1,91	0	171	nd	nd
0	156	35,0	1,91	0	183	nd	nd
0	162	35,2	1,83	0	187	nd	nd
0	168	35,5	1,74	0	196	nd	nd
5	101	nd	nd	5	117	nd	nd
5	107	nd	nd	5	131	nd	nd
5	112	32,3	2,69	5	138	nd	nd
5	122	33,6	2,33	5	141	35,4	1,76
5	127	35,0	1,91	5	145	nd	nd
5	138	35,1	1,87	5	153	35,7	1,68
5	150	33,6	2,31	5	156	nd	nd

5	159	34,0	2,19	5	166	nd	nd
5	164	nd	nd	5	199	34,8	1,96
5	169	35,0	1,90	5	209	35,2	1,82
10	102	nd	nd	10	120	nd	nd
10	110	nd	nd	10	125	35,5	1,75
10	116	35,4	1,77	10	133	nd	nd
10	126	nd	nd	10	134	nd	nd
10	129	nd	nd	10	143	nd	nd
10	136	nd	nd	10	160	30,1	3,36
10	140	34,4	2,08	10	168	nd	nd
10	144	33,9	2,23	10	172	31,7	2,88
10	148	35,5	1,74	10	193	nd	nd
10	165	35,4	1,76	10	205	nd	nd
15	105	36,0	1,60	15	126	nd	nd
15	115	nd	nd	15	128	35,7	1,70
15	120	nd	nd	15	129	nd	nd
15	121	nd	nd	15	174	36,0	1,60
15	128	35,8	1,65	15	176	nd	nd
15	137	35,6	1,72	15	180	34,1	2,15
15	142	33,2	2,43	15	186	nd	nd
15	147	31,9	2,83	15	190	36,0	1,60
15	153	34,0	2,19	15	201	nd	nd
15	163	33,5	2,34	15	206	nd	nd
25	108	21,8	5,84	18	137	20,1	6,37
25	119	19,6	6,52	18	151	27,9	4,04
25	123	20,9	6,12	18	161	23,4	5,37
25	132	22,6	5,60	18	173	33,3	2,39
25	133	25,8	4,64	18	179	19,8	6,45
25	141	20,8	6,15	18	184	35,6	1,73
25	143	20,0	6,40	18	195	nd	nd
25	154	21,1	6,05	18	197	nd	nd
25	157	22,0	5,78	18	203	nd	nd
25	167	nd	nd	18	207	20,2	6,35
40	103	19,1	6,66	20	119	nd	nd
40	113	35,7	1,69	20	132	26,6	4,41
40	118	20,1	6,35	20	140	20,2	6,34
40	125	21,3	5,99	20	148	20,2	6,33
40	131	21,3	5,99	20	155	33,9	2,23
40	135	20,5	6,24	20	164	nd	nd
40	146	26,1	4,56	20	175	35,5	1,74
40	151	19,9	6,41	20	181	34,9	1,93
40	160	19,7	6,49	20	189	20,1	6,35
40	166	19,6	6,52	20	194	20,1	6,35
55	114	nd	nd	30	118	19,9	6,41

55	149	20,3	6,31	30	130	nd	nd
55	155	20,0	6,39	30	144	20,2	6,33
55	158	34,9	1,93	30	152	21,3	6,02
55	161	23,7	5,28	30	159	20,4	6,27
70	104	23,5	5,34	30	169	nd	nd
70	106	20,0	6,40	30	185	23,9	5,22
70	109	19,7	6,48	30	192	24,9	4,92
70	111	20,6	6,22	30	204	25,5	4,76
70	170	28,2	3,95	30	211	nd	nd
<i>Muraya paniculata</i>				40	121	19,4	6,58
0	3	nd	nd	40	135	20,2	6,33
0	20	nd	nd	40	142	22,5	5,64
0	26	nd	nd	40	149	24,9	4,94
0	40	nd	nd	40	157	19,8	6,46
0	53	nd	nd	40	165	25,0	4,89
0	62	nd	nd	40	177	33,8	2,24
0	68	nd	nd	40	182	25,4	4,79
0	83	nd	nd	40	191	20,9	6,11
0	89	nd	nd	40	208	nd	nd
0	98	nd	nd	65	123	21,2	6,02
5	6	nd	nd	65	124	28,0	4,00
5	12	nd	nd	65	146	20,1	6,36
5	18	nd	nd	65	158	20,7	6,17
5	31	nd	nd	65	163	24,6	5,00
5	46	nd	nd	65	167	nd	nd
5	56	nd	nd	65	178	21,0	6,10
5	65	nd	nd	65	198	nd	nd
5	71	nd	nd	65	200	21,5	5,94
5	75	nd	nd	65	210	21,0	6,10
5	96	nd	nd	95	116	20,9	6,11
10	5	nd	nd	95	154	20,8	6,15
10	9	32,5	2,64	95	162	20,9	6,12
10	14	nd	nd	95	170	nd	nd
10	16	nd	nd	95	188	nd	nd
10	23	nd	nd	<i>Muraya paniculata</i>			
10	29	nd	nd	0	2	nd	nd
10	33	nd	nd	0	11	nd	nd
10	41	nd	nd	0	18	nd	nd
10	55	nd	nd	0	22	nd	nd
10	49	nd	nd	0	31	nd	nd
15	37	nd	nd	0	34	nd	nd
15	38	nd	nd	0	47	nd	nd
15	44	31,9	2,83	0	68	nd	nd
15	51	nd	nd	0	74	33,3	2,41

15	58	nd	nd	0	79	nd	nd
15	64	nd	nd	5	5	nd	nd
15	69	nd	nd	5	13	nd	nd
15	74	nd	nd	5	20	nd	nd
15	79	nd	nd	5	25	nd	nd
15	86	nd	nd	5	28	nd	nd
25	2	nd	nd	5	36	nd	nd
25	11	32,7	2,59	5	40	nd	nd
25	19	nd	nd	5	52	nd	nd
25	36	nd	nd	5	88	20,2	6,33
25	54	36,0	1,60	5	98	nd	nd
25	61	33,4	2,39	10	9	nd	nd
25	77	31,8	2,85	10	16	nd	nd
25	84	35,0	1,90	10	19	nd	nd
25	94	nd	nd	10	30	nd	nd
25	97	nd	nd	10	43	nd	nd
40	4	33,0	2,50	10	59	nd	nd
40	25	nd	nd	10	76	nd	nd
40	32	nd	nd	10	82	nd	nd
40	35	34,6	2,01	10	93	nd	nd
40	43	nd	nd	10	100	nd	nd
40	48	nd	nd	15	21	nd	nd
40	60	31,0	3,09	15	56	nd	nd
40	67	nd	nd	15	58	nd	nd
40	73	nd	nd	15	65	nd	nd
40	93	nd	nd	15	94	nd	nd
55	1	nd	nd	15	95	nd	nd
55	8	32,3	2,71	15	99	nd	nd
55	13	nd	nd	15	109	nd	nd
55	17	32,6	2,63	15	111	nd	nd
55	28	nd	nd	15	114	nd	nd
55	42	nd	nd	20	3	nd	nd
55	45	29,5	3,53	20	15	33,9	2,22
55	50	33,6	2,30	20	32	nd	nd
55	76	nd	nd	20	45	nd	nd
55	87	nd	nd	20	49	nd	nd
70	27	29,8	3,46	20	63	nd	nd
70	39	32,1	2,76	20	78	nd	nd
70	52	33,8	2,24	20	84	nd	nd
70	57	nd	nd	20	97	nd	nd
70	63	33,5	2,33	20	104	nd	nd
70	66	nd	nd	25	24	nd	nd
70	72	35,2	1,84	25	33	nd	nd
70	85	nd	nd	25	41	nd	nd

70	91	35,4	1,78	25	53	28,9	3,73
70	100	nd	nd	25	54	nd	nd
95	10	nd	nd	25	73	nd	nd
95	15	nd	nd	25	101	nd	nd
95	21	34,9	1,92	25	105	nd	nd
95	47	32,0	2,79	25	112	nd	nd
95	59	nd	nd	25	115	31,5	2,96
95	70	nd	nd	30	6	nd	nd
95	82	nd	nd	30	8	33,6	2,32
110	7	nd	nd	30	12	nd	nd
110	24	35,1	1,86	30	23	nd	nd
110	78	nd	nd	30	35	nd	nd
110	81	nd	nd	30	48	nd	nd
110	88	nd	nd	30	60	nd	nd
110	95	nd	nd	30	64	nd	nd
110	99	nd	nd	30	69	31,1	3,08
180	22	nd	nd	30	72	nd	nd
180	30	nd	nd	40	4	35,7	1,70
180	34	nd	nd	40	26	nd	nd
180	80	30,4	3,26	40	38	nd	nd
180	90	30,7	3,18	40	44	nd	nd
180	92	nd	nd	40	55	nd	nd
<i>Bergera koenigii</i>				40	62	nd	nd
0	215	nd	nd	40	70	nd	nd
0	215	nd	nd	40	75	nd	nd
0	215	nd	nd	40	83	nd	nd
0	223	nd	nd	40	87	35,2	1,83
0	223	nd	nd	65	10	nd	nd
0	223	nd	nd	65	37	32,9	2,52
0	228	nd	nd	65	46	nd	nd
0	228	nd	nd	65	61	32,5	2,66
0	228	nd	nd	65	77	nd	nd
0	233	nd	nd	65	85	nd	nd
0	233	nd	nd	65	90	35,1	1,87
0	233	nd	nd	65	96	nd	nd
0	236	nd	nd	65	106	nd	nd
0	236	nd	nd	65	113	29,1	3,67
0	236	nd	nd	95	1	nd	nd
0	245	nd	nd	95	17	nd	nd
0	245	nd	nd	95	29	nd	nd
0	245	nd	nd	95	42	nd	nd
0	247	nd	nd	95	57	nd	nd
0	247	nd	nd	95	80	nd	nd
0	247	nd	nd	95	89	nd	nd

0	251	nd	nd	95	103	nd	nd
0	251	nd	nd	95	110	nd	nd
0	251	nd	nd	170	27	nd	nd
5	212	nd	nd	170	51	nd	nd
5	212	nd	nd	170	66	nd	nd
5	212	nd	nd	170	81	35,9	1,62
5	217	nd	nd	170	91	nd	nd
5	217	nd	nd	170	102	nd	nd
5	217	nd	nd	170	108	nd	nd
5	219	nd	nd	<i>Bergera koenigii</i>			
5	219	nd	nd	0	10	nd	nd
5	219	nd	nd	0	10	nd	nd
5	221	nd	nd	0	10	nd	nd
5	221	nd	nd	0	12	nd	nd
5	221	nd	nd	0	12	nd	nd
5	230	nd	nd	0	12	nd	nd
5	230	nd	nd	0	17	nd	nd
5	230	nd	nd	0	17	nd	nd
5	231	nd	nd	0	17	nd	nd
5	231	nd	nd	0	25	nd	nd
5	231	nd	nd	0	25	nd	nd
5	238	nd	nd	0	25	nd	nd
5	238	nd	nd	0	42	nd	nd
5	238	nd	nd	0	42	nd	nd
5	242	nd	nd	0	42	nd	nd
5	242	nd	nd	0	62	nd	nd
5	242	nd	nd	0	62	nd	nd
15	214	nd	nd	0	62	nd	nd
15	214	nd	nd	0	78	nd	nd
15	214	nd	nd	0	78	nd	nd
15	220	nd	nd	0	78	nd	nd
15	220	nd	nd	0	86	nd	nd
15	220	nd	nd	0	86	nd	nd
15	222	nd	nd	0	86	nd	nd
15	222	nd	nd	0	97	nd	nd
15	222	nd	nd	0	97	nd	nd
15	225	nd	nd	0	97	nd	nd
15	225	nd	nd	0	98	nd	nd
15	225	nd	nd	0	98	nd	nd
15	227	nd	nd	0	98	nd	nd
15	227	nd	nd	5	22	nd	nd
15	227	nd	nd	5	22	nd	nd
15	234	nd	nd	5	22	nd	nd
15	234	nd	nd	5	24	nd	nd

15	234	nd	nd	5	24	nd	nd
15	240	nd	nd	5	24	nd	nd
15	240	nd	nd	5	43	nd	nd
15	240	nd	nd	5	43	nd	nd
15	248	nd	nd	5	43	nd	nd
15	248	nd	nd	5	51	nd	nd
15	248	nd	nd	5	51	nd	nd
30	213	nd	nd	5	51	nd	nd
30	213	nd	nd	5	65	nd	nd
30	213	nd	nd	5	65	nd	nd
30	218	nd	nd	5	65	nd	nd
30	218	nd	nd	5	69	nd	nd
30	218	nd	nd	5	69	nd	nd
30	226	nd	nd	5	69	nd	nd
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30	239	nd	nd	5	88	nd	nd
30	243	nd	nd	5	88	nd	nd
30	243	nd	nd	13	13	nd	nd
30	243	nd	nd	13	13	nd	nd
30	249	nd	nd	13	13	nd	nd
30	249	nd	nd	13	36	nd	nd
30	249	nd	nd	13	36	nd	nd
60	216	nd	nd	13	36	nd	nd
60	216	nd	nd	13	41	nd	nd
60	216	nd	nd	13	41	nd	nd
60	229	nd	nd	13	41	nd	nd
60	229	nd	nd	13	44	nd	nd
60	229	nd	nd	13	44	nd	nd
60	241	nd	nd	13	44	nd	nd
60	241	nd	nd	13	45	nd	nd
60	241	nd	nd	13	45	nd	nd
60	246	nd	nd	13	45	nd	nd
60	246	nd	nd	13	47	nd	nd
60	246	nd	nd	13	47	nd	nd
95	202	nd	nd	13	47	nd	nd

95	202	nd	nd	13	52	nd	nd
95	202	nd	nd	13	52	nd	nd
95	224	nd	nd	13	52	nd	nd
95	224	nd	nd	13	55	nd	nd
95	224	nd	nd	13	55	nd	nd
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95	237	nd	nd	13	87	nd	nd
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95	244	nd	nd	13	89	nd	nd
95	250	nd	nd	13	89	nd	nd
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50	90	nd	nd
50	90	nd	nd
50	90	nd	nd
50	96	nd	nd
50	96	nd	nd
50	96	nd	nd

^aCt: Cycle threshold.

^bLog: Las titer in log10 of amplicon copies per gram of plant tissue estimated based on a standard curve as described by Lopes et al., 2013.

^cnd: non-detected.

Supplementary Table 5. Data on the length (cm) values of each new shoots of *Citrus* × *sinensis*, *Murraya paniculata* and *Bergera koenigii* in acclimatized room at 25 °C, in experiment replicates 1 and 2.

