Morphological and physiological adaptations in the vascular system of infected fruit trees provide tolerance against phytoplasma diseases

Jannicke Gallinger[1], Kerstin Zikeli[1], Matthias Zimmermann[2], Louisa Goerg[1], Axel Mithöfer[3], Michael Reichelt[4], Erich Seemüller[1], Jürgen Gross[1], and Alexandra Furch[2]

[1]Julius Kühn-Institut Federal Research Center for Cultivated Plants
[2]Friedrich-Schiller-Universitat Jena
[3]Max-Planck-Institute for Chemical Ecology
[4]Max Planck Institute for Chemical Ecology

April 28, 2020

Abstract

The host-pathogen combinations - Malus domestica (apple)/ 'Candidatus Phytoplasma mali´, Prunus persica (peach)/'Ca. P. prunorum´ and Pyrus communis (pear)/'Ca. P. pyri´ show different courses of diseases although the phytoplasma strains belong to the same 16SrX group. While infected apple trees can survive for decades, peach and pear trees die within some weeks or years. To this date, neither morphological nor physiological differences caused by phytoplasmas have been studied in these host plants. In this study, phytoplasma-induced morphological changes of the vascular system as well as physiological changes of the phloem sap and leaf phytohormones were analysed and compared with non-infected plants. Unlike peach and pear, infected apple trees showed substantial reductions in leaf and vascular morphology, affecting phloem mass flow. In contrast, in pear mass flow and physicochemical characteristics of phloem sap increased. No changes in phytohormone levels were detected in pear but in apple and peach trees, where defence- and stress-related phytohormones increased. Compared with peach and pear trees, data from apple suggest that the long-lasting morphological adaptations in the vascular system, which likely cause reduced sap flow, triggers the ability of apple trees to survive phytoplasma infection. Some phytohormone-mediated defences might support the tolerance.

Keywords

Callose, '*Candidatus* Phytoplasma mali', '*Candidatus*Phytoplasma prunorum', '*Candidatus* Phytoplasma pyri', mass flow, phloem, phytohormones, plant-pathogen interaction

Introduction

The fruit tree diseases apple proliferation (AP), pear decline (PD) and European stone fruit yellows (ESFY), are of high economic significance, causing annual crop losses of around half a billion Euro in Europe, alone (Eurostat 2009; Strauss, 2009). Intra- and interspecific differences in the response of fruit trees to these phytoplasma diseases have been observed over the last decades under both experimental and natural infection conditions (Fiore et al., 2019; Marcone & Rao, 2019). Intraspecific differences are explained by varying susceptibility of tree species and genotypes (rootstocks and cultivars) to phytoplasmas as well as virulence of phytoplasma strains (Kison and Seemüller, 2001; Koncz et al., 2017; Richter, 2002; Seemüller & Schneider, 2007; Seemüller et al., 1986). Fundamental differences between these plant-phytoplasma systems might be based on the degree of adaptation. However, only few studies provide firm data on host response, host–pathogen interaction and on anatomical, physiological and molecular basis of resistance (Seemüller & Harries,

2010), which, thus, is still poorly understood (Marcone & Rao, 2019). While the host plants of the 16SrX phytoplasmas belong to the Rosaceae, the causing agents of the diseases '*Candidatus* Phytoplasma mali', '*Candidatus*Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum' are also phylogenetically closely related and believed to be indigenous to Europe (Jarausch et al. , 2019a; Seemüller & Schneider, 2004). Phytoplasmas are very small bacteria without a cell wall. They have small linear chromosomes, lacking many genes that encode important metabolic functions such as amino and fatty acid synthesis (Kube et al., 2008; Oshima et al. , 2013). Therefore, they need to consume essential metabolites from their plant hosts.

Phytoplasmas are restricted to the phloem sieve elements in their host plants (Seemüller, 2002; Zimmermann et al., 2015). The phloem serves as main route for the long and short-distance transport of mainly organic compounds (Hafke et al., 2005; van Bel, 1996). Sieve elements (SEs), companion cells (CCs) and phloem parenchyma cells (PPCs) are the three phloem cell types involved also in transport of defence- and stress related signalling molecules, such as RNA, proteins, and phytohormones (e.g. Dempsey & Klessig, 2012; Furch et al., 2014; Jung et al., 2009; Park et al., 2007). The sieve element sap is an energy-rich environment, sustaining phytoplasmas with nutrients and enabling them to distribute all over the plant. Therefore, upon phytoplasma infection the impairment of the phloem cells and the change in the phloem sap composition are most likely.

The distribution of secondary compounds plays a crucial role in plant communication and the induction of defence mechanisms against invading pathogens and attacking herbivores. It was previously shown that phytoplasmas produce and secrete effector proteins into phloem cells that induce physiological changes in infected host plants (Sugio et al. , 2011a). A number of non-specific symptoms, such as chlorosis, leaf yellowing, premature reddening, swollen leaf-veins, leaf curl and reduced vigor might be attributed to the impairment of the vascular system and the photosynthesis apparatus (Bertamini et al., 2002; Bertamini et al. , 2004; Maust et al., 2003). Additionally, abnormal growth, stunting, growth of witches' brooms, reduced root size and dwarf fruits occur in phytoplasma infected plants indicating a disturbed hormone balance (Dermastia, 2019). Phytohormones are induced in reaction to abiotic and biotic stresses and lead to the induction of defence responses (Walling, 2000). The influence of phytoplasma infections on salicylic acid, jasmonates, auxins, abscisic acid, ethylene and cytokinine biosynthesis and pathways was recently reviewed by Dermastia (2019), illustrating the diverse and complex interactions between the specialized pathogens and their host plants.

