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Chapter

Asymptomatic Phytoplasma Reveal a Novel and Troublesome Infection

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Abstract

Asymptomatic infections are by their nature challenging to study and even more difficult to monitor across broad geographical ranges, particularly as methods are reliant on expensive molecular techniques. The plant pathogen that causes Witches' Broom disease of lime (*Candidatus* Phytoplasma aurantifolia) is a major limiting factor in lime production across the Middle East and was recently detected in Brazil, but without the typical symptoms from the Middle East. Here, we discuss the difficulty of monitoring asymptomatic infections and highlight the threat posed by highlight future outbreaks. Asymptomatic infections have important implications for understanding the evolution of pathogens within perennial hosts. We use three model systems of asymptomatic infections: (i) a Phytoplasma and (ii) a bacterial infection of lime (*Citrus aurantifolia*) and (iii) an "out-group" Phytoplasma of Cassava (*Manihot esculenta*) to demonstrate consistency across divergent hosts. We found that although all plants in the study were intentionally infected, assays typically did not confirm this diagnosis. Emergent technologies monitoring gene expression could be used to both study novel biology associated with asymptomatic infections and develop monitoring technologies. We highlight the difficulty of monitoring asymptomatic infections in possible future outbreaks and have important implications for understanding the evolution of pathogens within perennial hosts.

Keywords: *Citrus aurantifolia*, acid lime, silent infection, phytoplasma, differentially expressed genes

1. Introduction

Vector-borne plant pathogens of perennial crop species provide an opportunity to study the impacts of long-term infections, in terms of epidemiology and vector ecology. Crop diseases directly threaten global food security; an estimated 16% of food production globally is lost despite our efforts to control crop diseases [1]. Perennial crops generally have advantages over annuals in terms of energetic efficiency; for example, constant canopy development increases photosynthesis efficiency [2], which results in 30% increases in carbon turnover than those maintained by annual crops [2]. Pathogens must evolve to infect and reproduce

within a single year in annual cropping systems, and thus typically demonstrate more aggressive pathologies [3], which often require multiple hosts, such as potato blight (*Phytophthora infestans*) and wheat rust (*Puccinia graminis*). As the host plant remains in situ after harvest, perennial cropping systems therefore theoretically allow for evolution of slower pathologies, which may be cryptic in nature.

Globally, plant pathogens are spreading faster than ever, due to climate change, increased crop and germplasm trading, failure of border biocontrol and associated spread of vector species. Here, we shall introduce and discuss a complex vector-borne plant pathogens of a perennial tropical cash-crop plant: *Citrus*. *Citrus* is the world's principal fruit crop, with about 60 million megatons grown annually [4]. Limes account for ~5% of global *Citrus* production [4]. Lime is cultivated in tropical, subtropical and temperate regions from 40°N to 40°S [5, 6]. Countries in the Middle East, as well as India, Pakistan, Brazil, Argentina and Mexico grow lime as a key part of their agricultural economies [7, 8].

The production of lime in the Middle East has been markedly impacted by Witches' Broom Disease of Lime (WBDL) [7]. Symptoms of witches' broom disease of lime (WBDL) were first observed in Oman in the 1970s [9]. Infected trees present with "witches' brooms": shoot structures characterized compactness and small, pale green leaves. In the advanced stages of the disease, leaves become dry, brooms become increasingly more prevalent, and fruits become significantly smaller and less marketable. Finally, the tree collapses within 4 or 5 years after infection [10].

Asymptomatic ("silent") infections have recently been detected in lime trees in Brazil [11] and Oman [12]. This silent infection was observed through molecular testing of plant material, yet the host plants themselves show no obvious visible symptoms. These infected trees do however, also collapse within the 5 year post infection period [13], making this asymptomatic variant potentially even more of a threat to global lime production.

Detailed research into this system has been limited, some suggest that the silent infection may be due to ultra-low pathogen titre levels within the host plant [12, 14] or due to different interactions with plant defences [15] or insect vectors [16, 17]. Silent infections are difficult to monitor and pose a significant risk to global food security, given that the limited knowledge we have suggests they may be as destructive as symptomatic [18], but we do not yet know the full extent of their range.

The Phytoplasma "*Candidatus* Phytoplasma aurantifolia" has been identified as the causative agent of WBDL [19]. Phytoplasma are wall-less gram-positive bacteria belonging to the class Mollicutes [20]. They are found in the phloem sieve tubes of plants and in the gut, salivary glands and other organs of Hemipteran insect vectors [21]. Phytoplasma are obligate biotrophic organisms, which lack many essential genes that encode for components of metabolic pathways; and they likely import metabolites such as nucleotides, amino acids, and fatty acids from the host plant [22]. Phytoplasma are the only known organisms that lack ATP-synthase subunits, which are thought to be essential for life [22]. Owing to the inability to culture them *in vitro* and their inaccessibility in host plants [19, 22], the molecular mechanisms that underlie Phytoplasma infections within host plants remain largely unknown [10]. Phytoplasma may be able to overcome plant defences by producing specific proteins: effectors [15]. The effectors (e.g., SAP11 and SAP54) may modulate host plant growth and interactions with the insect vectors [16, 23].

Although studies using proteomics [10, 24] and cDNA-amplified fragment length polymorphism (cDNA-AFLP) [25] have investigated differentially expressed genes (DEGs) in plants infected by "*Ca*. P. aurantifolia," these studies provide only a brief snapshot of gene expression and regulation during infection. Recent developments in high throughput "omics" based approaches now allow a detailed

examination of plant pathogen interactions, and these have been applied to symptomatic infections of "*Ca*. P. aurantifolia" in the Middle East [18, 26]. Our study examined DEGs in symptomatic and asymptomatic infections of the Phytoplasma in acid lime trees. Although asymptomatic infections have been linked to fitness benefits in the vectors of this pathogen [17], our knowledge of understanding of gene expression differences in an asymptomatic infection are extremely limited. One way to understand the effects and biology of asymptomatic infections is by developing our knowledge of these differentially expressed genes.