Day of pruning	Days post pruning	Plant number	Shoot Length (cm)	Day of pruning	Days post pruning	Plant number	Shoot Length (cm)
<i>Citrus x sinensis</i>				<i>Citrus x sinensis</i>			
04/07/2019	0	c1	0,05	21/10/2019	0	c11	0,05
04/07/2019	0	c2	0,05	21/10/2019	0	c12	0,05
04/07/2019	0	c3	0,05	21/10/2019	0	c13	0,05
04/07/2019	0	c4	0,05	21/10/2019	0	c14	0,05
04/07/2019	0	c5	0,05	21/10/2019	0	c15	0,05
04/07/2019	0	c6	0,05	21/10/2019	0	c16	0,05
04/07/2019	0	c7	0,05	21/10/2019	0	c17	0,05
04/07/2019	0	c8	0,05	21/10/2019	0	c18	0,05
04/07/2019	0	c9	0,05	21/10/2019	0	c19	0,05
04/07/2019	0	c10	0,05	21/10/2019	0	c20	0,05
27/07/2019	23	117	2,1	13/11/2019	23	122	1,9
27/07/2019	23	124	5,1	13/11/2019	23	127	2,7
27/07/2019	23	130	4,3	13/11/2019	23	136	2,2
27/07/2019	23	134	2,8	13/11/2019	23	139	3,5
27/07/2019	23	139	3,2	13/11/2019	23	147	2,8
27/07/2019	23	145	5,1	13/11/2019	23	150	3,1
27/07/2019	23	152	4,2	13/11/2019	23	171	2,7
27/07/2019	23	156	2,2	13/11/2019	23	183	2,7
27/07/2019	23	162	3,3	13/11/2019	23	187	2,5
27/07/2019	23	168	2,2	13/11/2019	23	196	2,5
01/08/2019	28	101	12,1	18/11/2019	28	117	2,8
01/08/2019	28	107	13,1	18/11/2019	28	131	7
01/08/2019	28	112	12,3	18/11/2019	28	138	5,8
01/08/2019	28	122	7,4	18/11/2019	28	141	7,6
01/08/2019	28	127	7,5	18/11/2019	28	145	7,1
01/08/2019	28	138	7,1	18/11/2019	28	153	6,5
01/08/2019	28	150	7,5	18/11/2019	28	156	7,8
01/08/2019	28	159	6,5	18/11/2019	28	166	4,8
01/08/2019	28	164	5,5	18/11/2019	28	199	6

01/08/2019	28	169	7,3	18/11/2019	28	209	5,9
06/08/2019	33	102	32	23/11/2019	33	120	13,4
06/08/2019	33	110	18,1	23/11/2019	33	125	7,3
06/08/2019	33	116	16,5	23/11/2019	33	133	7,9
06/08/2019	33	126	14,2	23/11/2019	33	134	14,9
06/08/2019	33	129	10,3	23/11/2019	33	143	4,8
06/08/2019	33	136	9,5	23/11/2019	33	160	14,1
06/08/2019	33	140	14,5	23/11/2019	33	168	11
06/08/2019	33	144	11,2	23/11/2019	33	172	10,1
06/08/2019	33	148	13,5	23/11/2019	33	193	1,7
06/08/2019	33	165	10,5	23/11/2019	33	205	11,6
11/08/2019	38	105	15,5	28/11/2019	38	126	9,8
11/08/2019	38	115	17,5	28/11/2019	38	128	7,3
11/08/2019	38	120	21	28/11/2019	38	129	7,7
11/08/2019	38	121	20,1	28/11/2019	38	174	8,5
11/08/2019	38	128	20,3	28/11/2019	38	176	10,5
11/08/2019	38	137	9,8	28/11/2019	38	180	7
11/08/2019	38	142	15,5	28/11/2019	38	186	13,9
11/08/2019	38	147	13,3	28/11/2019	38	190	15,5
11/08/2019	38	153	11,1	28/11/2019	38	201	20,1
11/08/2019	38	163	8,5	28/11/2019	38	206	7,8
21/08/2019	48	108	19,1	01/12/2019	41	137	9,8
21/08/2019	48	119	20,1	01/12/2019	41	151	4
21/08/2019	48	123	18,3	01/12/2019	41	161	19
21/08/2019	48	132	14,8	01/12/2019	41	173	8,9
21/08/2019	48	133	13,2	01/12/2019	41	179	12,6
21/08/2019	48	141	21,1	01/12/2019	41	184	15
21/08/2019	48	143	14,5	01/12/2019	41	195	19,5
21/08/2019	48	154	15,3	01/12/2019	41	197	3,4
21/08/2019	48	157	9,9	01/12/2019	41	203	6,8
21/08/2019	48	167	6,8	01/12/2019	41	207	11,3
05/09/2019	63	103	13,5	03/12/2019	43	119	11
05/09/2019	63	113	46,1	03/12/2019	43	132	10,5
05/09/2019	63	118	8,2	03/12/2019	43	140	8,4
05/09/2019	63	125	13,2	03/12/2019	43	148	13,5
05/09/2019	63	131	11,2	03/12/2019	43	155	12,4
05/09/2019	63	135	11,3	03/12/2019	43	164	12,5
05/09/2019	63	146	13,3	03/12/2019	43	175	15,6
05/09/2019	63	151	10,2	03/12/2019	43	181	10,4
05/09/2019	63	160	12,1	03/12/2019	43	189	6,1
05/09/2019	63	166	13,5	03/12/2019	43	194	7
21/09/2019	79	114	13,1	13/12/2019	53	118	5,1

21/09/2019	79	149	21	13/12/2019	53	130	10,2
21/09/2019	79	155	14,1	13/12/2019	53	144	12,1
21/09/2019	79	158	13,8	13/12/2019	53	152	10
21/09/2019	79	161	12,5	13/12/2019	53	159	5,9
07/10/2019	95	104	15,1	13/12/2019	53	169	13,9
07/10/2019	95	106	18,2	13/12/2019	53	185	14
07/10/2019	95	109	15,3	13/12/2019	53	192	8,6
07/10/2019	95	111	15,2	13/12/2019	53	204	12
07/10/2019	95	170	15,1	13/12/2019	53	211	11,5
<i>Murraya paniculata</i>				23/12/2019	63	121	14,1
04/07/2019	0	m1	0,05	23/12/2019	63	135	7,5
04/07/2019	0	m2	0,05	23/12/2019	63	142	7,5
04/07/2019	0	m3	0,05	23/12/2019	63	149	6,5
04/07/2019	0	m4	0,05	23/12/2019	63	157	15
04/07/2019	0	m5	0,05	23/12/2019	63	165	8,4
04/07/2019	0	m6	0,05	23/12/2019	63	177	6
04/07/2019	0	m7	0,05	23/12/2019	63	182	14,3
04/07/2019	0	m8	0,05	23/12/2019	63	191	11,5
04/07/2019	0	m9	0,05	23/12/2019	63	208	4,4
04/07/2019	0	m10	0,05	17/01/2020	88	123	17
27/07/2019	23	3	3,2	17/01/2020	88	124	10
27/07/2019	23	20	4,1	17/01/2020	88	146	9,5
27/07/2019	23	26	2,2	17/01/2020	88	158	9,8
27/07/2019	23	40	5,1	17/01/2020	88	163	10,5
27/07/2019	23	53	3,1	17/01/2020	88	167	4,6
27/07/2019	23	62	2,2	17/01/2020	88	178	7,5
27/07/2019	23	68	1,3	17/01/2020	88	198	11
27/07/2019	23	83	4,1	17/01/2020	88	200	8,4
27/07/2019	23	89	5,2	17/01/2020	88	210	11
27/07/2019	23	98	3,3	17/02/2020	119	116	11,5
01/08/2019	28	6	4,5	17/02/2020	119	154	11
01/08/2019	28	12	4,2	17/02/2020	119	162	11,5
01/08/2019	28	18	4,1	17/02/2020	119	170	10,5
01/08/2019	28	31	5,5	17/02/2020	119	188	13,5
01/08/2019	28	46	3,1	<i>Murraya paniculata</i>			
01/08/2019	28	56	3,2	21/10/2019	0	m11	0,05
01/08/2019	28	65	5,1	21/10/2019	0	m12	0,05
01/08/2019	28	71	4,2	21/10/2019	0	m13	0,05
01/08/2019	28	75	2,5	21/10/2019	0	m14	0,05
01/08/2019	28	96	3,5	21/10/2019	0	m15	0,05
06/08/2019	33	5	4,5	21/10/2019	0	m16	0,05
06/08/2019	33	9	5,1	21/10/2019	0	m17	0,05

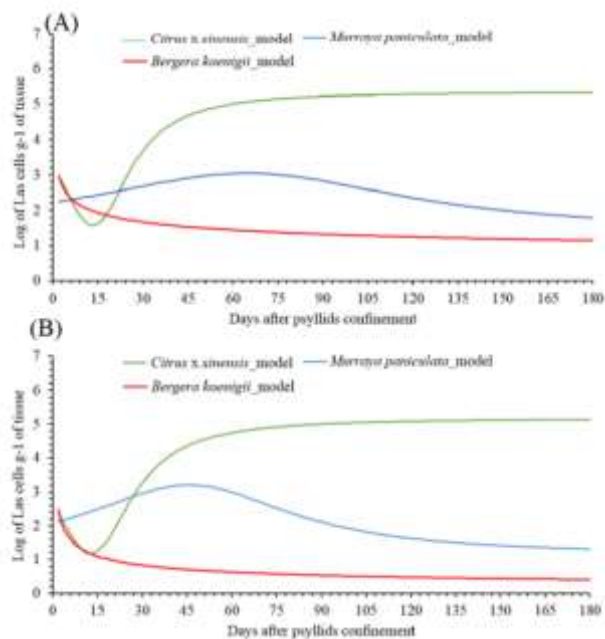
06/08/2019	33	14	4,9	21/10/2019	0	m18	0,05
06/08/2019	33	16	5,1	21/10/2019	0	m19	0,05
06/08/2019	33	23	7	21/10/2019	0	m20	0,05
06/08/2019	33	29	5,1	13/11/2019	23	2	1
06/08/2019	33	33	4,5	13/11/2019	23	11	0,6
06/08/2019	33	41	5,2	13/11/2019	23	18	0,9
06/08/2019	33	55	2,5	13/11/2019	23	22	0,5
06/08/2019	33	49	4,5	13/11/2019	23	31	0,9
11/08/2019	38	37	7	13/11/2019	23	34	0,5
11/08/2019	38	38	5,5	13/11/2019	23	47	0,9
11/08/2019	38	44	7,1	13/11/2019	23	68	2,3
11/08/2019	38	51	5,4	13/11/2019	23	74	1
11/08/2019	38	58	5,6	13/11/2019	23	79	1,6
11/08/2019	38	64	7,1	18/11/2019	28	5	1,4
11/08/2019	38	69	6,5	18/11/2019	28	13	1,2
11/08/2019	38	74	7,1	18/11/2019	28	20	2
11/08/2019	38	79	7,3	18/11/2019	28	25	1,5
11/08/2019	38	86	6,2	18/11/2019	28	28	1,7
21/08/2019	48	2	4,5	18/11/2019	28	36	1,5
21/08/2019	48	11	8,1	18/11/2019	28	40	2,1
21/08/2019	48	19	5,8	18/11/2019	28	52	1,1
21/08/2019	48	36	13,1	18/11/2019	28	88	1,7
21/08/2019	48	54	6,8	18/11/2019	28	98	3,3
21/08/2019	48	61	4,7	23/11/2019	33	9	2,7
21/08/2019	48	77	8,1	23/11/2019	33	16	2,6
21/08/2019	48	84	7,4	23/11/2019	33	19	2,2
21/08/2019	48	94	4,1	23/11/2019	33	30	3,2
21/08/2019	48	97	4,3	23/11/2019	33	43	2,3
05/09/2019	63	4	4,5	23/11/2019	33	59	3,8
05/09/2019	63	25	6,3	23/11/2019	33	76	3
05/09/2019	63	32	5,1	23/11/2019	33	82	1,3
05/09/2019	63	35	5,3	23/11/2019	33	93	4,6
05/09/2019	63	43	6,1	23/11/2019	33	100	5
05/09/2019	63	48	5,5	28/11/2019	38	21	2,1
05/09/2019	63	60	5,5	28/11/2019	38	56	2,2
05/09/2019	63	67	7,5	28/11/2019	38	58	4
05/09/2019	63	73	10,2	28/11/2019	38	65	3,1
05/09/2019	63	93	3,5	28/11/2019	38	94	6
21/09/2019	79	1	5,4	28/11/2019	38	95	2,1
21/09/2019	79	8	6,5	28/11/2019	38	99	1,8
21/09/2019	79	13	6,5	28/11/2019	38	109	2
21/09/2019	79	17	3,2	28/11/2019	38	111	5,3

21/09/2019	79	28	3,5	28/11/2019	38	114	3,8
21/09/2019	79	42	9,4	03/12/2019	43	3	2,8
21/09/2019	79	45	5,8	03/12/2019	43	15	2,3
21/09/2019	79	50	4,5	03/12/2019	43	32	3,3
21/09/2019	79	76	4,1	03/12/2019	43	45	2,7
21/09/2019	79	87	6,2	03/12/2019	43	49	3,6
07/10/2019	95	27	5,5	03/12/2019	43	63	2,6
07/10/2019	95	39	3,2	03/12/2019	43	78	2,5
07/10/2019	95	52	4,5	03/12/2019	43	84	4
07/10/2019	95	57	5,9	03/12/2019	43	97	2,8
07/10/2019	95	63	6,1	03/12/2019	43	104	3,5
07/10/2019	95	66	4,8	08/12/2019	48	24	2,2
07/10/2019	95	72	7,2	08/12/2019	48	33	4,1
07/10/2019	95	85	10,1	08/12/2019	48	41	2,6
07/10/2019	95	91	4,6	08/12/2019	48	53	3
07/10/2019	95	100	7,1	08/12/2019	48	54	1,5
30/10/2019	118	10	6,5	08/12/2019	48	73	3,4
30/10/2019	118	15	8,1	08/12/2019	48	101	5,5
30/10/2019	118	21	6,2	08/12/2019	48	105	4
30/10/2019	118	47	8,4	08/12/2019	48	112	3,5
30/10/2019	118	59	7,2	08/12/2019	48	115	4,4
30/10/2019	118	70	7,3	13/12/2019	53	6	3,4
30/10/2019	118	82	6,2	13/12/2019	53	8	3,1
15/11/2019	134	7	8,1	13/12/2019	53	12	2
15/11/2019	134	24	7,2	13/12/2019	53	23	2,2
15/11/2019	134	78	6,1	13/12/2019	53	35	1,1
15/11/2019	134	81	8,2	13/12/2019	53	48	5,2
15/11/2019	134	88	8,4	13/12/2019	53	60	4
15/11/2019	134	95	7,1	13/12/2019	53	64	3
15/11/2019	134	99	6,2	13/12/2019	53	69	2,5
27/01/2020	207	22	8,2	13/12/2019	53	72	3,7
27/01/2020	207	30	7,4	23/12/2019	63	4	3,5
27/01/2020	207	34	6,8	23/12/2019	63	26	3
27/01/2020	207	80	8,1	23/12/2019	63	38	5,2
27/01/2020	207	90	8	23/12/2019	63	44	3
27/01/2020	207	92	7	23/12/2019	63	55	1,4
<i>Bergera koenigii</i>				23/12/2019	63	62	2,8
21/10/2019	0	b1	0,05	23/12/2019	63	70	3,3
21/10/2019	0	b2	0,05	23/12/2019	63	75	3,4
21/10/2019	0	b3	0,05	23/12/2019	63	83	3,3
21/10/2019	0	b4	0,05	23/12/2019	63	87	3,6
21/10/2019	0	b5	0,05	17/01/2020	88	10	4,6

21/10/2019	0	b6	0,05	17/01/2020	88	37	3,3
21/10/2019	0	b7	0,05	17/01/2020	88	46	4,8
21/10/2019	0	b8	0,05	17/01/2020	88	61	5,4
13/11/2019	23	215	0,9	17/01/2020	88	77	3
13/11/2019	23	223	0,8	17/01/2020	88	85	3,6
13/11/2019	23	228	0,5	17/01/2020	88	90	3,5
13/11/2019	23	233	0,4	17/01/2020	88	96	5
13/11/2019	23	236	0,5	17/01/2020	88	106	2
13/11/2019	23	245	0,9	17/01/2020	88	113	3
13/11/2019	23	247	0,6	21/02/2020	123	1	6
13/11/2019	23	251	1	21/02/2020	123	17	3
18/11/2019	28	212	1	21/02/2020	123	29	3,1
18/11/2019	28	217	1,1	21/02/2020	123	42	1,5
18/11/2019	28	219	1,2	21/02/2020	123	57	5,4
18/11/2019	28	221	1,2	21/02/2020	123	80	3,9
18/11/2019	28	230	0,7	21/02/2020	123	89	5
18/11/2019	28	231	1,4	21/02/2020	123	103	7,4
18/11/2019	28	238	1	21/02/2020	123	110	5,5
18/11/2019	28	242	1	01/05/2020	193	27	4
28/11/2019	38	214		01/05/2020	193	51	3,8
28/11/2019	38	220	9,5	01/05/2020	193	66	4,1
28/11/2019	38	222		01/05/2020	193	81	4
28/11/2019	38	225	14	01/05/2020	193	91	5,6
28/11/2019	38	227		01/05/2020	193	102	5
28/11/2019	38	234	10,6	01/05/2020	193	108	5,5
28/11/2019	38	240	14	<i>Bergera koenigii</i>			
28/11/2019	38	248		10/08/2020	0	b9	0,05
13/12/2019	53	213	14	10/08/2020	0	b10	0,05
13/12/2019	53	218	15	10/08/2020	0	b11	0,05
13/12/2019	53	226	14	10/08/2020	0	b12	0,05
13/12/2019	53	232	10	10/08/2020	0	b13	0,05
13/12/2019	53	235	14	10/08/2020	0	b14	0,05
13/12/2019	53	239	15	10/08/2020	0	b15	0,05
13/12/2019	53	243	11	10/08/2020	0	b16	0,05
13/12/2019	53	249	22	10/08/2020	0	b17	0,05
17/01/2020	88	216	20	10/08/2020	0	b18	0,05
17/01/2020	88	229	15	02/09/2020	23	10	1,4
17/01/2020	88	241	20	02/09/2020	23	12	2,1
17/01/2020	88	246	18	02/09/2020	23	17	1,2
17/02/2020	119	202	10	02/09/2020	23	25	0,6
17/02/2020	119	224	20	02/09/2020	23	42	1,3
17/02/2020	119	237	20	02/09/2020	23	62	2,4