In the case of phytoplasmas, the impact on vector insects that are crucial for the distribution of phytoplasmas, has to be taken into consideration. So far, all phytoplasmas of the group 16SrX causing important fruit crop diseases are vectored by jumping plant lice (Hemiptera: Psylloidea) or succinctly psyllids (Jarausch et al., 2019b). Psyllids nymphs and adults feed on plant phloem and occasionally on xylem sap (Gallinger & Gross, 2018, 2020; Weintraub & Beanland, 2006). Therefore, morphological changes of the plant vascular system may affect psyllid feeding behaviour and suitability of plants as hosts of vector insects. Additionally, phloem/xylem components may influence host choice and oviposition behaviour of psyllids (Gallinger & Gross, 2018, 2020; Mayer et al., 2011). In addition, to detect appropriate host plants for feeding and reproduction, volatile signals are used by many vectoring psyllid species during migration (Gallinger et al., 2019, 2020; Gross & Mekonen, 2005; Mayer et al. , 2008a,b, 2009; Soroker et al. , 2005; Weintraub & Gross, 2013). As plant volatile emission is frequently regulated by phytohormones, their changes in concentrations play an important role on the interplay of vector insects, plants and phytoplasmas (Gross, 2016).

In the present study, we explored how infections with specific fruit tree phytoplasmas ('*Ca* .P. mali', '*Ca* . P. pyri' and '*Ca* . P. prunorum') of the 16SrX group (Seemuller & Schneider, 2004), changed important morphological and physiological parameters of their respective host plants, all Rosaceae (Potter et al., 2007). We measured various parameters such as leaf morphology, plant vascular morphology, and callose deposition, determined physical phloem parameters (mass flow velocity and volumetric flow rate, relative density and dynamic viscosity), and analysed the content of several phytohormones in leaf tissues of healthy and phytoplasma-infected plants. The importance of measured parameters for symptom manifestation as well as the impact on vector insects and phytoplasma spread is discussed.

Materials and methods

Plant material and phytoplasma inoculation

Apple trees (*Malus domestica*) cv. 'Gala Royal' were grown on clonal rootstock cv. 'M9' (non-infected control, n=14), pear trees (*Pyrus communis* L.) cv. 'Williams Christ' were grown on cv. 'Kirchensaller Mostbirne' rootstocks (non-infected control, n=5), and peach trees (*Prunus persica* (L.) cv. 'South Haven' were grown on peach seedlings cv. 'Montclar' (non-infected control, n=4). Additional apple, pear and peach plants were inoculated by grafting of two buds from trees infected with the respective phytoplasmas. Apple trees were infected with a virulent accession (3/6, n=13) in 2017 (Seemuller et al., 2010, 2011, 2013). Pear trees were infected with '*Ca.* P. pyri' (PD-W, n=5) in 2012. This strain causes mild symptoms but no quick decline (Seemuller et al., 1986), peach trees were infected with '*Ca.* P. prunorum' (ESFYQ06, n=4) in 2017. Experiments with pear and peach trees were conducted in 2018. Apple trees were investigated in 2019. All plants were grown under natural conditions in an insect safe environment.

DNA extraction of phytoplasmas

DNA from leaves and phloem scrapings was isolated using a CTAB (cetyltrimethylammonium bromide) extraction method modified from Doyle & Doyle (1990). Due to irregular distribution of '*Ca* . P. pyri' in the top (Seemuller et al. , 1984), in part infection status of pear trees was confirmed by extraction of phloem scrapings from shoots of PD-W inoculated trees, in addition to extraction of leaf tissue from mass flow measurements. Leaves and phloem scrapings were ground in preheated extraction buffer (60 degC, 2.5% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 1% (w/v) polyvinylpyrrolidone 40, 0.2% (v/v) 2-mercaptoethanol (with a tissue/buffer ratio 1:10; 0.1 g of tissue in 1 ml buffer) using a homogenizer (BIOREBA AG, Reinach, Switzerland) in extraction bags (BIOREBA AG). Homogenate (1 ml) was transferred into a microcentrifuge tube and incubated at 60 degC for 30 min. An equal volume of chloroform was added, the tube was briefly vortexed and shook for 5 min at room temperature. After a centrifugation step (10,000 *g* , 6 min at room temperature, Heraeus Fresco 17 Microcentrifuge, Thermo Fisher Scientific, Dreieich, Germany) the aqueous phase was transferred into a new centrifuge tube. For precipitation of nucleic acids an equal volume of isopropanol was added, the tube was inverted and incubated at 4 °C overnight. Precipitate was recovered by centrifugation at 10,000 *g* for 10 min at room temperature. Supernatant was discarded and the nucleic acid pellet was washed with 70% ethanol (centrifugation step at room temperature, 10,000 *g* , 10 min), air dried and resuspended in 50 µl high-performance liquid chromatography (HPLC) water (VWR International GmbH, Bruchsal, Germany). Unless explicitly stated elsewhere, laboratory chemicals were purchased from Carl Roth GmbH (Karlsruhe), Bernd Kraft GmbH (Duisburg) and Sigma-Aldrich Chemie GmbH (Taufkirchen), Germany, respectively.