Within this chapter, we shall discuss two studies on asymptomatic infections of crop plants [1]. Reliable detection of asymptomatic plant pathogens is the greatest limitation on controlling and limiting their global spread. We first discuss and test the potential for currently employed molecular tools to misidentify "healthy" plants. We study three asymptomatic infections (a Phytoplasma of lime, a Phytoplasma of cassava and *Citrus* Huanglongbing) and compare the rate of false-negatives detecting the disease [2]. Asymptomatic infections in Brazil represent a novel biology by the Phytoplasma infecting lime trees. This novel pathology needs to be explored to better understand the infection process, and also presents us with an opportunity to design superior detection tools. We compare the gene expression of infected symptomatic and asymptomatic plants using qPCR. These findings provide an important and novel examination of the nature of asymptomatic infections, a poorly understood, emerging area of plant pathology.

2. Pathogen detection in the absence of visible symptoms: study system

In order to comprehensively study the most ubiquitous methods used globally for asymptomatic infections of crop plants, we used three model systems: the aforementioned Phytoplasma causing Witches' Broom Disease of Lime (WBDL), a closely related Phytoplasma causing Cassava (*Manihot esculenta* L.) Witches' Broom, and an out group pathogen of lime—"*Candidatus Liberibacter* asiaticus," causative organism of Huanglongbing disease of lime. Data for the first two pathosystems was collected for the present study, whereas data from Huanglongbing came from a previously published study by *Citrus* [27].

2.1 Sample locations

Acid lime (*C. aurantifolia* L.) trees were grown on a *Citrus* orchard maintained at Universidade Federal de Viçosa (UFV), Brazil (S20°45′585″; W042°50′908″). The site was chosen as plant material there had previously been found to be infected with "*Ca*. P. aurantifolia," but showing no visible symptoms [17].

Leaf samples of cassava (*M. esculenta*) grown in a glasshouse at UFV and deliberately infected with a cassava witches' broom (Phytoplasma 16SrIII-A) were also taken. For details regarding this pathogen, please see [18]. Although this disease can display typical symptoms of witches' broom (e.g., stunting, leaf chlorosis, deformation, and reduced size), the infections in Brazil do not display symptoms until harvest, when it can cause 100% crop losses [18].

2.2 Plant material

Citrus leaf samples from Brazil were taken from four 15-year adult trees and 10 1-year saplings; for each adult tree 30 leaves were collected and for saplings 10 samples were collected in a semi-random fashion. Cassava leaf samples were collected from eight 1-year adult plants, 10 leaves were sampled from each cassava plant.

The sampling strategy for both lime Phytoplasma and cassava Phytoplasma aimed to collect a spatially diverse group of samples (orientated on x, y and z axes relative to the trunk), with the position of each leaf sampled noted with respect to its branches from the main trunk. For all sample types locations, leaf midrib samples (the larger vein along the midline of a leaf) were taken. The midribs were immediately frozen in liquid nitrogen after harvesting and then transported to the laboratory, where they were stored at −80°C until total DNA and RNA isolation.

2.3 Molecular detection of Phytoplasma

The presence/absence of the Phytoplasma in the leaf samples of both acid lime and cassava was analysed using PCR for Phytoplasma detection. To this end, total DNA was extracted from acid lime leaf samples using the DNeasy Qiagen Plant Mini Prep kit following manufacturer's instructions. Then, total DNA was extracted from the cassava leaf samples following the protocol of [28], with modifications that are detailed in [18].

We then used a nested PCR using universal primers for Phytoplasma detection. Extracted DNA of both *Citrus* and cassava Phytoplasma were amplified using 16S rRNA PCR primers P4 (5′-CAT CAT TTA GTT GGG CAC TT-3′) and 23rev (5′-CGT CCT TCA TCG GCT CTT-3′) in the initial reaction, and the resulting amplicon was diluted (1:10) and used as template DNA for nested PCR amplification using the P3 (5′-GGA TGG ATC ACC TCC TT-3′) and 23rev primers [18, 29, 30].

PCR amplification was carried out using a Loccus Biotechnologia TC9639 Thermal Cycler (LB, São Paulo, Brazil) in 20 µl volumes, such that each reaction contained the following: 2.0 µl (20 pmol) of each primer, 8.0 µl water (DNA-free water; Qiagen, SP, Brazil), 4.0 µl sample extracted DNA and 0.1 µl Invitrogen *Taq* DNA Polymerase (5 U/µl) (ThermoFisher Scientific, Brazil), 1.3 µl MgCl (50 mm) 2.6 µl dNTPs (10 mm), 2.0 µl PCR buffer (200 mm Tris-HCl pH 8.4, 500 mm KCl). For the first round PCR, initial denaturation at 95°C for 3 min, followed by 30 cycles of 95°C for 45 s, 55°C for 45 s and 72°C for 3 min, with a final elongation step at 72°C for 7 min. For the nested reactions, the conditions were 95°C for 3 min, followed by 32 cycles of 95°C for 45 s, 54°C for 45 s and 72°C for 3 min, with a final elongation step at 72°C for 7 min. The resulting amplicon was then visualised on agarose gel electrophoresis using SybrSafe DNA stain to confirm the presence/ absence of both Phytoplasma from each leaf sample of each plant host.

Data on the successful amplification of "*Candidatus* C. liberibacter" were obtained from the Coy et al. [27] study. Briefly, this study compares the efficacy of the current method of detection for C. *liberibacter asiaticus* within plant and insect samples is by a presence/absence PCR assay using a 16S rDNA gene target. Specifically they examined these methods for sensitivity to low bacterial titers or suboptimal PCR conditions that can result in false-negatives. This study concluded that the high incidence of false negatives using this system could contributes to the under-reporting of plant pathogen infections. Hence, the data paralleled our present study, and were used for direct comparison of this pathosystem with our own presented here.