17/02/2020	119	244	25	02/09/2020	23	78	2
17/02/2020	119	250		02/09/2020	23	86	3,7
				02/09/2020	23	97	4,5
				02/09/2020	23	98	3,5
				07/09/2020	28	22	3,4
				07/09/2020	28	24	2,1
				07/09/2020	28	43	4,8
				07/09/2020	28	51	1,9
				07/09/2020	28	65	1,8
				07/09/2020	28	69	2,8
				07/09/2020	28	70	2,4
				07/09/2020	28	82	5,1
				07/09/2020	28	83	13
				07/09/2020	28	88	4,2
				15/09/2020	36	13	2,9
				15/09/2020	36	36	2,9
				15/09/2020	36	41	4,6
				15/09/2020	36	44	12,3
				15/09/2020	36	45	3,3
				15/09/2020	36	47	1,2
				15/09/2020	36	52	24,4
				15/09/2020	36	55	17,4
				15/09/2020	36	87	11,9
				15/09/2020	36	89	15,4
				19/09/2020	40	3	6,2
				19/09/2020	40	7	11,3
				19/09/2020	40	19	15,3
				19/09/2020	40	29	17,3
				19/09/2020	40	35	5,4
				19/09/2020	40	59	16,5
				19/09/2020	40	74	19,4
				19/09/2020	40	81	18,1
				19/09/2020	40	92	20,4
				19/09/2020	40	95	17,6
				23/09/2020	44	1	18,8
				23/09/2020	44	5	14
				23/09/2020	44	15	8,4
				23/09/2020	44	27	22
				23/09/2020	44	31	15,6
				23/09/2020	44	39	15,4
				23/09/2020	44	48	11,9
				23/09/2020	44	57	12,6

23/09/2020	44	72	23,7
23/09/2020	44	80	29,9
05/10/2020	56	6	6,5
05/10/2020	56	16	29,6
05/10/2020	56	26	10,3
05/10/2020	56	37	21,9
05/10/2020	56	54	23,1
05/10/2020	56	63	35
05/10/2020	56	68	24,5
05/10/2020	56	76	13,5
05/10/2020	56	85	26,6
05/10/2020	56	99	18,4
22/10/2020	73	4	23,2
22/10/2020	73	11	19,2
22/10/2020	73	20	26,1
22/10/2020	73	30	21,2
22/10/2020	73	40	24,1
22/10/2020	73	58	23,1
22/10/2020	73	66	21,8
22/10/2020	73	75	12,5
22/10/2020	73	90	20,1
22/10/2020	73	96	21,2



Supplementary Figure 1. Lorentzian growth model for the population dynamics of ‘*Candidatus Liberibacter asiaticus*’ over time in new shoots of *Citrus × sinensis* and *Murraya paniculata* and the negative exponential model for the population dynamics of ‘*Candidatus Liberibacter asiaticus*’ over time in new shoots of *Bergera koenigii*, following a 48 h period of *Diaphorina citri* confinement in experiment replicates 1 (A) and 2 (B).

CHAPTER 4 – Susceptible and resistant genotypes transcriptomic response at early stages after ‘*Candidatus Liberibacter asiaticus*’ inoculation with infected *Diaphorina citri* feeding on young shoots

ABSTRACT

Huanglongbing (HLB) is the most destructive citrus disease mainly associated with the presence of the phloem-limited and non-culturable bacterium ‘*Candidatus Liberibacter asiaticus*’ (Las), which is naturally transmitted by the psyllid *Diaphorina citri*. Different approaches have been used trying to elucidate the pathogen-host interaction. However, most studies have focused on already infected and/or symptomatic plants, or using Las inoculations and plant maintenance systems different from the natural ones. After observation of Las dynamics on three species with different responses after Las challenging by *D. citri* in a controlled environment, the next step was to evaluate the key-time points of those interactions using a transcriptomic approach. Thus, we used RNA sequencing (RNA-seq) to compare transcriptional changes on new flushes from the susceptible sweet orange *C. × sinensis* ‘Valencia’, the transient host *M. paniculata* and the immune *B. koenigii*. When comparing transcriptomic differences in RNA samples from flushes exposed to Las+ insects vs. healthy insects we did not observe significant expression changes. However, it was observed that *B. koenigii* had not undergone any change in expression and essentially arrested their transcriptome due to infection until 10 days after being exposed to Las+ insects. Those results were coherent with the slightly slower growth of *B. koenigii* flushes exposed to Las+ insects vs. the healthy ones during the first infection phase. Consistent with that, GSEA results showed that photosynthesis-related gene expression was down-regulated in RNA samples exposed to Las+ insects at Time 0 and until Time 10 for *B. koenigii*. Taking together, it is possible to infer that a typical plant-biotroph or necrotrophy pathogen interaction does not occur in Las-

Aurantioideae pathosystems and that photosynthesis transcriptional reprogramming may be an early defense tool against Las in *B. koenigii*.

Keywords: *Bergera*, *Murraya*, citrus breeding, greening, HLB, RNA-seq

1. INTRODUCTION

Huanglongbing (HLB), also known as *greening*, is the most destructive *Citrus* disease worldwide (Bové, 2006). In Brazil, its occurrence is related to the presence of the phloem-limited, non-cultivable, endopathogenic and α -proteobacteria (Jagoueix et al., 1994; Bové, 2006) ‘*Candidatus Liberibacter asiaticus*’ (Las) and ‘*Candidatus Liberibacter americanus*’ (Lam) (Gottwald, 2010; Coletta-Filho et al., 2004; Teixeira et al., 2005). Las and Lam are both transmitted by the Asian citrus psyllid *Diaphorina citri* (Kuwayama) (Sternorrhyncha: Liviidae) (Capoor et al., 1967). However, Las is more resistant to high temperatures and easily transmitted by the insect vector (Lopes et al., 2009, 2013), which has made this specie the most destructive one, being present in more than 99% of field samples analyzed at Fundecitrus (Bassanezi et al., 2020).

Until now, no cure for HLB has been reported. Because of it, since its discovery, HLB has been managed in Brazil based on three strategies: the use of healthy plants from certified nurseries, elimination of positive plants from fields, and inspection and chemical control of the insect vector (Bassanezi et al., 2020). Those strategies have made it possible for Brazil’s orchards to continue to be important producers; however, their adoption is neither environmentally nor economically sustainable. Furthermore, HLB rate continues to increase, being present in more than 24.42% of symptomatic trees in the citrus belt of São Paulo and Minas Gerais states (Fundecitrus, 2022).

Although there is no resistant specie in the genus *Citrus* (Folimonova et al., 2009), some citrus relatives closer or distant phylogenetically within the family Rutaceae subfamily Aurantioideae have been reported as completely or partially resistant to HLB (Ramadugu et al., 2016; Beloti et al., 2018; Cifuentes-Arenas et al., 2019; Alves et al., 2021a; 2021b; 2022).

The monoembryonic *Murraya paniculata* (L.) Jack and *Bergera koenigii* L. species (previously called as *Murraya koenigii*), known as orange jasmine and curry

leaf, respectively, are commonly used in tropical and subtropical regions of the world as ornamental plants (Swingle and Reece, 1967). Although from the same subfamily, the Aurantiodeae, *M. paniculata* and *B. koenigii* do not have a good compatibility for grafting nor for pollination with species from the *Citrus* genera (Swingle and Reece, 1967; Raiol-Junior et al., 2022). Both species were described as good hosts for the insect vector *D. citri*, being even more attractive to females than sweet oranges (Damsteegt et al., 2010; Teck et al., 2011; Westbrook et al., 2011; Beloti et al., 2017; Tomaseto et al., 2019). However, *M. paniculata* was reported as a transient host for the HLB bacterium and *B. koenigii* as immune (Damsteegt et al., 2010; Beloti et al., 2018; Cifuentes-Arenas et al., 2019).

In general, phloem-limited pathogens such as Las intrigue researchers, as they are difficult to detect and present variable symptoms, which develop slowly (Bendix et al., 2018). HLB leaf symptoms include yellowing and asymmetrical blotchy mottle and enlarged veins (Bové, 2006); leaves become small and show aspects that resemble nutrient deficiency symptoms such as zinc and manganese (da Graça, 1991). Fruits are usually smaller, lighter, and highly acidic and with a reduced Brix ratio, and suffer a premature fruit drop (Bassanezi et al., 2009).

Las can be acquired by *D. citri* at any stage of life (Pelz-Stelinski et al., 2010). With a minimum latent period of ~2 weeks, an infected *D. citri* is capable of Las transmission (Canale et al., 2017) and passively introduces the bacterium into the plant phloem (Jiang et al., 2019; Haung et al., 2020). The insect vector is preferably attracted for feeding and reproduction on young flushes (Lopes and Cifuentes-Arenas, 2021; Pandey et al., 2021), on which Las remains until leaves are mature to turn into a source, moving through the phloem sap from sources to sinks (Pandey et al., 2021; Alves et al., 2021a; De Francesco et al., 2021; Raiol-Junior et al., 2021b). At late infection times, Las induce callose plugging of the sieve pore, reducing its diameters, and affecting phloem dysfunction, leading to starch accumulation in leaves and loss of sink growth (Achor et al., 2020; Bové, 2006). Without being mediated by flagella, Las moves through the phloem in an elongated form being the reduced diameters of the sieve pore ineffective in restricting the bacterium movement (Achor et al. 2020; Ammar et al. 2019).

Las preferentially migrate to new developing tissues (Raiol-Junior et al., 2021a) and irregularly colonizes the plant (Tatineni et al., 2008; Li et al., 2009). Because of it, and as being a phloem-limited pathogen, flush tissues are recommended for sampling diagnostic purposes as the proportion of phloem on this tissue is high (Cifuentes-Arenas et al., 2018).

After 48 hours of infected *D. citri* feeding on young shoots of a susceptible sweet orange variety, in the transient host *M. paniculata* and in the immune *B. koenigii*, Las titer drops until the 10-12th days. Following this, Las exponentially increase in the sweet orange and *M. paniculata* reaching, in the susceptible one, the stationary phase from the 40th day onward (Alves et al., 2021a) with a 2.9-3.8 cm day⁻¹ average speed of movement (Raiol-Junior et al., 2021b). On the other hand, on *M. paniculata*, between the 40th and 60th days, Las titer exponentially increases but at a lower rate than sweet orange, decreases gradually thereafter and becomes undetectable from the 160th day onward. In the case of the immune *B. koenigii*, Las titers decrease from the start and remain undetectable (Alves et al., 2021a).

As an evolutionary trait, herbivorous hemipteran as *D. citri* developed specialized stylets for piercing the plant tissue and accessing the nutrient-rich phloem sap. Prokaryote pathogens use this system to passively be delivered into the phloem by the feeding insects (Jiang et al., 2019). With a reduced genome size, those pathogens are unable to encode all core metabolic pathways, relying on this tissue to obtain essential nutrients and signals (Fagen et al., 2014).

The phloem is a vascular tissue consisting of sieve elements, companion cells, and parenchyma cells (Heo et al., 2014). How Las manipulates the host signaling being restricted only to the sieve elements that lack a nucleus and important organelles is still a question (Jiang et al., 2019). Can sieve cells alone mount an effective immune response?

Innate resistance in phloem-feeding insects is more related to the timing and magnitude of plant defenses rather than to qualitatively different responses. The recognition and signaling are rapid, fleeting, and highly localized to the feeding site (Thompson et al., 2006). Phloem insect-transcriptomic studies showed that resistant plants and susceptible cultivars does not have a huge different expression patterns (Zhang et al., 2004; Qubbaj et al., 2005; Park et al., 2006).

Different comparative omics approaches with less or more susceptible varieties have been tried to elucidate the HLB pathosystem. However, most previous transcriptomic studies evaluated plants Las-challenged by grafting (Albrecht and Bowman, 2008; Kim et al., 2009; Fan et al., 2011; Albrecht and Bowman 2012; Yu et al., 2017; Chin et al., 2020; Curtolo et al., 2020; Ramsey et al., 2020; Chin et al., 2021) or under the high amount of variations in the field (Rawat et al., 2017; Francesco et al., 2022). Furthermore, evaluations of the genes differentially expressed were done at late stages of infection (Kim et al., 2009; Fan et al., 2011; Albrecht and Bowman, 2012; Curtolo et al., 2020) and/or used *in vitro* studies without the presence of the root system (Wei et al., 2021).

Transcriptomic studies done with HLB plants after years of natural Las-exposition in the field show down expression of beta glucanases, DMR6-like genes, expansin and DET2, which are mentioned as being associated with the suppression of citrus immunity, while activation of NPR-1 like genes may induce the resistance response to HLB (Wang et al., 2016). However, the mechanisms triggering these gene suppression and activation are still unclear.

Similar observations are made when studying the transcriptomic profile of *Citrus* plants graft inoculated on Las late infection (Kim et al., 2009; Fan et al., 2011; Albrecht and Bowman, 2012; Curtolo et al., 2020). Compared with more tolerant varieties, the less susceptible ones show more induction of immune-related genes, cell wall-associated genes, transcriptional regulators, signaling cascades, and hormone signaling but lower effects on starch and photosynthesis than the susceptible genotypes (Curtolo et al., 2020). However, in the case of hybrids mentioned as resistant, only a few DEGs (differentially expressed genes) were observed in RNA-seq studies (Curtolo et al., 2020).

To gain more information on the early infection of Las, transcriptomics studies were done a few weeks after grafting inoculation (Albrecht and Bowman et al., 2008; Chin et al., 2020; Ramsey et al., 2020; Chin et al., 2021). Chin et al. (2020) evaluated the expression of 6 biological replicates of *C. × sinensis* after 8, 18, 26, and 46 weeks of graft inoculation, and only 246, 54, 664, and 1844 DEGs were obtained. However, no gene ontology (GO) was significantly over-represented among the DEGs, being only isolated up and down-expressed genes mentioned. Similar observations were

made by Ramsey et al. (2020) when studying the *C. × limon* genotype even in earlier times (2, 10, and 14 weeks after graft inoculation).

However, using a controlled inoculation system, a transcriptomic study was done mimetizing the natural infection by *D. citri* and using budwood sticks on Murashige and Skoog (MS) culture media (Wei et al., 2021) and almost 100 DEGs were observed only after 48 hours of psyllids feeding on *in vitro* flushes. Differences included plant hormone signal transduction, biosynthesis of secondary metabolites, glutathione metabolism, protein processing in the endoplasmic reticulum, and metabolic pathways, among others (Wei et al., 2021).

Aiming to understand better the initial bacterium dynamics after the delivery of Las into the phloem by *D. citri*, we studied the bacterial multiplication at the first stages post-inoculation by qPCR and RNA-seq. Using a well-controlled challenge system of Las inoculation that mimics the most natural method of Las delivery and plant aspects leads us to the establishment of the earliest events of Las infection on the susceptible, transient and immune species *Citrus × sinensis*, *Murraya paniculata* and *Bergera koenigii*, respectively. Knowledge of Las progression and the comparative defense responses of those three species may help to develop bacterial control strategies.

2. MATERIALS AND METHODS

2.1 Plant Material and Growth Conditions

Two-year-old plantlets of *Citrus × sinensis* (L.) Osbeck ‘Valencia’ grafted on ‘Rangpur’ lime (*Citrus × limonia* Osbeck), and 2-year-old seedlings of *M. paniculata* (orange jasmine) and *B. koenigii* (curry leaf) were used. Plants were grown in 300-mL conical tubes (6.5 x 5.9 cm, upper x lower diameter; 16 cm, height), filled with coconut fiber, and irrigated twice a week.

Rearing of Las-positive *D. citri* and plant maintenance during the entire experiment were carried out inside a controlled environmental room (CER) using shelves where the light source was 20 to 30 cm above the plants. The relative humidity ranged between 55% and 78%, the daily temperature varied from 24°C to 27°C, and

the photoperiod was set to 12 h of light at $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation and 12 h of darkness.

2.2 Rearing of Las-Infective Psyllids, Inoculation Procedure, and Sampling

For insect rearing and inoculation purposes, the same methodology described by Alves et al. (2021a) was used. Las-positive, symptomatic and 1-year-old plants of *C. × sinensis* grafted on ‘Swingle’ citrumelo (*Citrus × paradise* Macfad. × *Poncirus trifoliata* L. Raf.) growing in 4.7-l pots were used for rearing Las-infective *D. citri* insects. For Las presence confirmation, 4-5 leaves of each source plant were sampled and qPCR presented a Ct average of 21.82 ± 0.79 (Teixeira et al., 2005; Li et al., 2006). To stimulate the growth of new flushes, the source plants were pruned and when they reached the V2 stage of development, four healthy psyllids 10 to 20 days old were confined per shoot for 7 days of oviposition. The healthy insects were obtained from a colony kept at Fundecitrus, in which each insect is reared on healthy *M. paniculata* seedlings (Skelley and Hoy, 2004). Following this, adults were removed and plants were maintained to allow eggs to hatch and nymphs to develop into first-generation adults (F1). Three-five days after F1 emergence, a random sample of three insects per source plant was removed to confirm Las presence by qPCR, and the rest was maintained for up to 15-20 days before being used (Figure 1.A).