Real-time PCR

Quantitative PCR (qPCR) was performed with the Bio-Rad CFX96 Thermal Cycler (Bio-Rad Laboratories GmbH, Munich, Germany) using primer pair and probe of a TaqMan assay developed by Christensen et al. (2004) for the generic detection of phytoplasma DNA. The amplification of a part of the 16S rDNA gene was performed in 25 µl reactions containing 1 µl of DNA extraction, 0.625 U of FastGene Taq DNA Polymerase (Nippon Genetics Europe GmbH, Düren, Germany) with provided 10 x reaction buffer A (with 1.5 mM $MgCl_2$), 0.5 µl of dNTPs (10 mM each, Steinbrenner Laborsysteme GmbH, Wiesenbach, Germany), 1 µl of each primer (10 µM, Eurofins Genomics Germany GmbH, Ebersberg, Germany), 0.5 µl of TaqMan probe (10 µM, Eurofins Genomics Germany GmbH), and HPLC water (VWR International GmbH). Amplification parameters were 15 min at 95 °C followed by 46 cycles at 95 °C for 15 s and 60 °C for 1 min. Data analysis was performed with the BioRad CFX Manager 3.0 software (Bio-Rad Laboratories GmbH, Munich, Germany).

Calculation of phytoplasma titre

Phytoplasma concentration (number of copies of phytoplasma 16S rDNA gene per µl) was calculated automatically from the quantification cycle (C_q) values by the use of a cloned 16S rDNA gene standard ranging from 10^1 to 10^9copies in qPCR with the internal manufacturer's software (referred to as estimated qPCR

concentration). Samples with C_q values higher than 30 were considered as tested negative (see Table S1; value of control DNA from healthy trees maintained under insect-proof conditions). Calculation of phytoplasma cells per gram wet weight of extracted leaf tissue and phloem scrapings was performed by multiplying the assessed qPCR concentration by the applied volume of extraction buffer (Y) and the volume of HPLC water used for DNA resuspension, by dividing by the number of 16S rDNA operons (2) and the wet weight of extracted leaf tissue (X):

$$\text{Phytoplasma titer } \left[\frac{\text{cells}}{g}\right] = qPCR \textit{ concentration } \left[\frac{\varsigma o\pi\iota\varepsilon\varsigma}{\mu\lambda}\right] * (Y * 50 * \frac{1}{2} * \frac{1}{X}) \left[\frac{\mu\lambda}{}\right.$$

englishg

Experimental set-ups

From each tree, two (pear and peach) or four (apple) mature leaves were randomly selected. The mass flow measurements were done *in vivo* . Thereafter, the leaves were cropped, length and width of the leaf lamina were measured, cross sections were done to analyse the mass flow rate, the middle part of the midrib was fixed in a fixative (see below), and the rest of each leaf was used for phytoplasma titre determination. Leaves for phytohormone measurements were separately collected, immediately frozen in liquid nitrogen and stored at -20 °C until used.

Determination of symptoms and leaf morphology

Next to the general observation and assessment of the known symptoms following a phytoplasma infection the impact on the morphology of apple, pear and peach leaves was investigated with the determination of the length and maximum width of the leaf lamina. Photographs (Canon EOS 760D, Canon Deutschland GmbH, Krefeld, Germany) were taken to document characteristic symptoms at whole plant and leaf level.

Microscopic analyses of theplant vascular morphology and callose deposition

Cross sections of the midribs were created halfway from the base to the tip of each leaf. Therefore, pieces of about 1 x 1 cm were fixed in 2.5% (w/v) glutaraldehyde, 2% (v/v) paraformaldehyde in 0.1 M sodium-potassium phosphate buffer (pH 7.4, Merck KGaA, Darmstadt, Germany). Sections were cut at a thickness of 20 µm with a cryostat (Leica JUNG CM3000, Leica Microsystems, Wetzlar, Germany) at a chamber temperature of -26 °C and a specimen head temperature of -23 °C. Pieces were bound to a specimen disc by embedding them into plant tissue freezing medium (Jung, Leica Microsystems, Wetzlar, Germany) and then frozen at the quick freeze shelf for 10 min prior sectioning. Each cross section was stained for at least 30 min with 0.1% aniline blue solution (Sigma Aldrich, St. Louis, Missouri, USA) to visualize callose deposition at sieve plates.

Each cross section was imaged using an AXIO Imager.M2 (Zeiss Microscopy GmbH, Jena, Germany) equipped with a 10x objective (N-Achroplan 10x/0.3) and a 40x objective (W N-Achroplan 40x/0.75). The bright field and fluorescence images were recorded with a colour camera (AXIOCAM 503 colour Zeiss, Jena, Germany) by use of a DAPI (EM 445/50 nm) filter. Each digital image of infected and healthy cultivars was analysed with the determination of (1) the diameter of midribs, (2) the area of the vascular bundle, (3) the xylem area, (4) the phloem area and (5) the area of 10 sieve elements per section using the ZEN®software (Zeiss, Jena, Germany). The digital images were processed with ZEN® software and edited with Adobe®PhotoShop to optimize brightness, contrast and colouring. The intensity of aniline blue fluorescence was measured using ZEN®software by analysing the whole phloem area as region of interest (ROI) and ROIs of healthy and infected plants were comparatively evaluated.