3. Pathogen detection in the absence of visible symptoms: results and discussion

Detection of "*Ca*. Phytoplasma aurantifolia" by 23S-PCR on asymptomatic acid lime (*C. aurantifolia*) plants showed that all plants sampled in this study were technically infected (**Table 1**), meaning that each plant had at least one sample that positively detected the Phytoplasma. The proportion of samples that failed to detect the pathogen was, on average, in adult trees 38.5% ± 6.62 (n = 3), and in saplings

Tree	Infected	Detection likelihood (%)
A	21/31	67.74
B	24/44	54.55
C	28/45	62.22
SA	5/10	50.00
SB	6/10	60.00
SC	6/10	60.00
SD	6/10	60.00
SE	4/10	40.00
SF	3/10	30.00
SG	3/10	30.00
SH	10/10	100.00

Table 1.
Results of asymptomatic infections of "Ca. Phytoplasma aurantifolia" detection using 23S-PCR from adult (A–C) and sapling (SA-SH) Citrus acid lime plants.

46.25% ± 22.6 (n = 10). Within cassava (*Manihot esculenta*), false-negative rate was 48.75% ± 17.3 (n = 8). False-negative molecular tests have also been found in molecular techniques for detecting Huanglongbing infections in *Citrus* plants. Thus particular study found a false-negative rate of 54.9%, using a nested PCR assay [27], and identified that more sensitive molecular tests involving qPCR addressed this issue, albeit not in a manner applicable to growers and germplasm suppliers expecting to provide disease-free planting material [31].

The evidence for false-negative across multiple plant pathosystems has notable implications across the field. One of the base assumptions of plant pathology is the suitability of a biological sample to represent the entire host plant. These false-negatives mean that multiple biological samples per plant may be required to correctly identify the presence of a pathogen. A hypothetical plant with α leaves and a false-negative rate of β ± SD, to guarantee a correct identification (under P = 1.00) the minimum sample number (n) must be:

$$n = (\alpha \times \beta) + 1 \qquad (1)$$

$$\sum_{k=x}^{\alpha} \binom{\alpha}{k} \beta^k (1 - \beta)^{n-k} \qquad (2)$$

Due to the nature of additive probabilities (Eq. (2)), the probability of, for example, 38 continuous false-negatives on a tree of 100 leaves would be P = 4.83^{-22}. Consequently, a decision support system based on the likelihood of having an infected tree can be developed in order to determine the appropriate number of samples required to avoid a false-negative. For example, for P = 0.05, minimum sample number would be n = 4.19; for P = 0.005, n = 8.94; for P = 0.001, n = 12.25 (**Figure 1a**). For cassava similarly the minimum sample number for the same probabilities would be (in order): n = 3.55, n = 7.20 and n = 9.76 (**Figure 1b**).

Asymptomatic plant pathogens are particularly troublesome within perennial crops as they are not removed at the end of the growing season and act as reservoirs of infectious materials to be dispersed to new hosts by insects (and other vehicles). Persistence of asymptomatic infections in hosts may also cause problems through subtle direct damage or sublethal infections leading to plant-by-plant transmission
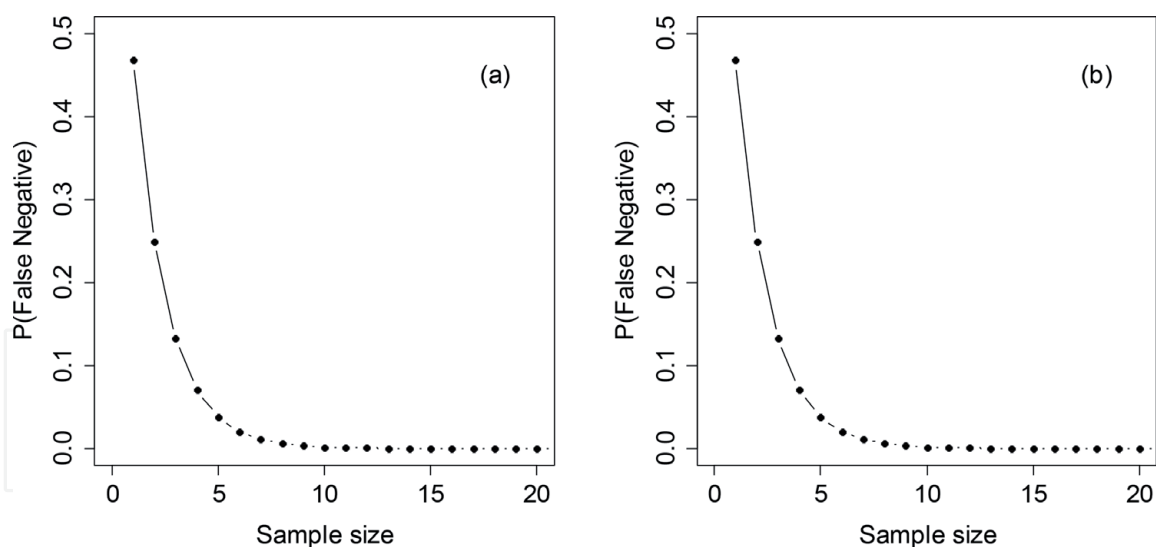
Figure 1.
Probability function for false negatives using PCR-based detection for asymptomatic Phytoplasma infections. The additive probability of sequential false negatives as the sample size increases in (a) Citrus Phytoplasma in adult Citrus trees (false negative rate = 38.5%); (b) cassava Phytoplasma (false negative rate = 48.75%).

[32]. The use of accurate and timely diagnostic methods is undoubtedly one of the best ways to monitor pathogen ranges in asymptomatic infected plants, and thus avoid dissemination to new hosts and ranges. Generally, traditional methods of identification based on visual symptoms and culturing in laboratories are time-consuming, labour-intensive, costly and have "very low sensitivity and specificity" [33, 34].