To be used in the experiment, the *C. × sinensis* plantlets were pruned at 15-25 cm above the substrate level, and *M. paniculata* and *B. koenigii* seedlings at 15-35 cm to promote the growth of a new flush (Figure 1.B). When they reached the V3 stage of development (Cifuentes-Arenas et al., 2018), five 15-20 day-old Las-exposed adults were confined to a single flush per plant for 48 h of inoculation access period (IAP). After 48 h, adults were removed and stored at -20°C and a random-stratified sampling was performed, where one of the five adults per new flush was selected for qPCR analysis. Plants were then sprayed with insecticide (Abamectin EC 7.2 g of active ingredient per 1 L of water) to eliminate any psyllid eggs or nymphs present. As a control treatment, the same methodology was used for rearing healthy psyllids on healthy *C. × sinensis* ‘Valencia’ grafted on ‘Rangpur’ lime.

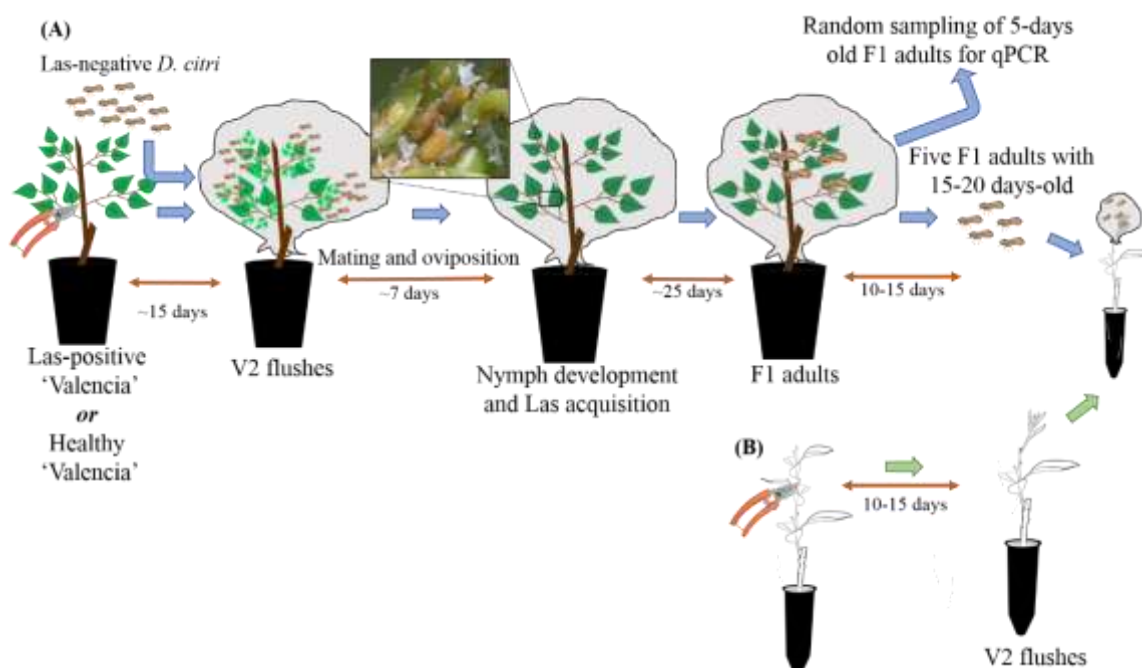


Figure 1. Experimental procedures for '*Candidatus Liberibacter asiaticus*' transmission by *Diaphorina citri*. The first sequence (A), indicated with the blue arrows, shows how infected or healthy *D. citri* were obtained. The second sequence (B), indicated with the green arrows, demonstrates how plants were prepared to be challenged.

2.3 Evaluation of Las multiplication and sampling procedure

Before confining the psyllids on the new flushes, three plants per genotype were randomly selected as controls without the contact with the insect (called here as *prior*). After 48 h of IAP and insect removal, 3 plants per genotype and treatment (exposed to Las-positive insects vs. healthy insects) were randomly detached from Time 0 (day of psyllids removal), 10, 20, 30, and 60 days after it (Figure 2). The entire detached flushes were immediately frozen in liquid nitrogen, triturated using a Tube Mill 100 control (IKA, Staufen, Germany) and designed for DNA and RNA extraction for qPCR and RNA-Seq, respectively. After data analysis, three more biological replicates from Time 10 of *C. × sinensis* and *B. koenigii* were destined for RNA extraction to validate the results observed. Moreover, data of length from the collected flushes were analysed and a one way ANOVA performed to determine if there were significant differences between the length of *C. × sinensis* and *B. koenigii* flushes after being exposed to Las+ insects vs. healthy insects on each flush detachment time point.

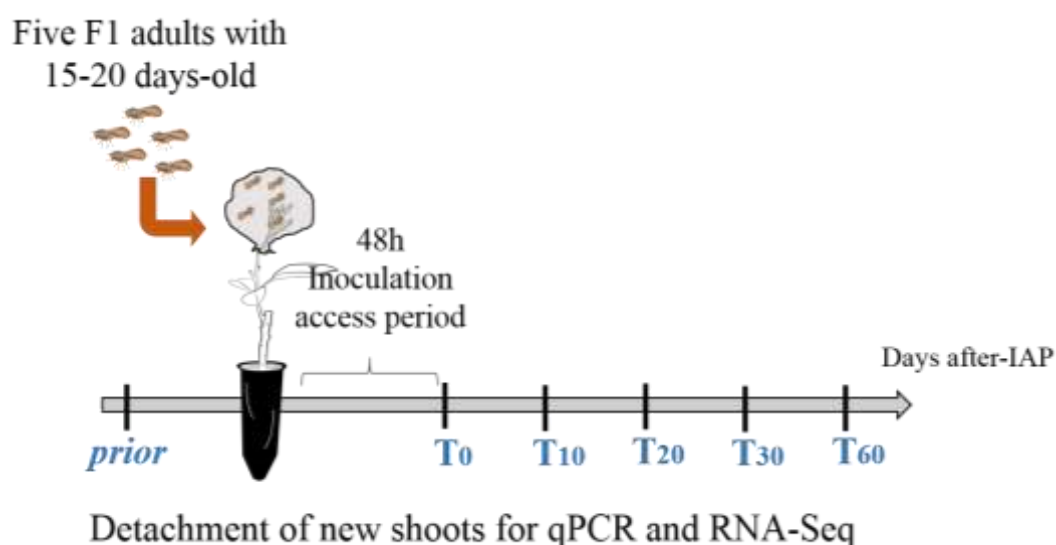


Figure 2. Experimental procedures for ‘*Candidatus Liberibacter asiaticus*’ inoculation by *Diaphorina citri* and evaluation scheme. After 48 hours of the inoculation access period, insects were removed and frozen. In the indicated selected times, flushes from *C. × sinensis*, *M. paniculata*, and *B. koenigii* were detached and immediately frozen for DNA and RNA extraction. The same approach was taken with the healthy insect treatment.

2.4 DNA Extraction and qPCR

Tissue-triturated frozen samples (0.05-0.1 g) were added to a 2 mL microtube and subjected to total DNA extraction. For psyllids DNA extraction, one insect and a 3 mm steel bead were used in a 1.5 mL microtube. Total DNA was extracted using the cetyltrimethylammonium bromide (CTAB) extraction buffer described by Murray and Thompson 1980, with adaptations mentioned by Alves et al. (2021a). After DNA precipitation with 0.6 V of isopropanol, DNA was washed twice with 70% ethanol, and resuspended in 50 μ L of Milli-Q® water. DNA quality was checked using a NanoDrop (Thermo Fisher Scientific, Massachusetts, EUA) (Desjardins and Conklin, 2010).

Real-time polymerase chain reactions (qPCR) for the identification of the 16S rRNA of Las was done with 1 μ L of total DNA (100 ng/ μ L), TaqMan® PCR Master Mix (1x) (Invitrogen, Carlsbad, CA, United States), and HLBas primer/probe (0.5 μ M/0.2 μ M) in a StepOnePlus thermocycler (Applied Biosystems, California, USA) (Li et al., 2006). A positive and a negative sample were included as quality controls during DNA extractions and qPCR assays. As an internal control, the mitochondrial gene

cytochrome oxidase (COX) and an insect *wingless* (Wg) gene region were used for the quality of plants and psyllid DNA, respectively (Li et al., 2006; Manjunath et al., 2008).

For Las quantification, the linear relationship between the cycle threshold (Ct) and the 16S rRNA log described by Lopes et al., 2013 was used. Ct values > 34.0, produce variable results (Lopes et al., 2013). Therefore, samples were considered Las-positive when the qPCR was lower than 34.0, suspected to be positive when Ct values were between 34.0 and 36.0, and negative when Ct > 36 and/or when undetermined.

2.5 RNA Extraction, Sequencing, and Data analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. A NanoDrop Spectrophotometer (Thermo Fisher Scientific, Massachusetts, EUA) and gel electrophoresis were used to assess RNA quality and quantity. RNA quality was further assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Waldbronn, Germany). Two µg of total RNA per sample was used for library generation using the Truseq stranded total RNA Library Kit plus Ribo-Zero and NEBNext kits for ribosomal RNA depletion. The Illumina NovaSeq6000 were used on a paired-end sequencing of 150pb. All procedures were performed according to Illumina's protocols. After quality trimming of raw reads and adapter removal with the command-line tool cutadapt, quality analysis of clean reads was performed using FastQC. Cleaned read pairs longer than 20 nucleotides were then mapped to a composite genome containing the nuclear and organelles genome assembly of *Murraya paniculata*, recently sequenced and annotated by the CREA in Italy (*unpublished*) and *C. × sinensis* (JJOQ00000000.1), plus the published genome of the JXGC strain of '*Candidatus Liberibacter asiaticus*' (Darolt et al., 2021; Accession NZ_CP019958) using STAR (Dobin et al., 2013), allowing up to 20 mismatches. The number of read counts mapped to one and only one gene of the composite genome (uniquely mapped) were obtained using STAR, and differential expression analysis using DESeq2 (Love et al, 2014). DEGs data were adjusted using *q* value (FDR) ≤ 0.05). Single and Gene Set Enrichment Analysis (ORA, GSEA) were performed using Blast2GO (<https://www.blast2go.com/>). Venn diagrams were obtained using the Bioinformatics & Evolutionary Genomics

(<https://bioinformatics.psb.ugent.be/webtools/Venn/>). The list of representative enriched GO (Gene Ontology) terms were analysed using Panther tools (Mi et al., 2013; www.pantherdb.org) and summarized for visualization with REVIGO (Supek et al., 2011; <http://revigo.irb.hr/>).

3. RESULTS AND DISCUSSION

3.1 Las transmission

At Time 0 (the day of psyllid removal after 48 h of IAP), the three *C. × sinensis* plants had Ct values lower than 34.0. The average Las titer in the new shoot at Time 0 was 2.22 ± 0.05 log cell/g of tissue. After this, there was an initial decrease in Las titers soon after psyllid removal until the 20th day, up to ~ 1.0 log cell/g of tissue. Subsequently, Las multiplied exponentially, presenting at the 30th day 3.97 ± 0.23 log cell/g of tissue (Figure 3), increasing to less than 1 log until the 60th day (4.82 ± 0.20 log cell/g of tissue), representing the stationary phase.

In *M. paniculata*, at Time 0, the three replicates showed an average titer of 2.82 ± 0.23 log cell/g of tissue. The initial decrease in Las titer up to the 20th day was less severe than that in *C. × sinensis*, with an average titer of ~ 1.5 log cell/g of tissue. The subsequent exponential increase was also less pronounced, and Las reached a maximum titer between the 30th and 60th days, with a population similar to that of the day of psyllid removal (~ 3 log cell/g of tissue) (Figure 3).

On the other hand, in *B. koenigii*, at Time 0, the three plants showed Ct values closer to or more than the limit of 34.0. The average of the Las titer in the new shoot at Time 0 was 1.68 ± 0.18 log cells/g of tissue. However, after this, a fast reduction in Las titer following psyllid removal was observed, reaching undetectable levels from the 10th day onward (Figure 3).

In the three species under study, Las growing dynamic followed the specifications made by Alves et al. (2021a), validating the selected key-time points sampled for the RNA-seq study. A representative figure of the aspect of plants and their respective new flushes used for *D. citri* confinement and detached at each time of evaluation in both treatments are demonstrated in Figure 4.

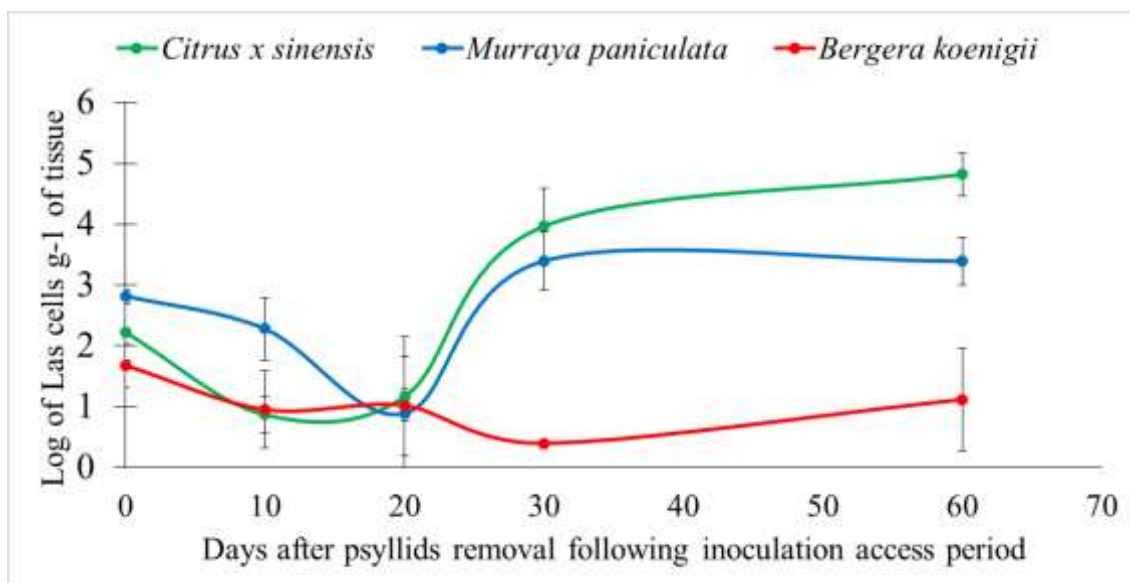


Figure 3. Evolution of the ‘*Candidatus Liberibacter asiaticus*’ population dynamics over time in new shoots of *Citrus × sinensis*, *Murraya paniculata*, and *Bergera koenigii*, following a 48 h period of Las-exposed *Diaphorina citri* confinement.

(A)





Figure 4. A representative aspect of (A) *Citrus × sinensis*, (B) *Murraya paniculata*, and (C) *Bergera koenigii* at each time of evaluation in both treatments: exposed to Las-positive insects vs. exposed to Healthy insects.

M. paniculata and *B. koenigii* are both considered as susceptible to *D. citri* (Beloti et al., 2017; Tomaseto et al., 2019) being the morphological, developmental, and chemical differences among the three species not influencing the ability of *D. citri* to access sieve elements through their stylets. After being released into the phloem, Las titer first dropped as observed by Alves et al. (2021a). This decline has been related to a dilution effect due to active flush growing after pruning, the time needed to adapt to the new phloem environment and be able to sequester metabolites from the

host and survive and/or as a consequence of defense responses induced by plant cells.

3.2 Transcriptomic analysis

Ninety libraries from the three genotypes, *C. × sinensis*, *M. paniculata* and *B. koenigii* were generated, which include the 6 times of evaluation (*prior*, 0, 10, 20, 30, and 60 days after psyllids removal) and the two treatments (exposed to Las+ vs. healthy insects).

Murraya paniculata and *Bergera koenigii* are relatives of *Citrus* from the Aurantioideae subfamily, which (presumably) have low genome diversity between them. To facilitate further downstream analysis and interpretation, the three transcriptome projects should be mapped against the same reference genome. The reference genome of *Citrus sinensis* is available (JJQ000000000.1), as well as the one from *Murraya paniculata*, recently sequenced and annotated by the CREA in Italy (*unpublished*). We then generated two composite genomes, including the nuclear (SWO.v1.0 <http://citrus.hzau.edu.cn/download.php>), chloroplast (accession NC_008334) and mitochondrial (accession NC_037463) genomes, plus the genome sequence of *Candidatus Liberibacter asiaticus*. Heterologous mapping using relaxed mapping parameters is a widely used strategy to map transcriptomes when a close relative is the only available genome, and allows high percentages of unequivocally mapped reads. We mapped all reads against both the *C. × sinensis* and the *Murraya paniculata* genomes, and read mapping rates for all samples in both genotypes were compared. Based on this, and within the limits of this similarity assessment based on global read mapping percentages, the two genomes do not appear dramatically influence in such a way as to hamper RNA-seq approaches substantially. After trimming, an average of 22 million and 33 million uniquely-mapped reads were obtained after mapping against the *Citrus* and *Murraya* composite genomes, respectively. Given the higher mapping percentage obtained with the *Murraya* genome, downstream analysis was performed using the *Murraya* mapping data.