Determination of the phloem mass flow velocity

The phloem mass flow rate was measured with the phloem mobile fluorochrome 5,6-carboxyfluorescein diacetate (CFDA) dye (ThermoFisher Scientific, Waltham, Massachusetts, USA). CFDA permeates the plasma

membrane in the non-fluorescent acetate form and is cleaved by cytosolic esterases generating membrane-impermeant fluorescent carboxyfluorescein (CF) (handbook from Molecular Probes, Eugene, OR, USA). CF is trapped inside SEs and transported by mass flow in the sieve tubes. A stock solution was prepared by solubilisation of 1 mg CFDA in 1 ml DMSO. A working solution of 1 µl stock solution in 1 ml buffer solution (containing 2 mol m^{-3} KCl, 1 mol $m^{-3}CaCl_2$, 1 mol $m^{-3}MgCl_2$, 50 mol m^{-3} mannitol, and 2.5 mol m^{-3} MES/NaOH buffer, pH 5.7) was applied at a cut leaf tip. After an inoculation period of 1 to 2 h at room temperature, each leaf was removed from the plant. Immediately, cross sections of the mid ribs were made by hand with a sharp and fresh razor blade in one centimetre intervals from the basal side of the leaf. Sections were covered with distilled H_2O, a cover glass and examined for appearance of fluorescence emitted from CF (emission 510-580 nm), by the means of an inverted fluorescence microscope (AxioVert S100, Carl Zeiss, Jena, Germany). The transport velocity was calculated by dividing the measured distance the CF moved within the sieve elements from the application side towards the leaf base by the exact inoculation time (from dipping one leaf tip into CFDA to removing of the specific leaf from the plant).

Calculation of the volumetric flow rate

The volumetric flow rate (J_V) was calculated by multiplying the measured phloem mass flow velocity (V_a) by the median area of ten measured sieve elements ($\tilde{A}_{SE}$):

$$J_v\ [\frac{cm^3}{h}] = V_a\ [\frac{\mathrm{cm}}{h}] *\ \tilde{A}_{\mathrm{SE}}\ [cm^2]$$

Determination of phytohormones

Four leaves were harvested of each tree, immediately frozen in liquid nitrogen, and stored at -20 °C. The leaves of each tree were pooled and 250 mg (per sample and two samples for each tree) were homogenized using a Geno/Grinder® (Spex SamplePrep, Stanmore, UK) at 1100 rpm for 1 min and extracted in 1.5 ml methanol containing 60 ng D4-SA (Santa Cruz Biotechnology, USA), 60 ng D6-JA (HPC Standards GmbH, Germany), 60 ng D6-ABA (Santa Cruz Biotechnology, USA), 12 ng D6-JA-Ile (HPC Standards GmbH), and D5-indole-3-acetic acid (D5-IAA, OlChemIm s.r.o., Olomouc, Czech Republic) as internal standards. Samples were agitated on a horizontal shaker at room temperature for 10 min. The homogenate was mixed for 30 min and centrifuged at 13,000 rpm for 20 min at 4 °C; the supernatant was collected. The homogenate was re-extracted with 500 µl methanol, mixed and centrifuged; the supernatants were pooled. The combined extracts were evaporated under reduced pressure at 30 °C and dissolved in 500 µl methanol.

Phytohormone analysis was performed by LC-MS/MS as in Heyer et al. (2018) on an Agilent 1260 series HPLC system (Agilent Technologies) with the modification that a tandem mass spectrometer QTRAP 6500 (SCIEX, Darmstadt, Germany) was used. Details of the instrument parameters and response factors for quantification can be found in Table S2.

Indole-3-acetic acid was quantified using the same LC-MS/MS system with the same chromatographic conditions but using positive mode ionization with an ion spray voltage at 5500 eV. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion - product ion fragmentations as follows: m/z 176 - 130 (collision energy (CE) 19 V; declustering potential (DP) 31 V) for IAA; *m/z* 181 -133 + *m/z* 181 -134 + *m/z* 181 -135 (CE 19 V; DP 31 V) for D5-IES.

Collection of phloem sap

Phloem saps were sampled applying centrifugation technique according to Hijaz and Killiny (2014). Briefly, the bark from young flush of *M. domestica* , *P. communis* and *P. persica* trees was manually removed with a clean scalpel and sliced into 2 cm pieces. After removing the bottom of a 0.5 ml Eppendorf tube, the tube was immersed in a second, larger tube (1.5 ml). Bark pieces were placed into the 0.5 ml tubes and centrifuged at 12,000 rpm at 4 °C for 10 min. The extracted phloem sap was collected and the refractive index, the density and the viscosity were determined.

Determination of the refractive index

The refractive index of the phloem sap was determined by the means of a handheld refractometer (type 45-81; Bellingham + Stanley Ltd., Tunbridge Wells, UK) and specified as °Brix. The refractometer was standardized for sucrose.

Δετερμινατιον οφ τηε δενσιτψ οφ ασσυλαρ σαπς (ρ)

The density of the phloem sap was measured using 0.5 µl glass capillaries (CAMAG®, Muttenz, Switzerland) allowing the distinct determination of bulk and volume. The bulk of empty and filled capillaries was separately measured (Analytical Balance, Sartorius Weighing Technology GmbH, Göttingen, Germany) and subtracted to determine the pure mass of the phloem sap. Simultaneously, the corresponding volume inside the capillaries was calculated and the density was converted to gram per litre (g l^{-1}).

Δετερμινατιον οφ τηε δψναμις ισχοσιτψ οφ ασσυλαρ σαπς (η)

The measuring instruction of the dynamic viscosity was described in Adam et al. (2009). The dynamic viscosity of the phloem sap was quantified with 0.5 µl glass capillaries (CAMAG®, Muttenz, Switzerland). The calibration required the determination of the specific viscometer constant ($\varkappa$ = mPa l g^{-1}) by using H_2O as reference solution with known dynamic viscosity (η_0 = 1.0087 mPa s) and density (ρ_0 = 997.9 g l^{-1}) for 20 °C. Following the measurement of elapsed time (t_0), the specific viscometer constant was calculated:

$$\kappa \; = \; \frac{\eta_0}{\rho_0 \; t_0} \; (\frac{\text{mPa l}}{g})$$

The dynamic viscosity of the phloem sap was calculated after the measurement of elapsed time (t) and determination of density (ρ):

$\eta = \varkappa * \rho * t$ (mPa s)

Statistics

All statistical analyses were performed using R version 3.5.1 (R Core Team, 2019). The data visualization was done with the package 'ggplot2' (Wickham, 2016).