Molecular methods are the mainstream alternative to symptomology and laboratory culture. The results of this present (and a previous) study [27] have demonstrated a potential flaw in molecular methods: the frequency of false-negatives. Whereas classical plant pathology can rely on a non-destructive inspection of the entire host plant, culture and molecular methods must only use a small "representative" destructive subsample of the plant. The major limitation to this is the quality of the representation of the host plant within this subsample. We have demonstrated here that a single biological sample from an infected plant may not be representative of the whole plant and therefore multiple samples from within the same host plant can result in different results from molecular testing for pathogens. We found false-negative rates between 38 and 49%, meaning that approximately a minimum of one in three samples would fail to detect a pathogen if taken alone. Although this calls into question the use of single biological samples for identifying pathogens by molecular methods, these methods have to strike a balance between precision and cost [35]. We calculate, based on these false-negative rates, minimum sample numbers (per plant) between 3 and 5 samples, which may make these methods prohibitively expensive for widespread use within agriculture.

By comparison, real-time PCR used to detect and quantify pathogens in symptomless plant tissues is a promising tool to improve our understanding of "silent" infections [36]. Different methods of DNA amplification that rely on conventional and quantitative PCRs have also been developed to detect and identify "*Ca.* Liberibacter" species associated with Huanglongbing (HLB) in *Citrus* [27, 37]. But other simpler methods, such as direct tissue blot immunoassay, have been used to facilitate detection of pathogens in asymptomatic plants of *Citrus* [38]. Molecular tools have been developed for identification of WBDL from field samples [7, 39], but remain prohibitively expensive for widespread implementation by growers. Much research effort and resources have been devoted to development of on-the-spot diagnostics in plant pathology, and have shown success in control and monitoring the spread of some plant diseases (e.g., *Potato Virus Y*), but do not exist for Phytoplasma

yet [40]. *In-situ* kits for testing Phytoplasma using immunofluorescence have been developed; but have not been adopted for widespread use yet [34, 41].

4. Novel asymptomatic infection biology: study system

Successful identification of asymptomatic infections by the Phytoplasma causing Witches' Broom Disease of Lime (WBDL) provide a unique opportunity to compare the pathology with its' symptomatic counterpart. A recent study by Mardi et al. [26] using a high-throughput genomics approach identified 2805 differentially expressed genes in symptomatically infected *Citrus* plants. This study revealed the key potential molecular pathways through which the Phytoplasma infects and parasitizes its host. Correspondingly, here we studied 25 of these that were differentially expressed by more than 128-fold and 4 further genes identified as significantly differentially expressed in recent infections found in Brazil (Alves et al. unpublished data). These genes allowed us to design a targeted study to understand how the symptomatic and asymptomatic infections differ, and potentially identify some of the "silent" symptoms in this newly emerged pathosystem.

4.1 Sample locations

Acid lime (*C. aurantifolia*) trees were grown at the same *Citrus* orchard at UFV mentioned previously. Lime leaves were also collected from cultivated areas in Muscat, Oman (N23°58′591″, E58°40′590″). Omani samples were collected from a farm with symptomatic infected trees (drastic reduction in growth, generalized leaf yellowing and necrosis) and uninfected (healthy tissue).

4.2 Plant material

Six *Citrus* plants were sampled each in Brazil and Oman (three symptomatic and three healthy plants), for three biological replicates. Samples from Brazil were confirmed for Phytoplasma by PCR (see Section 2), samples from Oman were confirmed by symptoms (drastic reduction in growth, generalized leaf yellowing and necrosis).

4.3 RNA extraction

Total RNA was extracted from the three biological replicates of limes infected with "*Ca*. P aurantifolia" and three healthy acid lime leaves (from both Brazil and Oman) using the RNeasy Plant Mini Kit (Qiagen, SP, Brazil). RNA quantity and quality were determined using a Nano-Drop ND 1000 spectrophotometer (Thermo Scientific, MA, USA). five hundred nanogram of total RNA from each replicate was reverse-transcribed in a 20 µl reaction using 1 µl of Invitrogen SuperScript® III Reverse Transcriptase (Thermo Scientific), 1 µl oligo(dT)$_{18}$ (100 nm), 1 µl dTT (100 mm), 2 µl dNTP (10 mm), 4 µl 5× first-strand buffer (250 mm Tris-HCl (pH 8.3), 375 mm KCl, 15 mm MgCl$_2$) and RNAse free water (Qiagen).

4.4 Gene expression

Gene specific primers were designed for 15 genes belonging to key pathways with possible implication in disease progression and resistance identified by Mardi et al. [26] and four by Alves et al. (unpublished). The sequence of primers, amplicon length, optimal primer and enzymatic efficiency for each primer pair is presented in **Table 2**. Mardi genes were amplified only for Brazilian samples,

Unigene	Forward primer	Reverse primer	Amplicon length (bp)
U352	TGGCTCTGGATGGCATTG	GTGCTTCTGGGATAGTGA	133
U2265	TGCTGCATTGGTTCTGTC	GACTGCAAAGGACTCCAAG	130
U27316	ATGCGATACACAACCCAATCT	CGGCCATGAGACCAAAACT	126
U75775	GAAGGAGCTGACGTTTTC	CTTCTGCCTCTTCCCTCTC	160
U26576	GATTGTCCGCCCAGTAGTG	CACGCGATCAGCCAAACTC	174
U72184	CAAAGAGATGGGCAAAGAG	GCCAAATTACAAACCAAACGA	121
U59125	TATGGGGATAAGGGGTGT	TGCCACAACTAACCTCCTC	182
U68165	CTGCTGAGATTACATGGTT	CTCTTCAGGGAATTGCAC	147
U68593	GACTCTCTTTCAATGCCA	TTGAAAGCACAGGTTCCGA	119
U77887	CATGCCATCCTCTTCACT	GGGTTGGGTTGAGTATCT	123
U3869	CTCCTCCTCCTCCTCCAAAG	GCGAACCCATCACACTACAT	117
U17275	AACACCCATTTGCATTCTC	GGTTTGTATGCCTTCGATG	130
U41653	GAGAGTAGCAAGACCTCAAG	TATCACCAGCCTCACTTCAC	114
U17606	CTCACCGCAGATTTTGAACCAC	ACATCCGTCTTCTCATCCACA	158
U24969	GCCTCCGTTTCCAATTCTC	GATACCGAGGATTTCATGGC	131
WRKY33	GATGATGAAAATGAACCTGATGCT	CAATTCTTGGCTCCCTCACAGT	144
WRKY70	AGACCGGAGAGGATGCTACAAG	CCCATATTTCCTCCATGCAAA	152
MYBR1	AATGGATCCAACTTGGTTTTGAA	ATCCAAACTCGCCCTGGTT	110
JAZ6	ACAATGATGCAACCCCACTTC	TGCTGCAGCCCTTTCTTTTC	120

Table 2.
Primer sequences for potential infection related differentially expressed genes in Citrus aurantifolia used in qPCR.

whereas we were able to study the smaller number Alves genes were amplified for both Brazilian and Oman samples.