For all the three species, some reads were mapped to the *Liberibacter* genome only in infected samples. Unfortunately, the low coverage obtained did not allowed the use of these data to get a glimpse of the expression patterns of the bacteria in every

host. The Dual-seq methodology provides the simultaneous detection of the response of both the host and the pathogen (Westermann et al., 2022). However, until now, transcriptomic analysis of the genes from the bacterium *in planta* has only been reported with success when using an enrichment protocol for Las isolation from midribs leaves (Francesco et al., 2022).

3.2.1 Constitutive differences between *C. × sinensis*, *M. paniculata*, and *B. koenigii* new shoots

Morphological, developmental, and chemical differences among the three Rutaceae species have been described (Beloti et al., 2018; Cifuentes-Arenas et al., 2019; Tomaseto et al., 2019). Although they did not apparently influence the ability of psyllids to access sieve elements and release the bacterium into the plant (Alves et al., 2021), the possibility of a different response by the bacteria once it is into the plant due to biochemical and molecular differences between these three hosts, cannot be neglected. With this aim, we first compared transcriptomes of *Citrus*, *Murraya*, and *Bergera* in the absence of *Diaphorina citri* using samples of new shoots collected before the onset of the experiment (called as *prior*). Despite having similar developmental stages, new shoots of the three species present a dramatically different transcriptome, as shown by the principal component analysis in Figure 5. Moreover, despite being a confined experiment, a higher variability was observed in *M. paniculata* replicates.

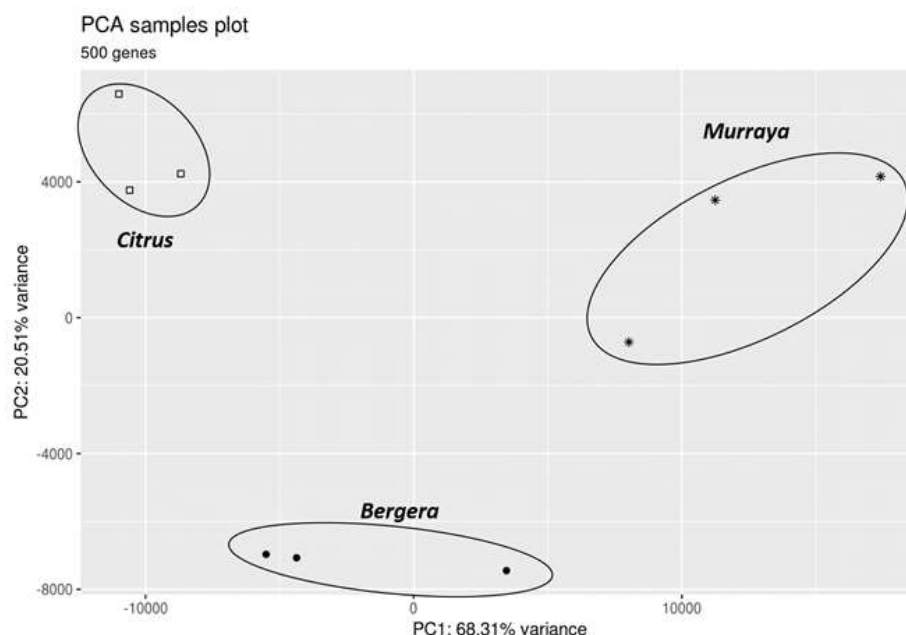


Figure 5. Principal component analysis (PCA) of *Citrus × sinensis*, *Murraya paniculata*, and *Bergera koenigii* new shoots collected before the onset of the experiment.

When comparing the new shoots collected before the onset of the experiment, 7600, 8430, and 3561 DEGs were obtained from “*Citrus* vs. *Bergera*”, “*Citrus* vs. *Murraya*”, and “*Murraya* vs. *Bergera*”, respectively (Supplementary Table 1, 2 and 3). Venn diagrams from DEGs “*Citrus* vs *Bergera*” and “*Citrus* vs *Murraya*” are shown in Figure 6. From those analyzed intersections, unique answers were observed, enabling the assumption of some important aspects.

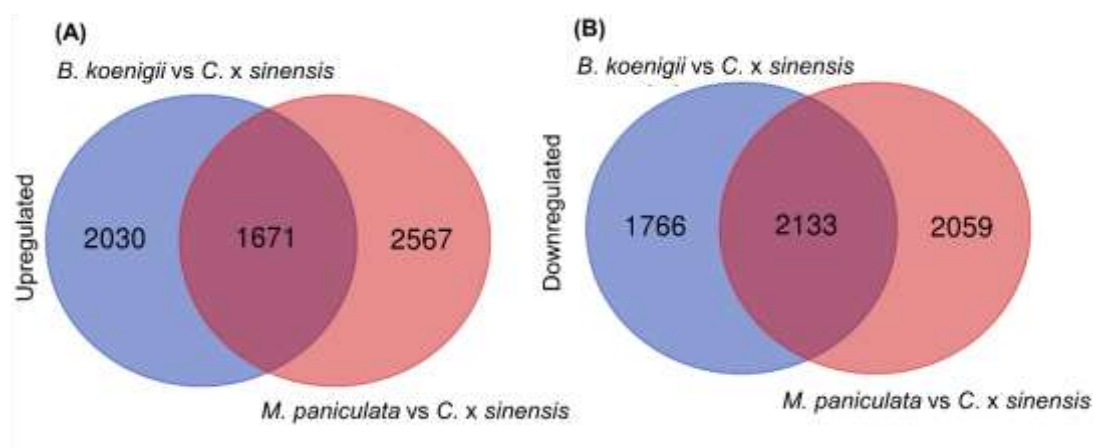


Figure 6. Venn diagram from DEGs (A) up-regulated on “*Citrus* vs. *Bergera*” when compared with the up-regulated on “*Citrus* vs. *Murraya*” and (B) down-regulated on “*Citrus* vs. *Bergera*” when compared with the down-regulated on “*Citrus* vs. *Murraya*”.

As an example, it is possible to assume that *C. × sinensis* might express something beneficial for Las that is not expressed in *M. paniculata* nor in *B. koenigii*. These genes would be more in the *C. × sinensis* than *B. koenigii* and/or in *C. × sinensis* than in the *M. paniculata* list of genes. Similarly, we could assume that *B. koenigii* and/or *M. paniculata* express something detrimental to Las infection, which would be less present in *C. × sinensis* than *B. koenigii* and/or in *C. × sinensis* than *M. paniculata* list of genes. Given that *B. koenigii* and *M. paniculata* showed a very different transcriptomic profile, the possibility that *B. koenigii* and *M. paniculata* share genetic components is less likely, although all possibilities could be contemplated.

In general aspects, it is interesting to note that *C. × sinensis* has a more active metabolism of monocarboxylic acids than *B. koenigii* and *M. paniculata* (Supplementary Table 4 and Table 5; Figure 7.A and 7.B), organic acids that are necessary for Las growing. Las has a reduced genome size and is dependent on the phloem sap as a carbon source (Huang et al., 2020), the pyruvate and malate, for example, are essentially for Las (Merfa et al., 2019). In this study, the gene Malate synthase was 9 times more expressed in *C. × sinensis* than in *B. koenigii* and *M. paniculata*. As observed by Alves et al. (2021a) and reinforced in this study, once released into the phloem, is possible that Las needs a time for Las to adapt to this new environment and use its carbon source (Fagen et al., 2014). The more active metabolism of monocarboxylic acids in *C. × sinensis* than *B. koenigii* and *M. paniculata* suggest that the phloem does not provide the necessary metabolites for Las as observed by Killiny (2016) on comparative metabolomics study of the phloem sap of those species. This kind of observations has already been reported in others pathosystems as spiroplasmas (André et al., 2005).

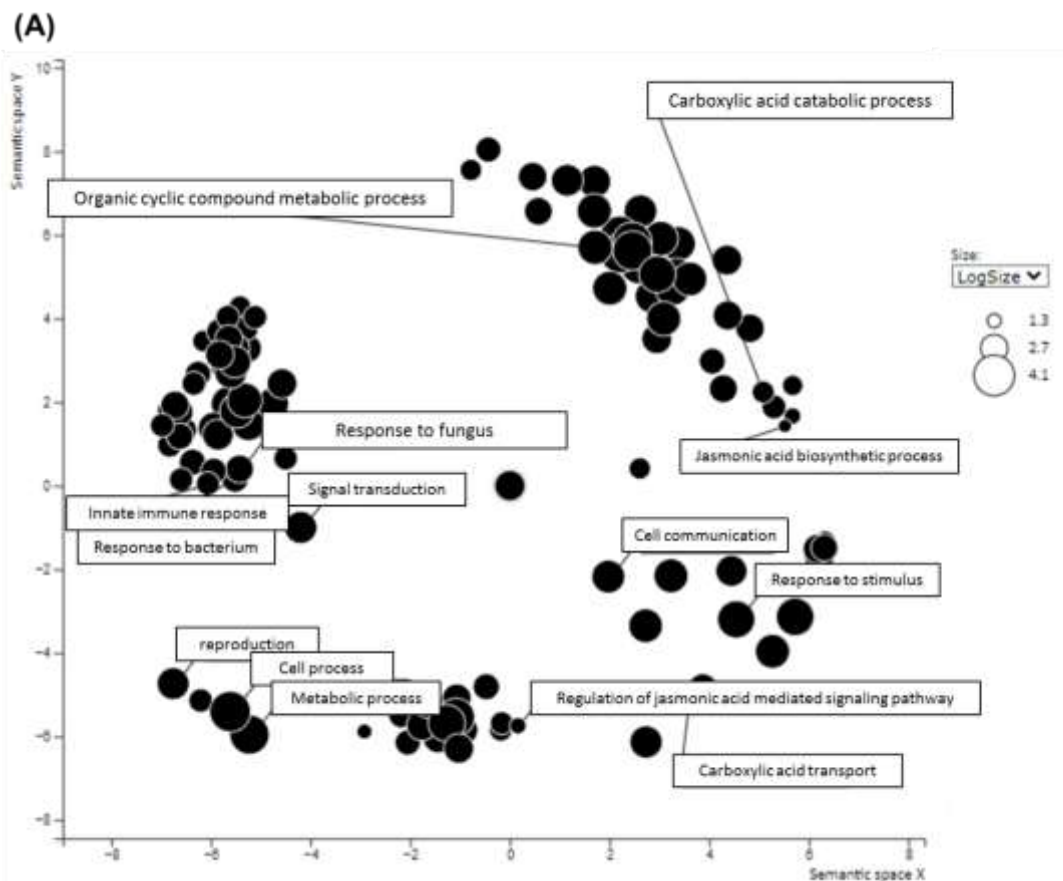
Another interesting aspect to remark on is that, despite being the susceptible genotype, these analyses showed that *C. × sinensis* has more expression of genes from the synthesis and pathway of jasmonic and salicylic acid than *B. koenigii* and *M. paniculata* (Supplementary Table 4 and Table 5; Figure 7.A and 7.B). This observation indicates that a higher basal expression of these genes does not necessarily imply greater resistance if the method of entry of the bacterium into the plant is through a vector that passively introduces the pathogen into the phloem as in Las pathosystem (Jiang et al., 2019; Haung et al., 2020).

C. × sinensis showed more genes related to leaf senescence than *M. paniculata* (Supplementary Table 5; Figure 7.B), which could be indicative of more amino acids being loaded into the phloem. During senescence, leaf protein undergoes proteolysis and the amino acids released are remobilized from source leaves to sink tissues as young leaves, young roots, and seeds by the phloem (Masclaux-Daubresse et al., 2010). A higher number of genes from leaf senescence, nitrate transporters, and amino acids can also indicate that *C. × sinensis* has a richer phloem than *M. paniculata* which is also in agreement with the metabolomic study from Killiny (2016). Furthermore, *M. paniculata* and *B. koenigii* presented a higher expression of genes related to amino acids activated by t-RNA than *C. × sinensis* (Supplementary Table 6 and 7; Figure 7.C and 7.D), which could mean that more amino acids are used for translation, being a lower amount available for Las metabolism (Banerjee et al., 2010).

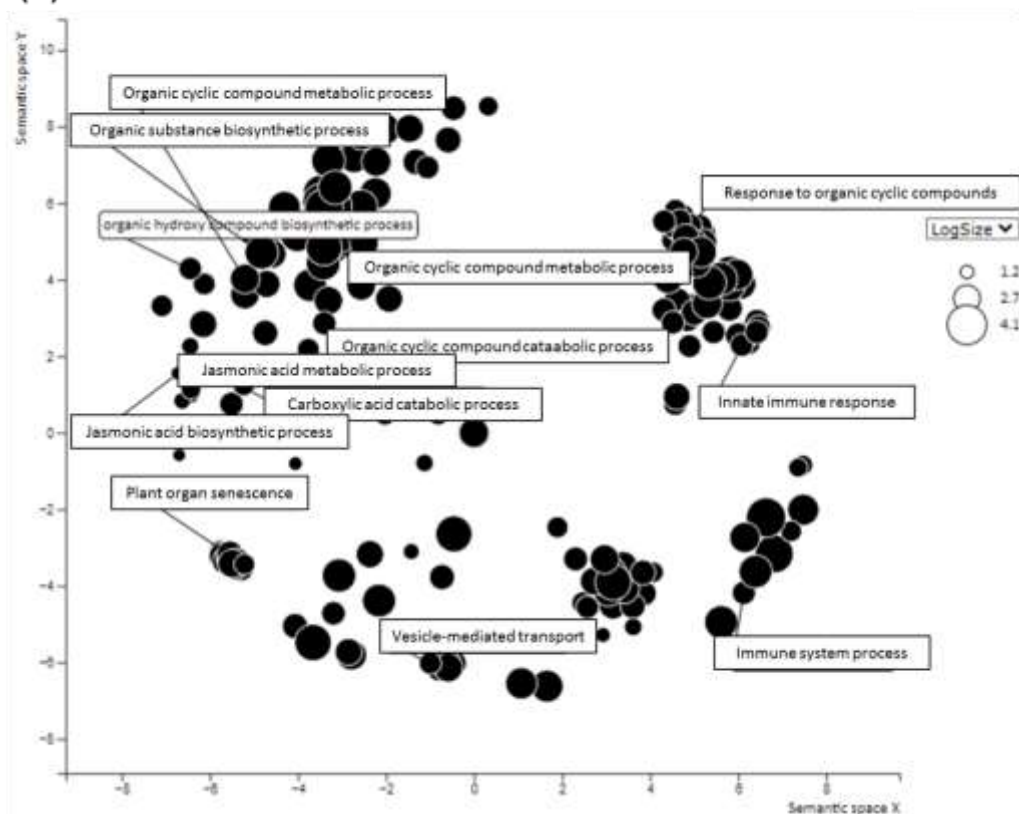
In general, Las infected sweet orange leaves show excessive accumulation of starch, a consequence of the phloem dysfunction after callose plugging of the sieve pores (Fan et al., 2013; Achor et al., 2020; Bové, 2006), even in varieties mentioned as less susceptible to HLB (Fan et al., 2012; Deng et al., 2019). However, when compared with susceptible genotypes, lower effects on starch were observed (Curtolo et al., 2020; Fan et al., 2012; Wang et al., 2016; Yu et al., 2017). In addition, it is also known that this plugging of the sieve pores is not efficient in restricting the bacterium movement (Achor et al., 2020; Ammar et al., 2019). In our study, a higher starch metabolism was observed in *B. koenigii* compared with *C. × sinensis* (Supplementary Table 7; Figure 7.D). Most related up-regulated genes were from starch/glucan/cellular polysaccharide biosynthesis and involved in the regulation of starch biosynthetic process. How this constitutive observation can influence *B. koenigii* less susceptibility to Las remain unknown.