Mass flow, volumetric flow rate, morphology data and functional/physiological parameters: Linear mixed effect (LMM) or generalized linear mixed models (GLMM) were used to determine the effect of phytoplasma infection on phloem mass flow, volumetric flow rates and morphological and functional parameters (phloem sap viscosity and density) in *M. domestica* , *P. persica* and *P. communis* leaves. To account for non-independent errors, which may occur due to repeated measurements at each tree, trees were specified as a random factor in all models. Models with different error distributions and link-functions were compared by AICc (Akaike information criterion with correction for small sample size) with the *AICctab* function from the 'bbmle' package (Bolker & R Development Core Team, 2017). Models with the lowest AICc values were used if model assumptions were valid. LMMs were fitted with the *lmer* function from the 'lme4' package (Bates et al. , 2015), and Type III analysis of variance (ANOVA) with Satterthwaite's method, which was calculated with the*Anova* function from the 'lmerTest' package (Kuznetsova et al. , 2017). GLMMs were fitted with the *glmer* function from the 'lme4' package, and Type II analysis of variance was calculated with the *Anova* function from the 'car' package (Fox & Weisberg, 2019) to determine treatment effects. Used error distribution, link-function and ANOVA results are specified in the Tables S3-S5 in the Supporting Information.

Phytohormone data and Brix values: Linear models were fitted to determine the influence of phytoplasma infections on the concentration of phytohormones and the relative density of phloem sap in *M. domestica* , *P. persica* and *P. communis* plants. In case of non-normality of the residuals the data was log, square root or box-cox transformed as specified in Table S6. Variance heterogeneity was detected in abscisic acid content in samples from *P. communis* . In this case the generalized least squares method (GLS) was applied

with the *gls* function of the 'nlme' package (Pinheiro et al., 2019). The different variance in the treatments was incorporated into the model with the varIdent variance structure. Treatment effects were calculated by Type I analysis of variance.

Callose deposition: Linear models were fitted with the GLS method, to model the different variance structures of the data with the varIdent function. Treatment effects were calculated by Type I analysis of variance (Table S7).

General procedure: For all models, the estimated marginal means (EMMs) and corresponding 95% confidence intervals were calculated and used to determine differences between treatment levels with the 'emmeans' package (Lenth, 2019). All model assumptions were validated graphically as recommended by Zuur et al. (2009).

Results

Phytoplasma infection affects leaf and vascular morphology.

We first investigated and compared the effects of phytoplasma infection on leaf and vascular morphology. The successful infections resulted in known visible disease symptoms: witches' broom and enlarged stipules in apple trees, premature foliar reddening in pear trees and chlorosis and suberization in peach trees (Figure 1). The symptoms indicated impairments in the leaf development, which was further examined by a comparison of the leaf lamina, midrib sizes and their ratios among infected and healthy plants. It was found that leaves of AP-infected apple trees were significantly ($p<0.05$) smaller (length -17% and width -22%) and the diameter of midribs were significantly reduced (-27%) compared to those of healthy plants (Table 1). The phytoplasma infection in apple trees did not affect the leaf size ratio and the midrib ratio (Figure 1a). In pear, basing upon a significant increase of the leaf width (+8.5%), a significant decrease of the leaf size ratio of nearly 9% was observed, but no changes for the midrib ratio were found (Figure 1b). In contrast to apple and pear plants, phytoplasma infected peach trees exhibited a significant rise of the leaf size ratio of +13% and the midrib ratio of +16% (Figure 1c). No significant changes were found for leaf length, width and midrib diameter (Table 1). All morphological results demonstrated the heterogeneity of the symptoms and indicated differences in the individual host-pathogen interactions.

The specific impact of the phytoplasma infection on the vascular morphology was investigated by analysing the areas of vascular bundle, xylem, phloem and SEs as well as the ratios of xylem to phloem and SE to phloem (Figures 2-4). For apple, the phytoplasma infection exhibited significantly ($p<0.05$) degraded areas of the vascular bundle (-39.1%), xylem (-49.8%), phloem and SE (-33.7%) in comparison to healthy plants, whereas the ratio of SE to phloem was not affected (Figure 2b). Phytoplasma infected pear trees did not show any changes (Figure 3), whereas in peach trees infected with ESFY, the mean sieve element area (-26%) and the ratio of SE to phloem (-46.9%) decreased significantly (Figure 4b). Confirming the heterogeneity of the morphological results (Figure 1), different disease patterns were also found on the cellular level of the vascular system for apple, pear and peach (Figures 2-4).

The translocation situation and phytohormone distribution are different in the individual host-pathogen-systems.

We next examined the consequences of the morphological changes (Figures 2-4) on the physiological situation within the sieve elements. In apple leaves the phloem mass flow velocity and the calculated volumetric flow rate decreased significantly ($p<0.05$) in infected leaves in comparison to healthy ones by - 25% and -58%, respectively (Figure 5a). In pear leaves the phloem mass flow velocity and the volumetric flow rate increased significantly by +32.6% and +46.6%, respectively (Figure 5b). In peach leaves the phloem mass flow velocity was not affected, but the volumetric flow rate decreased significantly (-30.8%; Figure 5c).