The selected genes were quantified using the Applied Biosystems StepOne™ Real Time PCR system (Thermo Scientific). qRT-PCR was performed in a 10-μl reaction containing 5 μl of SYBR Green PCR Master Mix, 4 μl of each primer mix (**Table 1**), 50 ng of template cDNA. The thermal cycling conditions consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s, and a final extension step at 72°C for 5 min.

The detected expression of selected transcripts was measured using the absolute quantification method. We prepared standard curves for each target gene (0.01– 10 ng μl^{-1}) in order to quantify each genes expression relative to a standard internal control gene. Cycle threshold (C_T) value of each gene relative to the internal control gene was used to estimate gene expression in RNA concentration values of ng μl^{-1} [42]. Triplicate reactions were used for each sample.

Ubiquitin 1 and Tubulin alpha were used as internal reference genes, with primer sets UBi-IF (5′-TTT CTT CCT CAA CTT CAC TTG TAT CC-3′), UBi-IR (5′-TGG TCA TAG GCT GTT CGA TCA C-3′), α-tub-F (5′-CTG CAA GGG TTC TTG GTG TTC-3′) and α-tub-R (5′-GAT AGG CGT TCC AGT AAC AAC GA-3′), respectively. Standard curves for each gene were examined in the amplification plot and the standard curve plot was prepared in ABI 7500 software v.2.0.6. Reaction efficiency, R square and slope values were calculated by the ABI 7500 software v.2.0.6 program (**Table 3**) and were used to determine the copy number of infection-related RNA in each sample.

Unigene	Slope	−1/slope	E	E (%)
U352	−4.69701	0.212901	1.159017	115.9
U2265	−4.99484	0.200206	1.148863	114.9
U27316	−4.17641	0.23944	1.180534	118.1
U75775	−1.01071	0.989403	1.985363	198.5
U26576	−1.69178	0.591093	1.506388	150.6
U72184	−4.35679	0.229527	1.17245	117.2
U59125	−4.19476	0.238393	1.179678	117.9
U68165	−4.67463	0.213921	1.159836	115.9
U68593	−1.68595	0.593137	1.508523	150.8
U77887	−1.32334	0.755662	1.688406	168.8
U3869	−4.3699	0.228838	1.171891	117.2
U17275	−2.41499	0.41408	1.332449	133.2
U41653	−4.77939	0.209232	1.156072	115.6
U17606	−3.94001	0.253806	1.192349	119.2
U24969	−5.796	0.172533	1.127035	112.7
WRKY33	—	—	—	112.8
WRKY70	—	—	—	132.5
MYBR1	—	—	—	119.1
JAZ6	—	—	—	102.7

Table 3.
qPCR efficiency values for Phytoplasma related genes in C. aurantifolia.

4.5 Statistical analysis

Analyses of differential gene expression in asymptomatic Phytoplasma infections of acid lime were performed using the *R* statistical software v3.3.2 [43]. Non-metric multidimensional scaling (NMDS) was used to analyse differential gene expression and partition variation between symptomatic/asymptomatic and infected/uninfected groups across all genes [44]. Here, we analysed normalised gene copy number by NMDS using the "*metaMDS*" function [45]. NMDS was performed using the Bray-Curtis dissimilarity index on two ordinal scales for optimal NMDS stress values. Interactions between these and infection type were tested and assigned significance using the "*envfit*" function. Significant differences in DEGs between asymptomatic infected and healthy *Citrus* plants were tested using Student's *t*-tests. Matched gene expression data between Brazil and Oman were further analysed using *post-hoc* Tukey HSD tests to test differential expression based on sample location and symptom type.

5. Novel asymptomatic infection biology: results and discussion

Gene expression profiles were determined by qPCR for 15-disease related genes identified previously for infections of "*Ca*. Phytoplasma aurantifolia" in *Citrus aurantifolia* adult trees by Mardi et al. [26]. NMDS showed that a two-dimensional solution was sufficient to achieve low stress values to enable us to interpret disease-related gene expression (stress = 0.049). Infection status (asymptomatic/uninfected) of leaf samples was significantly correlated with the NMDS analysis of gene expression (**Figure 2**, R^2 = 0.533, P < 0.001), demonstrating clear differences in
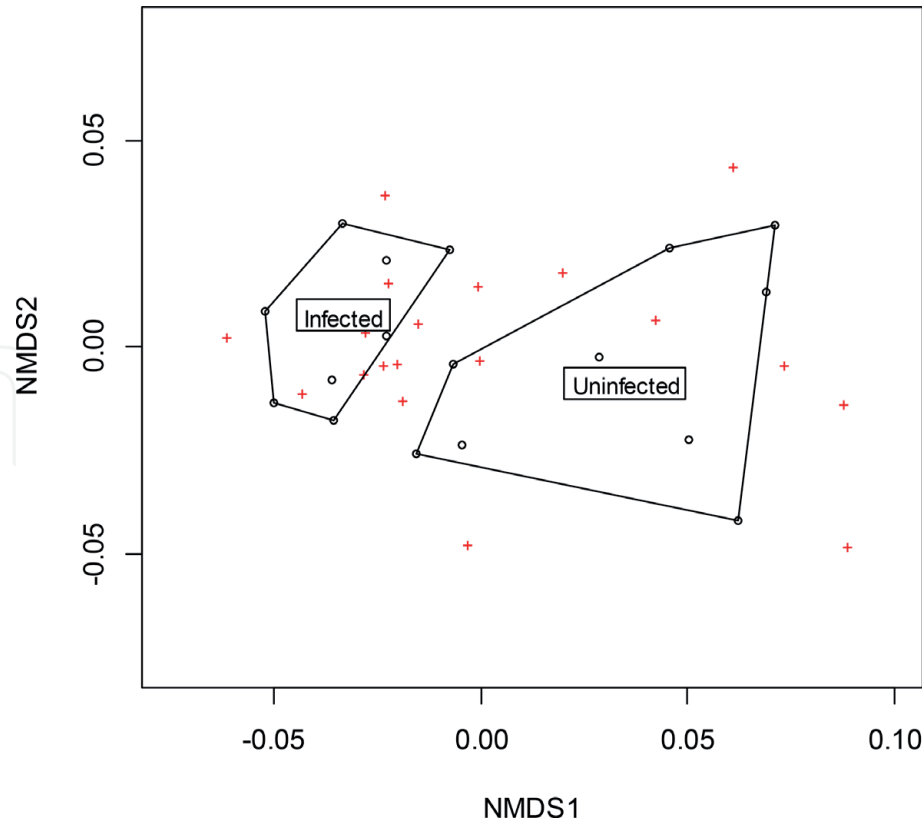