Another observation is that *M. paniculata* and *B. koenigii* showed a higher expression of genes related to increased photosynthesis (Supplementary Table 6 and 7; Figure 7.C and 7.D). A decline in photosynthetic rates following attack by insects or pathogens is well-reported in the literature (Welter, 1989; Schenk et al., 2000; Sasaki et al., 2001; Sasaki-Sekimoto et al., 2005; Uppalapati et al., 2005; Zou et al., 2005; Aldea et al., 2006; Major and Constabel 2006; Shimizu et al., 2007; Vogel, Kroymann and Mitchell-Olds, 2007; Yang et al., 2007; Bozso et al., 2009; Nabity et al., 2009). If

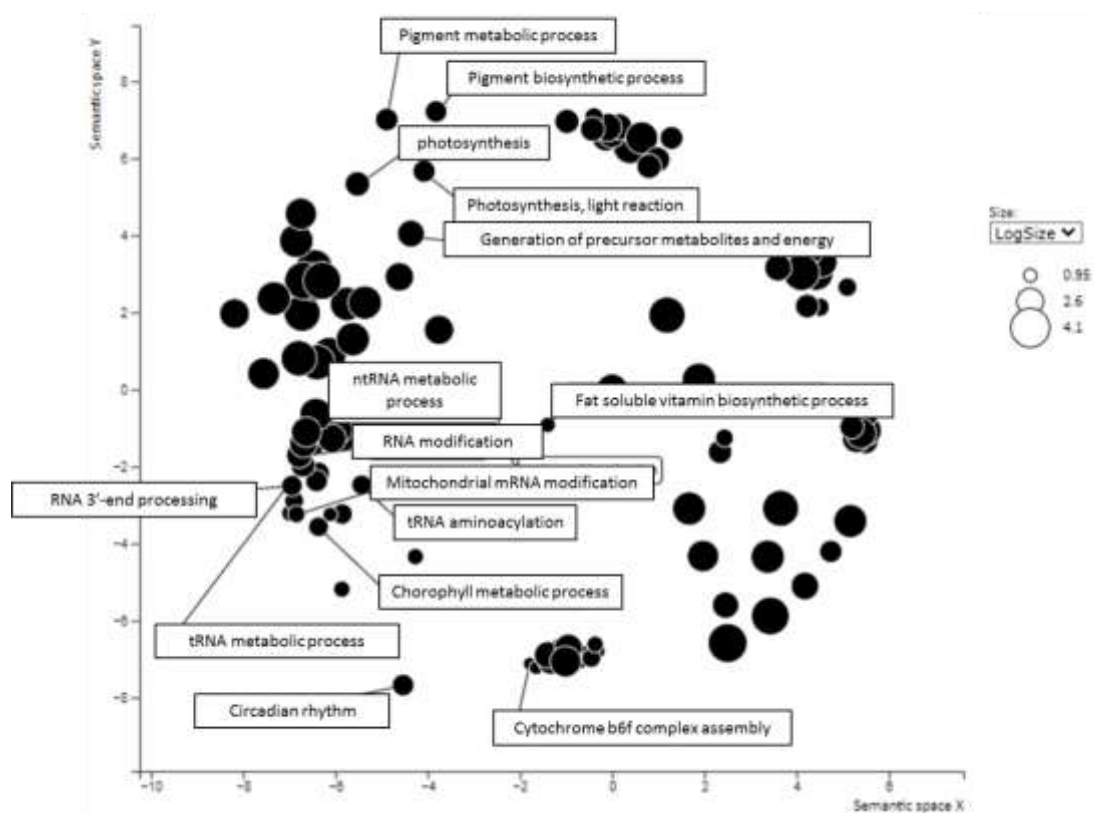
these constitutive higher rates of photosynthesis can be related to the lower susceptibility of *M. paniculata* and *B. koenigii* to Las became a question to be investigated.



(B)



(C)



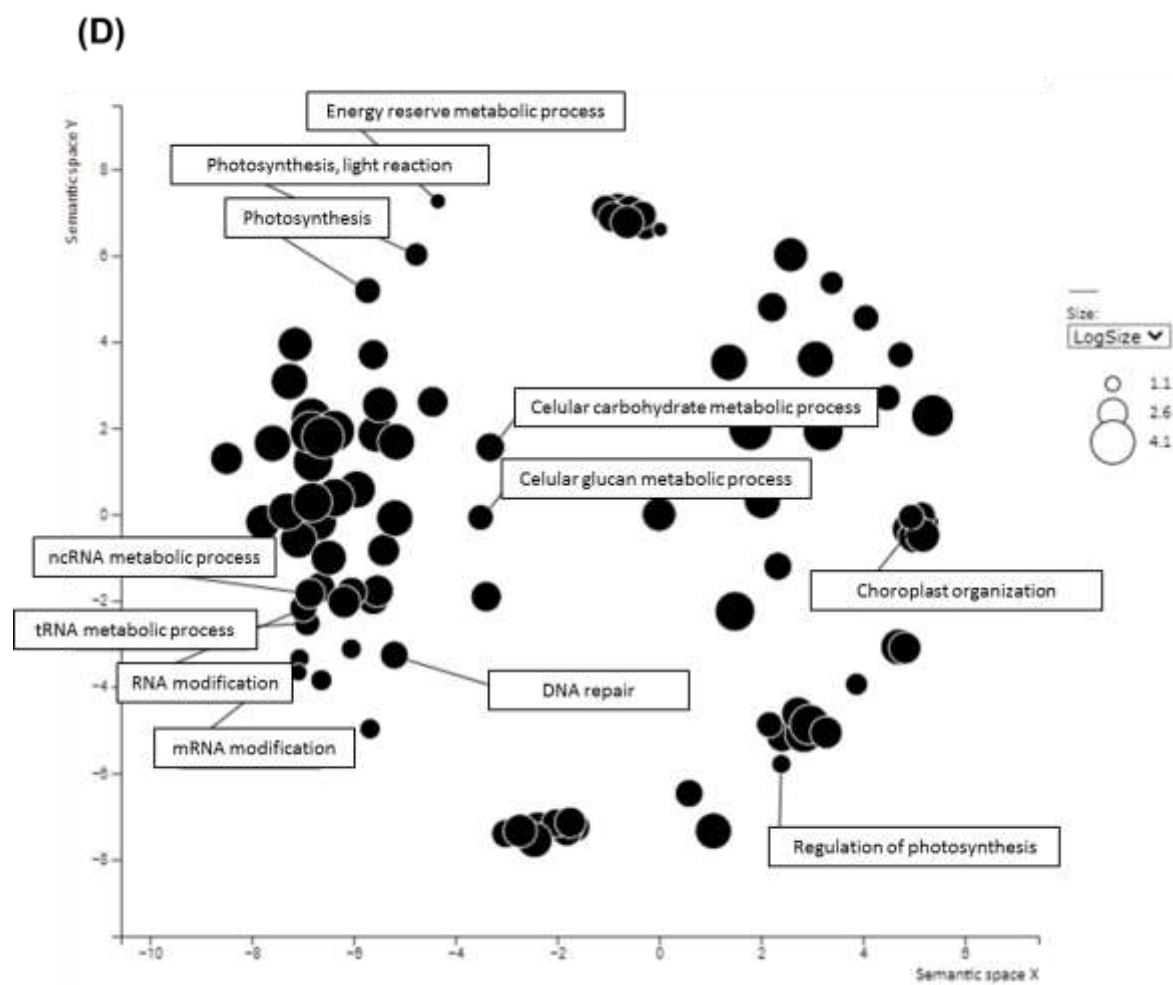


Figure 7. Gene Ontology summarized for visualization with REVIGO after removing redundant GO terms of constitutive differences between *C. × sinensis*, *M. paniculata*, and *B. koenigii* new shoots after comparisons between (A) *C. × sinensis* and *B. koenigii*; (B) *C. × sinensis* and *M. paniculata*; (C) *M. paniculata* and *C. × sinensis*; and (D) *B. koenigii* and *C. × sinensis*.

3.2.2 Differential expression between *C. × sinensis*, *M. paniculata*, and *B. koenigii* inoculated with healthy or infective-psyllids.

Aiming to gain more information on Las interaction at the early stage of infection, we studied at the transcriptomic level three genotypes with different responses to the HLB-bacterium as soon after being exposed to infective vs. healthy psyllids for 48 h (Time 0, day of psyllids remove), and 10, 20, 30 and 60 days after removing the insects.

The principal component analysis (PCA) of *C. × sinensis*, *M. paniculata*, and *B. koenigii* (Figure 8) showed that the samples tended to be clustered more according to the morphological stage of the flush than according to the treatments under study

(exposed to Las+ vs. healthy insects). This observation suggests that there is not a big difference in the transcriptomes of the three species by being attacked by healthy vs. Las+ psyllids. These results also show that later sampling times exhibit higher variability between the biological replicates, especially at late times (30 and 60), and more evidenced in *M. paniculata*, making interpretation of results very difficult. A lower genetic variability was expected as *C. × sinensis* plants were propagated by grafting, an asexual technic. In the case of *M. paniculata* and *B. koenigii*, propagation was done by seed as they are not compatible with *Citrus* for grafting. As monoembryonic species and less compatible for pollination with other Aurantioideae species (Swingle and Reece, 1967), a higher homozygous level was expected even existing a diversity of *M. paniculata* and *B. koenigii* described in the literature (Ciampi et al., 2013; Nguyen et al., 2019).

To confirm the observation in the PCAs, when comparing the DEGs obtained from the comparison done at each time point between the treatments exposed to Las+ insects vs. healthy insects, a low number of differentially expressed genes (Figure 9) were observed in all comparisons in the three species. The HLB-susceptible genotype *C. × sinensis* ‘Valencia’ showed only 121, 73, 4, 9, and 31 DEGs in the 0, 10, 20, 30, and 60 days, respectively (Figure 9.A; Supplementary Table 8) compared with 8, 46, 70, 87 and 118 from *M. paniculata* (Figure 9.B; Supplementary Table 9) and 114, 4, 35, 13, and 713 from *B. koenigii* (Figure 9.C; Supplementary Table 10). Unfortunately, given the use of only three replicates, we cannot speculate on the biological significance of these expression differences, which could be reduced (or increased) if the number of replicates increases. In addition, this conclusion is supported by the fact that no consistent GO categories are enriched among the few DEGs genes observed in all cases.

These results indicate that the inoculation of Las on the phloem sap by *D. citri* on *Citrus* and related species avoids triggering a clear transcriptome response by the hosts. It is important to remember that the particular way of *D. citri* passively introduce Las directly into the phloem can enable it to avoid triggering the activation of some otherwise potentially effective plant defensive machinery. Similar observations have been made when studying aphids (Zhu-Salzman et al., 2004; Couldridge et al., 2007). Despite that Las genome is able to encode genes for the presence of flagella

and a type IV pili (Kearns, 2010), low expression of those genes in plant host has been reported (Andrade et al. 2019; Andrade and Wang 2019), besides that, Las lacks genes involved in type 3 and most part of type 2 and 4 secretion systems (Fagen et al., 2014). Taking together, it is possible to infer that Las does not induce a typical positive plant-pathogen interaction, being passively released in the phloem by the insects, and only being able to stay there because it is a compatible environment with enough and available source of nutrients to survive, being its composition a limiting aspect for Las establishment in *M. paniculata* and *B. koenigii* as discussed before.

Some transcriptomics studies done on early infection after Las graft-inoculation also did not observe a high number of DEGs nor did gene ontology (GO) significantly over-represented among the DEGs (Chin et al., 2020; Ramsey et al., 2020). It is important to mention that inoculation by grafting is an aggressive methodology of inoculation where it is necessary to damage the tissue for budwood connection. In addition, the load of bacterium injection is higher when using this not natural methodology (Lopes et al., 2009; Lopes and Cifuentes-Arenas, 2021). In contra part, almost one hundred DEGs were observed only 48 hours of psyllids feeding on *in vitro* sweet orange flushes (Wei et al., 2021). However, comparisons were made between plants exposed to psyllids raised on Las+ sweet orange plants vs. psyllids raised on curry plants. If the feeding habits can or can not influence the fitness of the insects is something still unknown. Moreover, *in vitro* plants were not rooted. Instead, flushes from budwood sticks on Murashige and Skoog (MS) culture media were used and contamination of the culture media mentioned by the authors, aspects that may influence the results obtained.

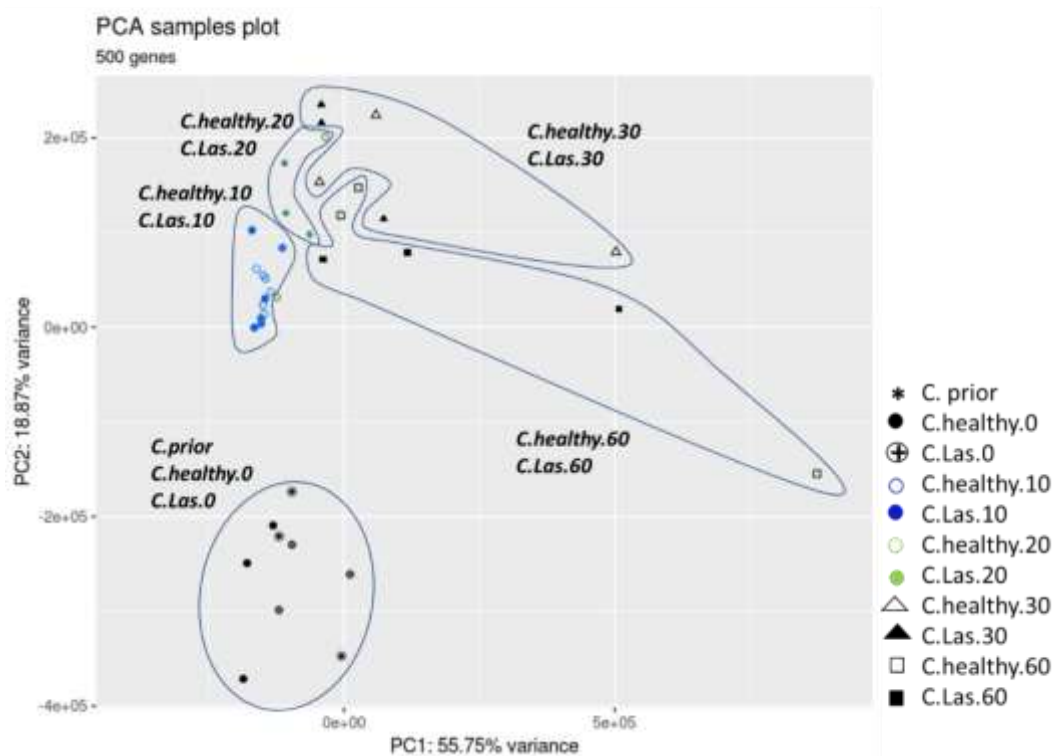
However, an interesting observation was done on the samples on Time 10 and after this, six more samples from *B. koenigii* and *C. × sinensis* at Time 10, 3 from shoots exposed to healthy psyllids and 3 against Las+ psyllids, were sequenced to reinforce it. The PCA plots include, then, six replicates for these time points. Whereas in *C. × sinensis*, the 12 samples at Time 10 cluster together (Figure 8.A), indicating that there are no significant differences between samples coming from plants exposed to healthy or Las+ psyllids, in *B. koenigii* (Figure 8.B), the principal component analysis (PCA) showed that only two of the six plants from Time 10 exposed to Las+ insects (B.Las.10.2 and B.Las.10.4) were grouped with the six plants from the healthy

treatment of the Time 10, whereas the other four plants from Time 10 exposed to Las+ insects grouped with the samples from Time 0 (exposed to Las+ insects and healthy insects).