The varying effects for the phloem mass flow indicated changes in the flow properties of the phloem sap. Thus, the dynamic viscosity, density and refractive index of phloem sap obtained by bark tissue centrifugation were measured (Table 2). No changes of the refractive index for apple and peach were found. In contrast, the dynamic viscosity in infected pear plants was doubled (+104%) and the relative density increased strongly

(+97.7%). Unfortunately, the peach plants did not deliver enough phloem sap volume for a complete analysis. For this reason, only the relative density was determined without any significant changes. Furthermore, a comparative analysis of the phloem's relative density between apple, pear and peach revealed significant differences, illustrating a plant specificity of the phloem sap composition regarding total sugar content. The measured/calculated phloem mass flow parameters again exhibit heterogeneous effects of a phytoplasma infection (Figure 5, Table 2) and confirmed the variability of previously shown anatomical/morphological results (Figures 1 to 4).

To obtain indications for the changed mass flow translocation of the individual plant-phytoplasma variations, the callose deposition in the SEs was visualized and its intensity analysed (Figure 6). No differences of callose depositions were found for the phytoplasma infection, in comparison to healthy apple trees (Figure 6a). In contrast, an increase of callose deposition was found in peach (+300%) and pear (+67%; Figures 6b+c), showing a stronger impact on the anatomical and physiological balance in comparison to apple trees.

The stress level of the plants was explored by the measurement of salicylic acid (SA), jasmonic acid-isoleucine (JA-Ile), jasmonic acid (JA), abscisic acid (ABA), 12-oxo-phytodienoic acid (cis-OPDA) and indole-3-acetic acid (IAA) in leaves (Figures 7 and S1; Table S8). Upon an infection with the virulent accession 3/6, in apple, SA (+109%), ABA (+55%) and JA-Ile (+78%) increased significantly ($p<0.05$) whereas a significant decrease of cis-OPDA (-45%) and no changes of JA and IAA were observed (Figures 7a and S1). No significant changes for the several measured phytohormones were detected in pear trees due to infection (Figure 7b). In peach trees, SA (+192%), JA-Ile (+345%) and IAA (-40%) were significantly changed in infected plants, whereas JA and ABA did not show any significant changes (Figures 7b and S1). Moreover, the basic level of SA, ABA and cis-OPDA differed among healthy apple, pear and peach plants. For example, ABA was 6-fold higher in pear and 3-fold higher in peach compared to apple.

In accordance with the obtained morphological and functional results, also the effect of phytoplasma infections on the stress-related phytohormone contents revealed different patterns among particular host-pathogen combinations.

Discussion

Although the phytoplasmas '*Candidatus* Phytoplasma mali´, '*Ca.* P. prunorum´ and '*Ca.* P. pyri´ belong to the same 16SrX group, their pathogenicity is quite different in their respective host plants *Malus domestica* (apple), *Prunus persica*(peach) and *Pyrus communis* (pear). Apple trees can survive a phytoplasma infection for decades, whereas phytoplasma-infected peach and pear trees often die after a few years and sometimes even after a few weeks (quick decline of pear) (Fiore et al*.,* 2019; Marcone et al., 2010; Marcone & Rao 2019; Seemüller et al., 1986, 2018). This indicates a higher tolerance resulting in better survival rate for phytoplasma-infected apple compared with pear and peach; however, the underlying reason is not known yet. To address this open question, we collected a comprehensive dataset (summarized in Table 3) covering anatomical and physiological responses of each plant species to its particular phytoplasma infection, supporting a co-evolutionary impact. Based on these results we are discussing several implications thereof in the following paragraphs.

Phytoplasma infections affect the vascular morphology of apple trees more than peach and pear trees.

Although apple trees survive phytoplasma infection for decades (Seemüller et al., 2018), various significant reductionsin size were found in leaves (width, length, midrib), tissues (vascular bundle, phloem and xylem) and cells (sieve elements) when compared to non-infected plants (Figures 1 and 2). In contrast, pear and peach trees showed less significant differences between healthy and phytoplasma-infected leaves; if any, we found significant increases for leaf size and midrib ratio for peach and leaf width for pear (Table 3). That seems surprising as it could be expected that plants with a higher tolerance and survival rate would show a lower rate of symptoms than plants demonstrating a higher mortality (Marcone & Rao 2019). Additionally, the recovery phenomenon, describing the remission of symptoms and the disappearance of phytoplasmas in the crown, was observed for both, apple and apricot trees (*Prunus armeniaca*), but not for pear trees (Carraro et al., 2004; Musetti et al., 2013). Hence, the morphological and physiological changes can be considered for

representing the ability to handle a phytoplasma infection and might be the result of a selective adaptation. Intraspecific comparisons of these parameters between plant genotypes of different sensitiveness to the same phytoplasma isolate could be used in future studies to confirm or refuse this hypothesis. In particular, the *P. communis* /'*Ca.* P. pyri´–system is of high scientific interest, as disease severity shows huge variance from mild symptoms, such as premature foliar reddening, to severe growth depression and the quick decline of infected trees (Seemüller et al., 1986).

The pear and peach phloem reactions are very sensitive to phytoplasma infections whereas apple is coping with the infection .