Figure 2.
Surface NMDS ordinations of differential gene expression from samples of "Ca. Phytoplasma aurantifolia" asymptomatic infected and uninfected (healthy) acid lime trees from Brazil denoted by open circles; their position is determined by where they fall on ordinal axes 1 and 2. Red names are species centroids for each Unigene. Polygons indicate clustering of each infection type, which are interpreted as how each gene (and the overall gene expression composition) correlates with the infection properties.

host plant gene expression in response to infection by this asymptomatic infection. When examining the direction and significance of differential expression of each of these 15-disease related genes, several significant decreases were found (**Table 4**). Expression of four genes related to stress tolerance, cell replication, energy production and protein production (CRT/DRE binding factor; NAC domain-containing protein 71; beta-galactosidase 3; nitrite reductase) were significantly decreased in asymptomatic infected plants. Genes related to immune response (mitogen-activated protein kinase 1; cyclic nucleotide-gated ion channel 1; brassinosteroid insensitive-1-associated receptor kinase) were not significantly differentially expressed however.

Unigene ID	Transcript	Uninfected expression (ng μl^{-1})	Asymptomatic expression (ng μl^{-1})	Differential expression	Functional characterisation
U24969	Probable LRR receptor-like serine/threonine-protein kinase	11.22	13.67	NS (p = 0.321)	ABA-signalling
U72184	Zinc finger A20 and AN1 domain-containing stress-associated protein 3	8.15	7.82	NS (p = 0.537)	Abiotic stress tolerance
U59125	CRT/DRE binding factor	12.32	7.76	↓ (p = 0.050)	Abiotic stress tolerance
U27316	NAC domain-containing protein 71	32.74	15.30	↓ (p = 0.011)	Cell Replication
U352	Beta-galactosidase 3	9.74	8.47	↓ (p < 0.001)	Energy production
U68165	Ent-copalyl diphosphate synthase	7.54	7.38	NS (p = 0.664)	Growth regulation
U77887	Gibberellin 2-oxidase	10.24	8.20	NS (p = 0.195)	Growth regulation
U41653	LRR receptor-like serine/threonine-protein kinase GSO1	21.27	18.40	NS (p = 0.140)	Growth regulation
U26576	Mitogen-activated protein kinase 1	27.04	22.42	NS (p = 0.118)	Immune response
U68593	Cyclic nucleotide-gated ion channel 1	17.12	16.97	NS (p = 0.918)	Immune response
U17606	Brassinosteroid insensitive-1-associated receptor kinase	20.04	21.12	NS (p = 0.453)	Immune response
U3869	Jasmonate ZIM domain-containing protein 6	15.42	15.73	NS (p = 0.659)	JA-signalling
U17275	Phytochrome-interacting factor 3	6.42	6.52	NS (p = 0.623)	Light response
U2265	Amino acid transporter	7.85	8.38	NS (p = 0.051)	Protein production
U75775	Nitrite reductase	17.27	12.58	↓ (p = 0.001)	Protein production

Significant differences were tested by students T test.

Table 4.
Functional characterisation of DEGs expressed in response to infection by "Ca. P. aurantifolia" and mean differential expression between asymptomatic infected and healthy C. aurantifolia plants.

The genes MYBR, JAZ6, WRKY37 and WRKY70 were targeted for amplification from samples from both Oman and Brazil (Alves et al. unpublished). MYBR gene expression was not significantly different between Brazil and Oman (F = 3.725, P = 0.067 **Figure 3a**); *posthoc* tests showed no significant difference between infected/uninfected in Oman (P = 0.998) or Brazil (P = 0.354). JAZ6 expression was significantly different between Brazil and Oman (F = 24.016, P < 0.001 **Figure 3b**); *posthoc* tests showed a significant difference between infected/uninfected in Oman (P = 0.043), but not Brazil (P = 0.588). WRKY70 expression was significantly different between Brazil and Oman (F = 50.002, P < 0.001 **Figure 3c**); *posthoc* tests showed a significant difference between infected/uninfected both in Oman (P < 0.001), and Brazil (P = 0.004). WRKY37 expression was significantly different between Brazil and Oman (F = 9.617, P = 0.004 **Figure 3d**); *posthoc* tests showed a significant difference between infected/uninfected both in Oman (P < 0.001), and Brazil (P < 0.001).