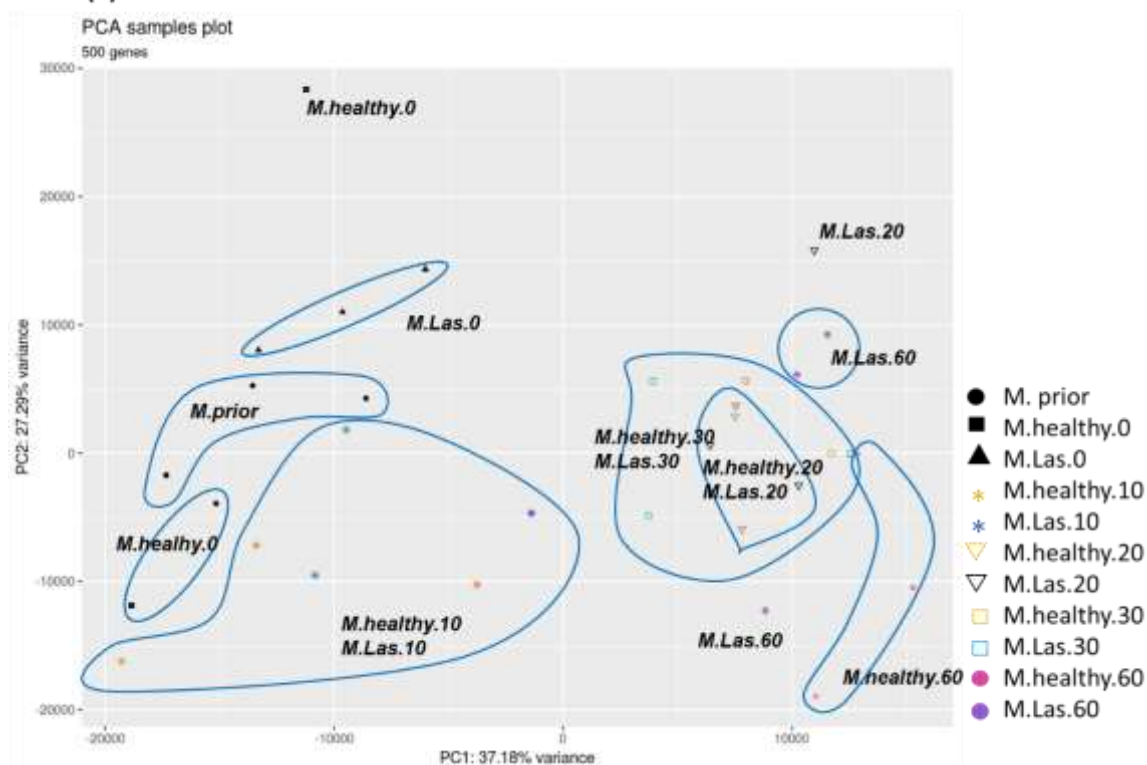
To explain the inconsistency between the six challenged samples of *B. koenigii*, we first discarded the possibility of a mistake during the sampling. Flushes collected at Time 10 have a very different morphology from flush in Time 0 (Figure 4.C) (there are 10 days of interval between them). Because of that, the possibility of samples from Time 10 being in fact from Time 0 is totally discarded.

Once Las is delivered in the phloem by *D. citri*, an initial decrease in Las titers soon after psyllid removal is observed. Later on, in the case of *B. koenigii*, Las reaches undetectable levels from the 10th day onward. Because of it, we believe that the two samples (B.Las.10.2 and B.Las.10.4) that grouped with the six plants from the healthy treatment of the Time 10 escaped from Las inoculation, which means, *D. citri* was not capable of inoculate Las and establish an infection. Escaping from Las inoculation is not surprising when using graft inoculation or the psyllids (Lopes et al., 2009; Albrecht et al., 2014; Lopes and Cifuentes-Arenas, 2021). Furthermore, times 0, 10, and 20 are difficult to be analyzed by qPCR for Las detection once the Ct is closer to the detection limit, as it can be observed on Figure 3 and by Alves et al. (2021a). PCA analysis from other studies with *Citrus*, *Poncirus*, and hybrids, also observed variability with the global expression landscape more related to the different genotypes than the analyzed condition (infection by Las) (Curtolo et al., 2020). Taken together, these observations suggest that the four samples from Time 10 exposed to Las+ insects grouping together are real infected samples, and the two samples grouping together with the ones from Time 10 exposed to healthy insects are, in fact, escapes and should not be considered as exposed to Las+ insects.

(A)



(B)



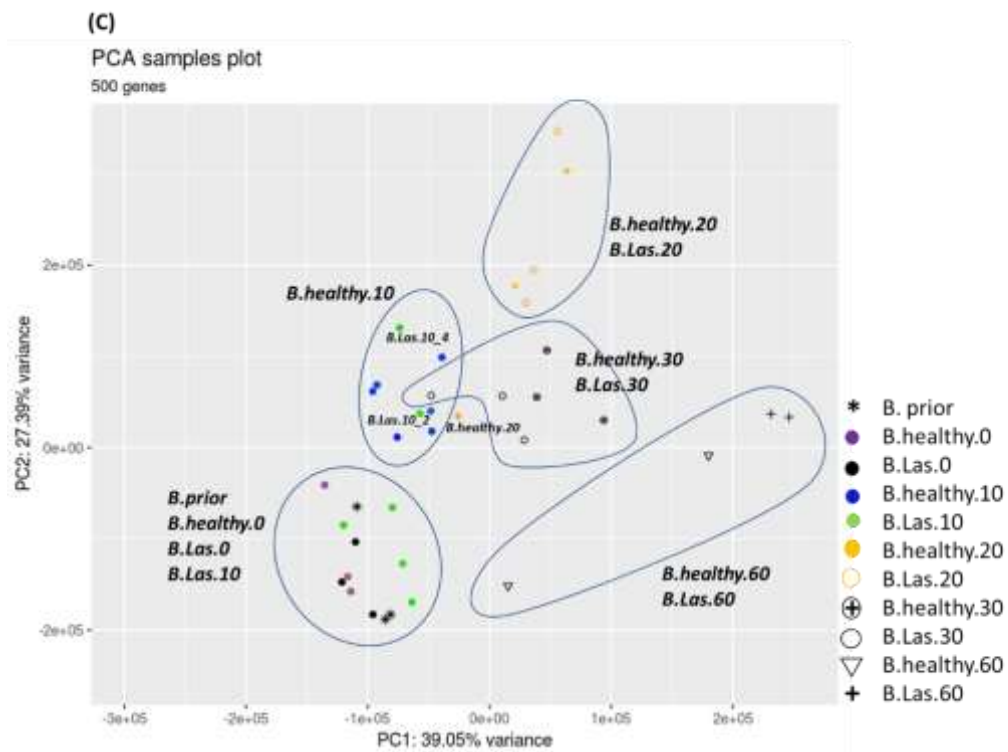


Figure 8. Principal component analysis (PCA) of each of the biological replicates of (A) *Citrus × sinensis*, (B) *Murraya paniculata*, and (C) *Bergera koenigii* at each time of evaluation in both treatments: exposed to Las-positive insects vs. exposed to Healthy insects.

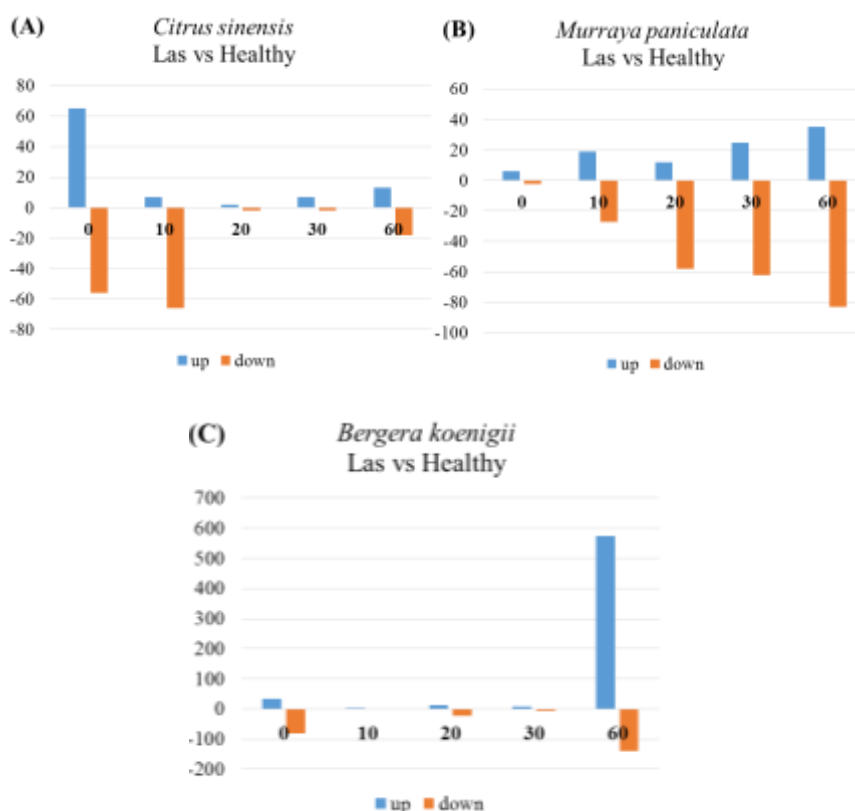


Figure 9. Number of DEGs adjusted using q value (FDR) ≤ 0.05 obtained by the comparison done in each time between the treatments exposed to Las+ insects and healthy insects per genotype.

To investigate the observation from *B. koenigii* in more detail, the comparisons between the *prior* vs. Time 0 and *prior* vs. Time 10 from both treatments exposed to healthy insects and exposed to Las+ insects were investigated to see which genes were differentially expressed. The same was done for the samples of *C. × sinensis* and *M. paniculata*. As shown in Figure 10, while *C. × sinensis* and *M. paniculata* (Supplementary Table 11 and 12) at time 10 induce and repress a very high number of genes in both treatments, in *B. koenigii* (Supplementary Table 13) the healthy samples also induce and repress a high number of genes when compared with the initial moment of the experiment (*prior* time). However, the samples from *B. koenigii* at time 10 exposed to Las+ insects have not undergone massive changes in expression, which suggest that *B. koenigii* largely arrest their gene expression due to infection.

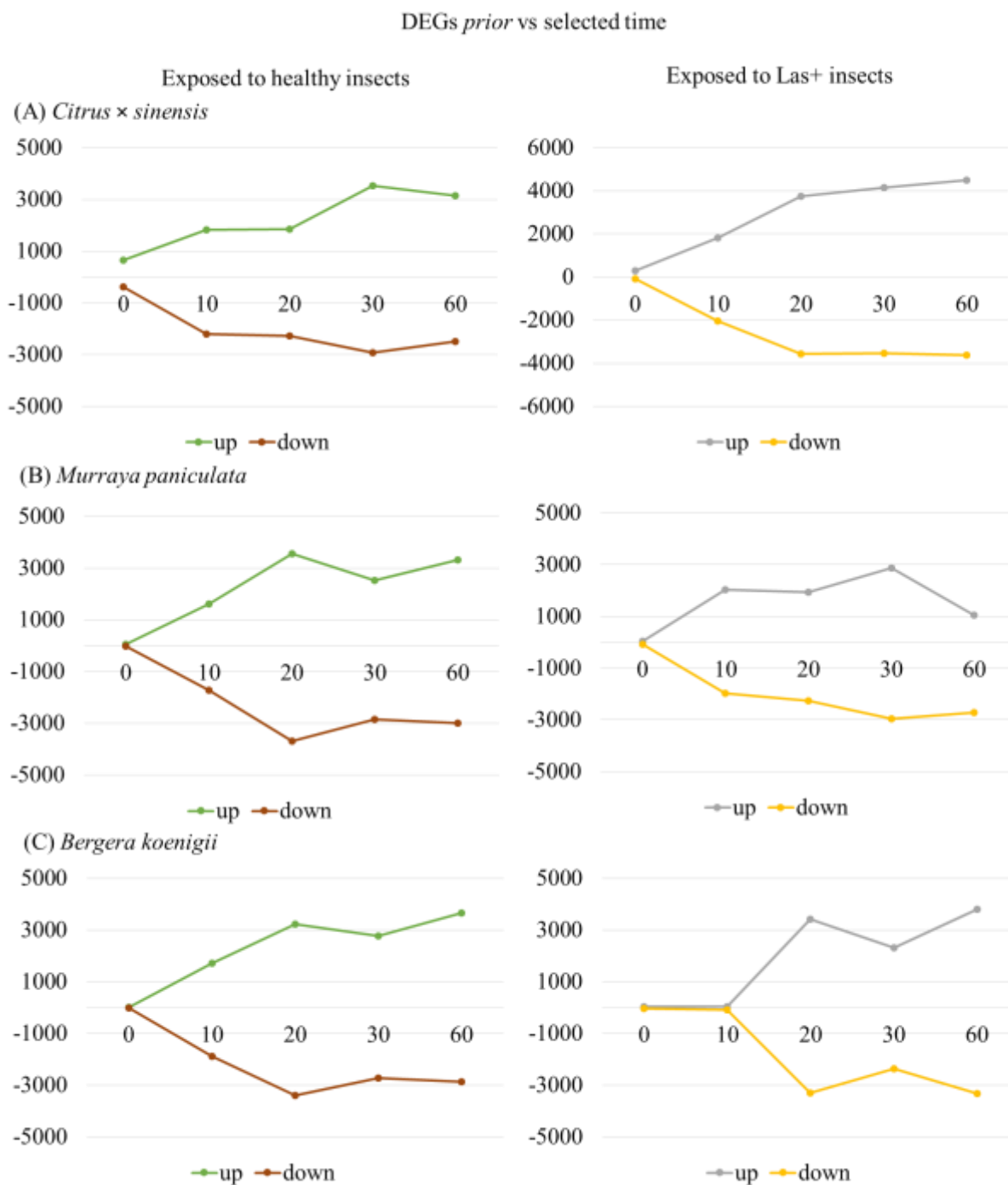


Figure 10. Number of DEGs adjusted using q value (FDR) ≤ 0.05 obtained by the comparison between the time *prior* (without insect) vs. the times 0, 10, 20, 30, and 60 for both treatments studied, exposed to Las+ insects and healthy insects.

To advance the biological significance of this transcriptional arrest, the transcriptomic samples from *B. koenigii* of Time 10 exposed to healthy insects and Las+ insects were compared for differential processes using the GSEA tool, which is

not limited to DEGs genes alone, but to expression trends between the two samples. As shown in Figure 11.A, occurred an increase in photosynthesis and a reduction in translation categories and cell cycle in Time 10 exposed to healthy insects when compared with the *prior*. However, the comparison between healthy vs. Las+ treatments from Time 0 and 10, reveals that genes related to photosynthesis category were enriched when exposed to healthy *D. citri* and the DNA replication category enriched when exposed to Las+ *D. citri* (Figure 11.B).

Aiming to compare these observations with what happens on *C. × sinensis* and *M. paniculata*, we also analyzed the GSEA from Time 0 and 10 of the healthy vs. Las+ comparison. The comparison between Time 0 healthy vs. Time 0 Las+ of *C. × sinensis* (Figure 12.A) and *M. paniculata* (Figure 12.B) showed the same tendency as in *B. koenigii*: genes related to photosynthesis category were much more present in samples exposed to healthy insects being reduced in the Las+ ones. However, contrary to the observed trend in *B. koenigii*, this lower photosynthesis was also persistent until Las+ samples of Time 10 in *M. paniculata* but not in *C. × sinensis*, in which no categories related to photosynthesis, translation, ribosomes, and DNA replication were observed.

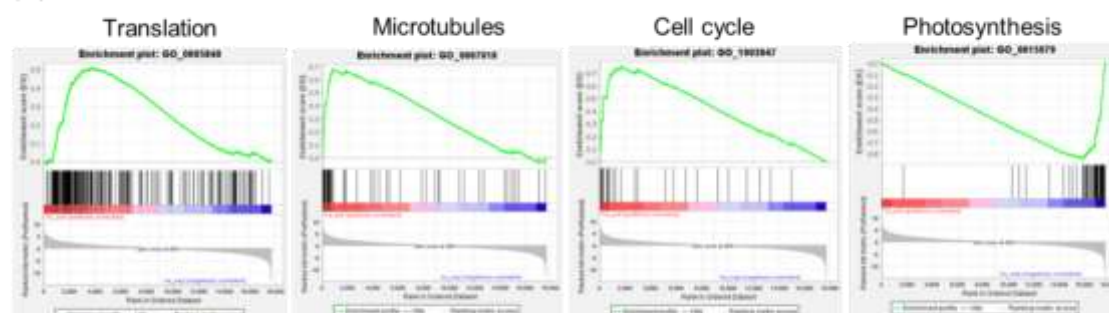
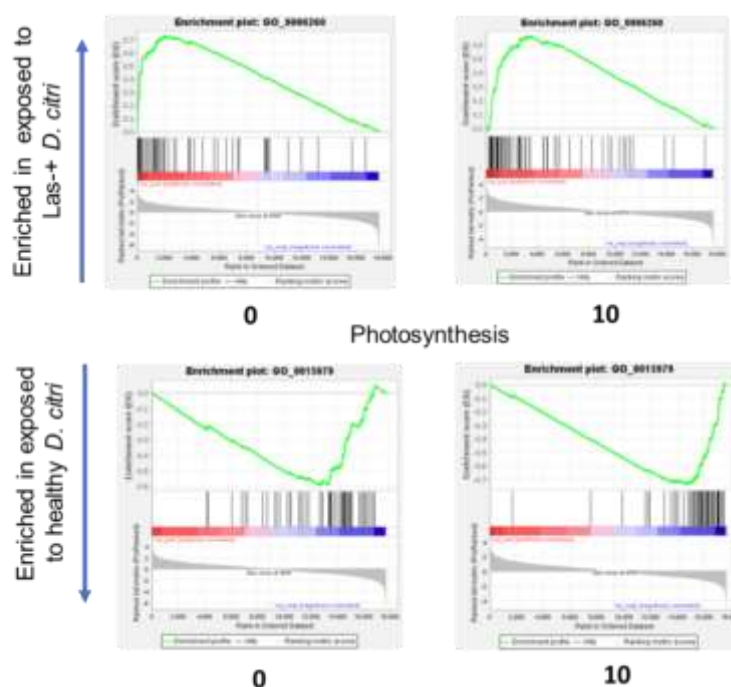
GSEA of *Bergera koenigii* responses to(A) *prior* vs the time 10 of the treatment exposed to healthy insects(B) Time 10 exposed to healthy insects vs Time 10 exposed to Las + insects and Time 0 exposed to healthy insects vs Time 0 exposed to Las + insects
DNA Replication

Figure 11. GSEA analysis of *Bergera koenigii* response to the comparison (A) *prior* vs. Time 10 of the treatment exposed to healthy insects. Blue means more in Time 10 and Red more in the prior; (B) Time 10 exposed to healthy insects vs. Time 10 exposed to Las+ insects and Time 0 exposed to healthy insects vs. Time 0 exposed to Las+ insects. Blue means more in healthy and red more in Las+.