All observed results regarding the particular morphological (Figures 1 to 4) and functional measurements (Figure 5) illustrate well the consequences of a phytoplasma infection for a plant: However, depending on the individual host-pathogen system, they are heterogeneous between the systems and specific within. One reason for the heterogeneity might be found in the formation of plant defence. A general defence response to several (a)biotic stresses is an elevated Ca^{2+}-dependent deposition of callose that was already reported for phytoplasma infections (Chen & Kim, 2009; Musetti et al., 2013). We were able to show that *P. communis* and *P. persica* trees responded to phytoplasma infections by blocking sieve plates with callose. Phytoplasma effectors may cause regulating of Ca^{2+} channels, which leads to sieve-tube occlusion with dramatic effects on photoassimilate distribution as indicated by the reduced volumetric flow rate in *P. persica* trees. Surprisingly, the mass flow of infected *P. communis* trees was increased though a simultaneous increase of phloem sap viscosity, which reflects an increased sugar content. The reason may be an increased pressure gradient (we calculated ˜6.5 bar using the van-'t-Hoff equation) in infected trees, which drives the mass flow against the resistance of the SEs. Thus, *P. communis* trees have to take major effort with increased energy supply, which eventually causes negative feedback. As the callose deposition in response to phytoplasma infections never results in a restriction of the bacteria it suggests that callose deposition only is a costly non-functional leftover of general defence mechanisms. Strikingly, the infection with '*Ca* . P. mali' in apple trees did not lead to an increased callose deposition. This might be due to the particular apple cultivar, phytoplasma strain, specific defence mechanisms or an evolutionary adaptation to the phytoplasma infection. The apple-phytoplasma interaction might be older and better adapted compared to peach and pear. Whether the callose deposition is directly or indirectly induced by phytoplasmas, is an issue for future studies.

Callose deposition also is a defence mechanism against phloem-feeding and is induced by phloem feeding insects (Hao et al. , 2008; Will et al., 2013). Therefore, callose concentrations are of great importance for phloem-feeding vector insects carrying phytoplasmas. The occlusion of sieve tubes inhibits the phloem flow and affects the feeding of piercing-sucking insects on the phloem tissue of host plants (Will et al. , 2009). Nevertheless, the brown plant hopper*Nilaparvata lugens* is able to overcome this plant defence by activating and secreting a hydrolysing enzyme, which induces the degradation of callose in SEs (Hao et al. , 2008). Whether psyllid species transmitting AP, PD and ESFY (AP: *C. picta* ; PD: *C. pyri, C. pyrisuga* and *C. pyricola* ; ESFY: *C. pruni*) have evolved such mechanisms to overcome this particular plant defence is still unknown. Yet, it was shown that phloem ingestion of *C. pruni* was not influenced by phytoplasma infection of its host plants (*P. persica* and *P. insititia*), indicating that callose deposition in infected peach plants does not affect vector feeding (Gallinger & Gross, 2020).

In general, sugars (e.g. sucrose) are known to stimulate feeding of phloem-feeding insects such as aphids (Arn & Cleere, 1971; Mittler & Dadd, 1963). Thus, the detected higher sugar concentration in infected pear phloem could increase probing and feeding behaviour of psyllids and, therefore, increase the acquisition and spread of phytoplasmas in pear orchards. However, a recent detailed phloem composition analysis of*Prunus* trees revealed no major differences in the phloem metabolite composition between ESFY infected and healthy trees (Gallinger & Gross, 2020).

Apple trees are able to restrict the phytoplasma infection via phytohormone signalling.

Besides the direct and local defence mechanisms, an activation and systemic distribution of signalling compounds such as phytohormones via the SEs, may be induced by phytoplasmas and may have an impact

on the plant's defence. Moreover, typical symptoms, such as development of witches' broom, smaller fruits, reduced leaf and vascular morphology of diseased apple trees, can be explained with an infection-induced imbalance of phytohormones. In apple and peach trees, SA and JA-Ile levels significantly increased in infected trees, indicating the involvement of defence pathways to phytoplasma colonization. Furthermore, the content of ABA in apple leaves increased as well. Commonly, SA plays the central role for the interaction between biotrophic pathogens and host plants (Ma & Ma, 2016; Robert-Seilaniantz et al. , 2011) and an increase of SA in apple treesafter*Ca.* P. mali infection was found earlier (Zimmermann et al*.,* 2015). In contrast, the jasmonic acid pathway is induced in response to wounding, herbivore attack and necrotrophic pathogens (Heil & Ton, 2008). The development of different pathways in reaction to different threads enables plants to respond more specifically and is therefore more resource-efficient. An antagonistic crosstalk between JA/ABA and SA was detected in several plant species (Flors et al. , 2005; Zimmermann et al., 2015). Not surprisingly, some bacteria species evolved the production of effector proteins that interfere with SA regulated defence responses by activating JA pathway (Chisholm et al. , 2006). This mechanism was also detected for phytoplasmas in Aster yellows-witches' broom phytoplasma (AY-WB). AY-WB produces the SAP11 effector that down-regulates the plant defence response by reducing lipoxygenase2 expression and JA production (Sugio et al. , 2011b). This down-regulation of defence mechanisms in AY-WB infected plants is advantageous to vector fitness (Sugio et al. , 2011b). Recently, an SAP11-like protein was detected in '*Ca.* P. mali' that affected JA, SA and ABA pathways (Siewert et al. , 2014; Janik et al. , 2017). Jasmonic acid plays a central role in induced plant defence e.g. by regulating the biosynthesis of herbivore-induced plant volatiles (Heil & Ton, 2008). Moreover, exogenous application of JA can be used to elicit plant defence responses similar to those induced by biting-chewing herbivores and mites that pierce cells and consume their contents. A low-dose JA application results in a synergistic effect on gene transcription and an increased emission of a volatile compounds involved in indirect defence after herbivore infestation (Menzel et al., 2014). The induction of JA defence mechanisms in apple, pear and peach in response to psyllid feeding has not yet been proven. However, infestations of *Citrus*plants with Asian citrus psyllid (ACP, *Diaphorina citri*) led to an upregulation of genes involved in the JA-pathway (Nehela et al. , 2018). Additionally, the infection of Citrus trees with the phloem dwelling proteobacterium *Candidatus* Liberibacter asiaticus induced the SA-pathway (Nehela et al. , 2018) and resulted in an increased emission of methyl salicylate from infected plants (Martini et al. , 2018). Auxins (IAA and IBA) were shown to induce the recovery of periwinkle plants from '*Ca.* P. pruni' and '*Ca.*P. asteris' infections (Curković Perica, 2008), illustrating the importance of IAA in plant-pathogen interactions. Interestingly, the IAA concentration in infected *P. persica* plants was significantly lowered compared to healthy peach trees. A reduced auxin content was also detected in leaves of lime infected with '*Ca.* P. aurantifoliae' (Zafari et al. , 2012). Imbalanced auxin concentrations might be responsible for abnormal growth of infected peach trees (Figure 1c).