Disease symptoms are, taken at their most literal, an observable change in host homeostasis in response to the presence of a pathogen. The mechanism underlying symptoms (or lack thereof) within the host plant is broad, but mostly resides in genetic changes (host immune response, genomic mutations, RNA silencing) in either the host or pathogen. The nature of asymptomatic infections is complex and poorly understood. Some may express pathogenesis genes at a lower level and be kept in the host without causing overt symptoms [46].
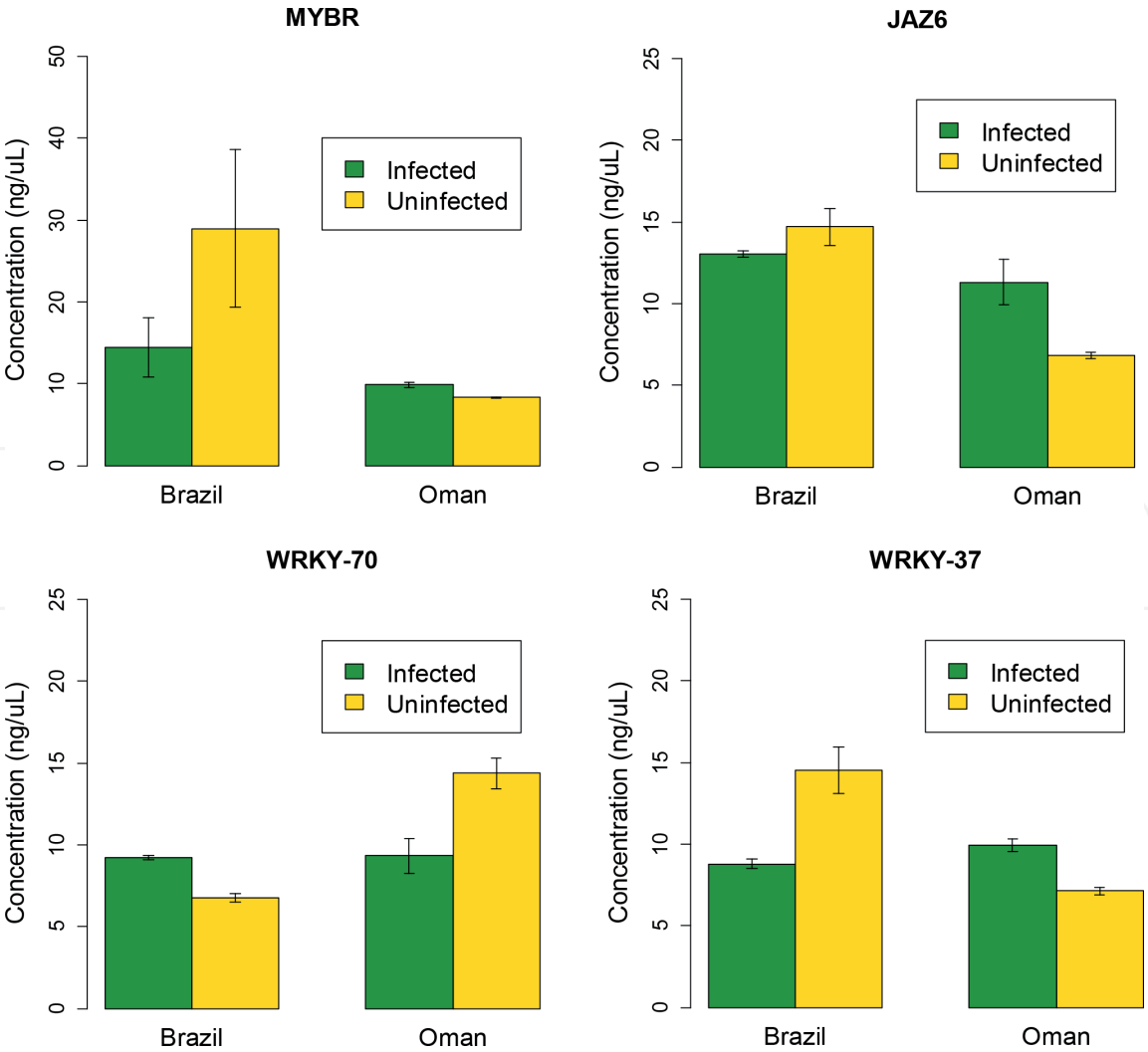

Figure 3.
Differential gene expression of disease-related genes amplified in Brazilian (asymptomatic) and Omani (symptomatic) acid lime trees infected with the Phytoplasma "Ca. Phytoplasma aurantifolia."

We examined a group of host plant (*C. aurantifolia*) infection related genes identified by [26] in the context of an asymptomatic infection. This previous study established by next generation sequencing of host plant RNA expression (RNAseq) that 2805 genes are differentially expressed in symptomatic infected compared with healthy uninfected plants. Of these, 71 genes were significantly deregulated; of them, 52 were upregulated and 19 down-regulated in response to Phytoplasma infection [26]. Here, using quantitative PCR methods, we studied a subset of these genes that were expressed by more than 128-fold and their differential expression in relation to healthy vs. asymptomatic infected lime plants in Brazil (**Table 4**). We demonstrate that the asymptomatic infection does result in detectable changes in host plant gene expression (**Figure 2**). Specifically, however, no significant change in expression of *Citrus* immune response genes was found here, which would be expected given the asymptomatic nature of the infections (**Table 4**).

Certain genes related to stress tolerance, cell replication and energy production had their expression significantly reduced in infected plants (**Table 4**). The latter may be the best candidate for a "symptom" of these "silent" infections: Phytoplasma are obligate biotrophic organisms and their parasitism may be through host ATP-synthase subunits [22]. When comparing these results with those of [26], the stress tolerance gene also shows a significant reduction in expression in symptomatic infected lime plants. However, cell replication and energy production genes were significantly deregulated in the asymptomatic infections, which was distinct to the symptomatic infection in [26]. This may be one of the first accounts of a significant alteration of gene expression by a host plant infected by an asymptomatic plant pathogen. The demonstrated response by the plant clearly indicates that these are not truly "silent" infections, and perhaps opens up new routes for detecting these pathogens.