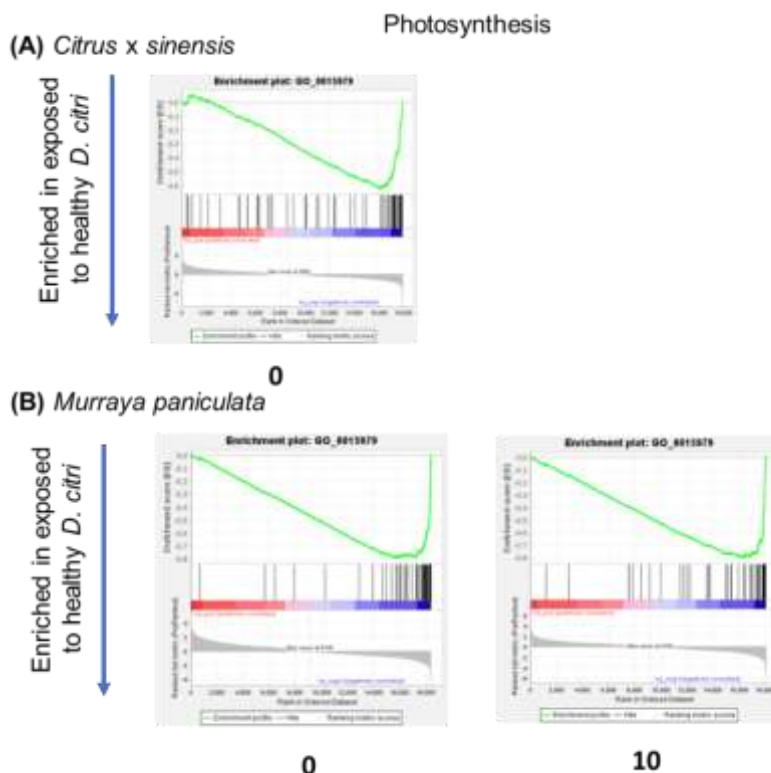


Figure 12. GSEA of (A) *Citrus × sinensis* and (B) *Murraya paniculata* responses to Time 10 exposed to healthy insects vs. Time 10 exposed to Las+ insects and Time 0 exposed to healthy insects vs. Time 0 exposed to Las+ insects. Blue means more in healthy and red more in Las+.

Photosynthesis is a universal process that provides a material basis and energy supply for multiple physiological and metabolic processes in plants. Different biotic and abiotic stress, including the phloem-feeding planthopper *Nilaparvata lugens* in rice and the aphid greenbug *Schizaphis graminum* in sorghum, cause the suppression of photosynthesis (Watanabe and Kitagawa, 2000; Park et al., 2006). Lower photosynthesis rates have also been reported in tomato after inoculation of *Fusarium oxysporum* f. sp. *lycopersici* or *Verticillium albo-atrum* (Lorenzini et al., 1997) and on maize after the infection with *Colletotrichum musae* and *Fusarium moniliforme* (Costa Pinto et al., 2000).

When comparing resistant and susceptible barley varieties, a photosynthesis decrease is observed after inoculation with powdery mildew, being more intense in the resistant plants than in the susceptible ones (Swarbrick et al., 2006). Similar observations were made in the interactions between tobacco and *Phytophthora*

nicotianae (Scharte et al., 2005) and between *Arabidopsis* and *Pseudomonas syringae* (Bonfig et al., 2006). Changes in photosynthesis rates in the early stage after a pathogen infection could be a causal factor influencing acquired resistance, while those in the late stage could be the result of resistance formation (Yang and Luo, 2021).

Genes related to photosynthesis have been reported as directly regulating the immune defense response. For example, transgenic tobacco lines overexpressing the glycolate oxidase (GOX2), serine glyoxylate aminotransferase (SGT), and serine hydroxyl methyltransferase (SHMT1) genes, which are involved in photorespiration, increased the defense against *P. syringae* (Sørhagen et al., 2013). The ferredoxin-I (FD-I), an electron receptor protein, is involved in the photosynthetic process related to ROS production and induction of hypersensitive response (Huang et al., 2007). Rice overexpressing a ferredoxin-like protein increased resistance to *Xanthomonas oryzae* pv. *oryzae* (Tang et al., 2001). Moreover, *Oncidium* orchids expressing sequences of this ferredoxin-like protein showed more resistance to soft rot disease (Liau et al., 2003).

Aiming to investigate if this transcriptional arrest due to infection was strongly related with the development of the flush, we evaluated the aspect from *C. × sinensis* and *B. koenigii* flushes on both treatments. However, we were not able to conduct more deeply investigations on the morphology and neither photosynthesis rates as sampling of flushes were a destructive analysis. Luckily, length of each flush was accessed at the moment of sampling and analyzed. It was possible to observe (Figure 13) that flush growth on *C. × sinensis* exposed to healthy and Las+ insects followed similar values having a very active growth until the Time 20 each agreed with the Las+ exposed sweet orange flushes studied by Alves et al. (2021a). At the different time points after flush detachment, there was no difference in the flush length between *C. × sinensis* ($p > 0.05$) exposed to Las+ insects vs. healthy insects. Regarding *B. koenigii* the flush growth rate was lower than sweet orange, however, the Las+ exposed flushes growth was even lower than the healthy one. At the different time points after flush detachment, there was no differences between *B. koenigii* length from Time 20, 30 and 60, however, statistic differences ($p > 0.05$) were observed between the flush length

exposed to Las+ insects vs. healthy insects from Time 0 and 10, being in agreement with the transcriptomic results.

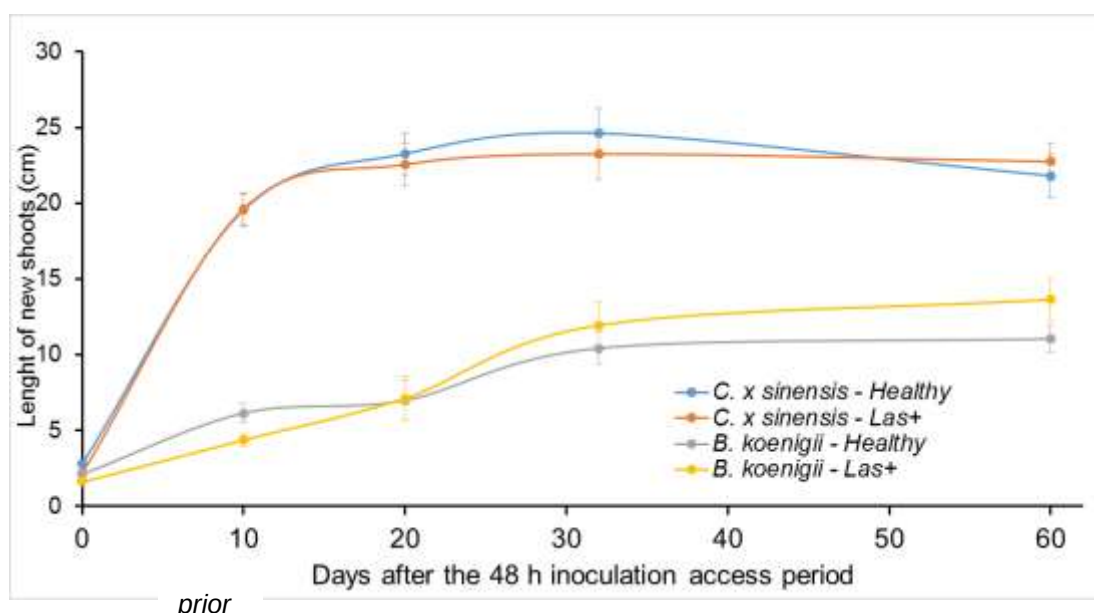


Figure 13. New shoot growth (cm) of *Citrus × sinensis* and *B. koenigii* in an acclimatized room at 25°C following a 48 h period of Las-exposed *Diaphorina citri* confinement and Healthy-exposed *Diaphorina citri* confinement.

4. CONCLUSIONS

This is the first report assessing the early transcriptomic dynamic responses of Las-resistant citrus relatives from the subfamily Aurantioideae, family Rutaceae, after challenge inoculation with Las-infected *Diaphorina citri* adult insects. While *Bergera koenigii* is full-resistant to Las infection, its phylogenetically close relative *Murraya paniculata* is a transient host of the bacterium. In parallel bioassays, sweet orange was used as a susceptible host (Alves et al., 2021a).

When comparing transcriptomic differences in RNA samples from the flushes exposed to Las+ insects vs. healthy insects we did not observe significant expression changes. Literature also supports this finding of no gene ontology (GO) categories significantly over- or under-represented at early stages of Las infection among DEGs from transcriptomics studies performed with other *Citrus* species (Chin et al., 2020; Ramsey et al., 2020). As Las is passively introduced into the phloem by *D. citri* with no damage to host plant cells (Jiang et al., 2019; Haung et al., 2020), it is conceivable that plant defensive arms could be avoided by the psyllids, as also apparently occurs in

studies performed with aphids in other plants (Zhu-Salzman et al., 2004; Couldridge et al., 2007). Moreover, Las lacks genes involved in type 3 and most part of type 2 and 4 secretion systems and shows largely incomplete flagella and pili structures *in planta* (Fagen et al., 2014; Andrade et al. 2019; Andrade and Wang 2019), suggesting that it does not secrete any pathogenicity effector to the phloem of the host plant. In any case, if it eventually exudes nucleic acids or metabolites, the transcriptomes from the three species investigated here indicate that none of them trigger any significant change in host gene expression at early stages of infection. Taking together, it is possible to infer that a typical plant-biotroph or necrotrophy pathogen interaction does not occur in Las-Aurantioideae pathosystems.

Las is a phloem-restricted intracellular bacterium which does not infect neither attaches nucleated cells, so it associates exclusively with the phloem vessels from the host. Our results show that once released into the phloem, Las need a time (about ten to 12 days in all three cases investigated) to adapt to this new environment and use it as a metabolites source for survival and multiplication (Fagen et al., 2014; Alves et al., 2021a). While sweet orange phloem constitutes a proper medium for Las to take the basic nutrients for efficient multiplication, dynamic transcriptomic results in the three species suggest that *M. paniculata* and *B. koenigii* phloem does not provide the necessary nutrientes for Las establishment, being this in agreement with previous metabolomics studies (Killiny, 2016).

Furthermore, our results showed that until Time 10, *B. koenigii* flushes exposed to Las+ insects did not undergone changes in gene expression and largely arrested transcriptome responses likely due to Las perception in phloem tissues. Those results were coherent with the slightly slower growth of *B. koenigii* flushes exposed to Las+ insects vs. the healthy ones during the first infection phase. Consistent with that, GSEA results showed that photosynthesis-related gene expression was down-regulated in RNA samples exposed to Las+ insects at Time 0 and until Time 10 for *B. koenigii*. Photosynthesis suppression has been also reported in other plant systems and intended to be a cellular reaction against different biotic and abiotic stresses (Watanabe and Kitagawa, 2000; Bonfig et al., 2006; Park et al., 2006; Scharte et al., 2005; Swarbrick et al., 2006). We propose that photosynthesis transcriptional reprogramming may be an early defense tool against Las in *B. koenigii*. However, the

molecular mechanisms underlying this type of defensive response remains unclear (Tang et al., 2001; Liau et al., 2003; Huang et al., 2007; Sørhagen et al., 2013; Yang and Luo, 2021). As foreseeable if associated to Las resistance, downregulation of photosynthesis activity was not consistent in the partial resistant *M. paniculata* host and was lacking in the susceptible *C. × sinensis*.

5. CONTRIBUTIONS TO THIS CAPHTER

This work received the contribution and is going to be published by:

Mônica Neli Alves^{1,2}, Juan Cifuentes-Arenas¹, Everton Carvalho^{1,3}, Laudecir Lemos^{1,2,3}, Javier Forment⁵, Jesus Ferro², Nelson Wulff¹, Berta Alquezar⁴, Leandro Peña^{1,4} and José Gadea⁵

¹Fundo de Defesa da Citricultura, Araraquara, Brazil,

²Faculdade de Ciências Agrárias e Veterinárias (FCAV), Universidade Estadual Paulista (UNESP), Jaboticabal, Brazil,

³Empresa Brasileira de Pesquisa Agropecuária, Cruz das Almas, Brazil,

⁴Instituto de Biología Molecular y Celular de Plantas – Consejo Superior de Investigaciones Científicas, Universidad Politécnica de Valencia, Valencia, Spain

⁵Universidad Politécnica de Valencia, Valencia, Spain

Mônica Neli Alves, Leandro Peña, Jesus Ferro and José Gadea conceptualized and designed the work. Mônica Neli Alves, Juan Cifuentes-Arenas, Everton Carvalho and Laudecir Lemos collected the data. Mônica Neli Alves performed DNA and RNA extractions and qPCR. Javier Forment conducted the bioinformatics analysis. Nelson Wulff, Berta Alquezar and all the mentioned authors, contributed to data analysis and interpretation.

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8. SUPPLEMENTARY MATERIAL

The Supplementary Material for this chapter can be found online at:

https://1drv.ms/u/s!AnDFYjGd5J46goo8jmr9P2JRxo_Huw?e=ZbgHiL

Supplementary Table 1. Differentially expressed genes (DEGs) obtained from the *C. × sinensis* vs. *B. koenigii* constitutive comparison. DEGs adjusted using *q* value (FDR) ≤ 0.05 .

Supplementary Table 2. Differentially expressed genes (DEGs) obtained from the *C. × sinensis* vs. *M. paniculata* constitutive comparison. DEGs adjusted using *q* value (FDR) ≤ 0.05 .

Supplementary Table 3. Differentially expressed genes (DEGs) obtained from the *M. paniculata* vs *B. koenigii* constitutive comparison. DEGs adjusted using *q* value (FDR) ≤ 0.05 .

Supplementary Table 4. PANTHER overrepresentation comparing constitutive differences between *C. × sinensis* and *B. koenigii* new shoots.

Supplementary Table 5. PANTHER overrepresentation comparing constitutive differences between *C. × sinensis* and *M. paniculata* new shoots.

Supplementary Table 6. PANTHER overrepresentation comparing constitutive differences between *M. paniculata* and *C. × sinensis* new shoots.

Supplementary Table 7. PANTHER overrepresentation comparing constitutive differences between *B. koenigii* and *C. × sinensis* new shoots.

Supplementary Table 8. Differentially expressed genes (DEGs) obtained from the comparison done at each time point between the treatments exposed to Las+ insects vs. healthy insects in *C. × sinensis*. DEGs adjusted using q value (FDR) ≤ 0.05 .

Supplementary Table 9. Differentially expressed genes (DEGs) obtained from the comparison done at each time point between the treatments exposed to Las+ insects vs. healthy insects in *M. paniculata*. DEGs adjusted using q value (FDR) ≤ 0.05 .

Supplementary Table 10. Differentially expressed genes (DEGs) obtained from the comparison done at each time point between the treatments exposed to Las+ insects vs. healthy insects in *B. koenigii*. DEGs adjusted using q value (FDR) ≤ 0.05 .

Supplementary Table 11. Number of DEGs adjusted using q value (FDR) ≤ 0.05 obtained by the comparison between the time *prior* (without insect) vs. the times 0, 10, 20, 30, and 60 for both treatments studied, exposed to Las+ insects and healthy insects in *C. × sinensis*.

Supplementary Table 12. Number of DEGs adjusted using q value (FDR) ≤ 0.05 obtained by the comparison between the time *prior* (without insect) vs. the times 0, 10, 20, 30, and 60 for both treatments studied, exposed to Las+ insects and healthy insects in *M. paniculata*.

Supplementary Table 13. Number of DEGs adjusted using q value (FDR) ≤ 0.05 obtained by the comparison between the time *prior* (without insect) vs. the times 0, 10, 20, 30, and 60 for both treatments studied, exposed to Las+ insects and healthy insects in *B. koenigii*.