Previous research into symptomatic infections of "*Ca*. P. aurantifolia" infecting acid lime has indicated production of several metabolites significantly altered during infection. In Iran, infections are associated with catechin and epicatechin production in leaves [47, 48]. Amino and organic acid concentrations (such as proline, arginine, glutamate, citrate and salicylate) are also significantly increased immediately after inoculation [47]. Studies have shown that "*Ca*. P aurantifolia" also alters the concentration of limonene, ocimene and trans-caryophyllene [7]. Much like the DEGs we have identified in the present study, each of these chemicals could act as measurable indicators to diagnose the infected lime at the early stages of the WBDL progression.

A distinct host plant genomic response to infection by this asymptomatic infection has significant implications for the diseases' insect vectors. Management strategies for insect-vectored pathogens specifically target the vector-plant interactions, relying on monitoring and suppressing these vectors in order to reduce the frequency and severity of disease outbreaks [49]. Many vector-borne plant diseases alter host plant phenotypes in ways that can influence their vectors biology and behaviour [50–52], with significant implications for disease transmission.

Infected plants are often better for their vectors than uninfected in terms of vector growth rates, reproduction and longevity [17, 53]; although the opposite is certainly true in some pathosystems [53] and some vectors actively avoid infected hosts that represent inferior hosts [54]. We have previously demonstrated that an asymptomatic infection results in significant increases in vector life history traits (reproduction and growth rates) than with a symptomatic infection [17]. In future studies, the distinct expression profile detected within the plant host here could be usefully explored in relation to differential gene expression in the insect host, in order to fully understand this vector-host-pathogen complex [16, 23].

We also specifically consider differences between two agricultural loci—the Middle East and South America—by examining a gene set directly related to the plant-pathogen (Phytoplasma) interaction. Four genes (JAZ6, MYBR, WRKY70 and WRKY33)

are modulated during Phytoplasma infection in lime trees (**Figure 3**). Interestingly, an inverse expression profile for this gene set could be verified by comparing infected lime trees from Brazil and Oman (**Figure 3**). While JAZ6 and WRKY33 are up-regulated in infected (symptomatic) Omani samples, the same genes present lower gene expression in infected (asymptomatic) Brazilian samples (**Figure 3**). The same inverse relation can be verified for WRKY70, which is down-regulated in infected Omani samples, but presents a significantly higher expression in Brazilian samples (**Figure 3**). Such expression profiles of this gene set represent a signature of symptomatic and asymptomatic Phytoplasma infected plants, which can be used to distinguish earlier Phytoplasma infections. This specific expression profile can be associated to the distinct "*Ca*. P. aurantifolia"-related strains responsible for different infections (symptomatic and asymptomatic) in lime trees. The differential expression of plant transcriptional regulation-related genes reflects the possible action of strain-specific Phytoplasma effectors, as verified for other plant-Phytoplasma interactions [16].

Finally, we should also address the previously reported benefits of asymptomatic infections for their host plants. Asymptomatic infections may result in induced systemic resistance (ISR) [55]: pathogens acquired at low titres elicit a set of systemic plant defences (i.e., oxidative burst, phytoalexins and pathogenesis-related proteins) which prepare hosts to more successfully resist later, more severe infections [56–58]. The use of ISR to induce resistance in plants by application of exogenous (chemical or organic) inducers, has been used in integrated programs of disease management. Pre-inoculation of sour orange (*Citrus aurantium*) seedlings with a hypovirulent isolate of *Phytophthora Citrus* root rot protected them from later infections [59]. Li et al. [60] have also demonstrated the effects chemical inducers on resistance of *Citrus* groves to HLB disease of *Citrus*. Over-expression of an *Arabidopsis* gene (a positive regulator of ISR) in transgenic "Duncan" grapefruit and "Hamlin" sweet orange increased their resistance to *Citrus* canker [61]. Although ISR may be a useful alternative for disease control, it has to be cautiously assessed. In some cases the use of ISR compounds may not provide the expected protection against disease: for example, spraying ISRs onto sweet orange plants did not reduced incidence of *Citrus* canker [59].

6. Conclusions

This study has addressed two key questions regarding the nature of asymptomatic infections: [1] that being invisible or "silent" infections (and the consequent reliance on molecular tools for detection) makes them inherently challenging to monitor; and [2] that this organism interacts with its plant host in a distinct manner that we have observed in the present study. The key findings are that asymptomatic infections from three case studies all demonstrate high rates of false-negative discovery; meaning that repeated testing of the same plant can give both negative and positive results and that a single positive result is taken as meaning the plant is infected. We also demonstrate that infection by the Phytoplasma "*Ca*. P aurantifolia" is associated with significantly different genetic expression by its acid lime host, giving a first unique insight into the biology of a "silent" infection.

The Phytoplasma "*Ca*. P aurantifolia" is the aetiological agent of Witches' Broom Disease of Lime (WBDL). Although in the Middle East this disease causes high economic impact on lime production, in Brazil emerging infections are notably symptomless [17]. Asymptomatic infections are not particularly rare in plant pathology. *Colletotrichum* fungi, for example, are symbionts that interact with a range of plants as either symptomatic pathogens or asymptomatic endophytes [62]. Yet we do not understand whether the symbiont can use both strategies or if certain strains display the pathogenic or endophytic strategy. Asymptomatic infections also exists for plant

viruses: Pelargonium line pattern virus (PLPV; family Tombusviridae) can be asymptomatic when infecting geranium, which may be due to plant defences such as RNA silencing [63]. In some cases, a resource allocation trade-off mechanism between replication and virulence factor production may explain the emergence of asymptomatic modes of a pathogen, for example, in *Ralstonia solanacearum* populations [64].

As "*Ca.* P. aurantifolia" and other asymptomatic plant pathogens like it spread to novel sites of infection globally [31], and as these infections become more difficult to detect [11], new rapid detection methods will be required in order to effectively detect sources of pathogen and monitor its evolution. This study has presented both the difficulties in monitoring "silent" infections using PCR based methods, but has also identified target genes that behave consistently and distinctly during infection by this Phytoplasma.

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Conflict of interest

The authors declare no conflict of interest.

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