Overall, it has to be considered that such measurements merely represent a snapshot of time. Indeed, various results from phytohormone analysis in AP-infected apple plants are reported in the literature (Janik et al., 2017; Zimmermann et al. , 2015), indicating that reactions to phytoplasma infections depend on season, cultivar and environmental conditions. Consequently, exact phytohormone and phloem sap analysis over a longer period (season) in correlation to respective phytoplasma titres are needed to draw reliable conclusions about symptom development.

The vector of apple proliferation behaves more adapted to phytoplasma infection than the other vectors.

By analysing VOCs (volatile organic components) emitted by the leaves of apple trees, it was shown that '*Ca* . P. mali' changed the VOCs composition of infected trees compared to healthy ones by inducing the sesquiterpene β-caryophyllene (Mayer et al. , 2008a,b). The main vector of '*Ca* . P. mali', the apple psyllid *Cacopsylla picta* , reproduces on apple and overwinters on conifers. The adults of the new generation (emigrants) are attracted by β-caryophyllene and lured to infected apple trees (Mayer et al. , 2008b), before migrating to their overwintering host. This behaviour increases the number of psyllids, which are able to acquire '*Ca* . P. mali'. By returning in early spring to apple trees, they prefer healthy apple trees for oviposition, in order to avoid detrimental effects of the phytoplasma on the offspring development (Mayer et al., 2011). As they need to feed before oviposition, they transmit the phytoplasma to healthy apples. This is

a perfectly balanced transmitting system, which improves the spread of the phytoplasma without negatively impacting the vector. A similar adaptation of *C. pruni* , the migrating vector of '*Ca* . P. prunorum' is unknown yet. This species did not distinguish its host plants by odour but phloem constitution (Gallinger et al*.,* 2019; Gallinger & Gross, 2020). As the development on *P. persica* infected by '*Ca* . P. prunorum' had no detrimental effects on the vector (Gallinger & Gross, 2020), there was no selection pressure on distinguishing between infected and uninfected host plants as observed in apple (Mayer et al., 2011). It has to be understood that the two main vectors of '*Ca* . P. pyri', *C. pyri* and *C. pyricola*do not migrate between different host plant species, therefore volatile signals might be less important in host choice of this psyllid species (Jarausch et al. , 2019a).

Conclusion

In the three investigated fruit crops the infection with specific phytoplasmas induced different morphological and physiological responses in the particular host plants. As apple trees generally survive a phytoplasma infection more often and much longer than peach and pear, some unique apple-specific responses are most interesting and indicative features that could explain how a plant might become tolerant against phytoplasma. Based on the results obtained, the long-lasting changes in the structure of the vascular system with all physiological consequences on the sap flow found in apple trees provides a promising step towards a deeper understanding of host plant defence against phytoplasma. Despite the growing understanding of this pathosystem, it seems clear that the complexity of these interactions is not fully elucidated, yet, and many open questions remain: Does the plant perceive a phytoplasma infection at all? If so, what does the plant recognize? Is there a MAMP/DAMP/effector present that induces an increased defence response in the SEs? What are the specific events during infections in the host on spatio-temporal and intensity level? How do the antagonists interact on the molecular level? All these questions require more investigations on the molecular level including for example RNAseq and transgenic approaches.

Acknowledgements: We thank Sebastian Faus and Katharina Piwowarczyk (JKI, Dossenheim, Germany) for excellent assistance in the laboratory. We thank Felix Hergenhahn (JKI, Dossenheim, Germany) for grafting and cultivation of the plants. We thank Andrea Lehr (MPI for Chemical Ecology, Jena, Germany) for technical support. We are grateful to Dr. Eva Gross (Schriesheim, Germany) for language editing. This work was supported by the Deutsche Forschungsgemeinschaft (Grant FU 969/2-1). Jannicke Gallinger was supported by a fund of the "Landwirtschaftliche Rentenbank" number 28RF4IP008.

References

Adam G., Läuger P. & Stark G. (2009). Physikalische Chemie und Biophysik. Springer Berlin Heidelberg.

Ambrožič-Dolinšek J., Camloh M., Žel J., Kovač M., Ravnikar M., Carraro L. & Petrovič N.(2008). Phytoplasma infection may affect morphology, regeneration and pyrethrin content in pyrethrum shoot culture. *Scientia Horticulturae,* 116, 213–218.

Arn H. & Cleere J.S. (1971). A double-lable choice-test for the simultaneous determination of diet preference and ingestion by the aphid*Amphorophora agathonica* . *Entomologia Experimentalis et Applicata,* 14, 377–387.

Bates D., Mächler M., Bolker B. & Walker S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software,*67, 1–48.

Bertamini M., Grando M.S., Muthuchelian K. & Nedunchezhian N. (2002). Effect of phytoplasmal infection on photosystem II efficiency and thylakoid membrane protein changes in field grown apple (*Malus pumila*) leaves. *Physiological and Molecular Plant Pathology,* 61, 349–356.

Bertamini M., Grando M.S. & Nedunchezhian N. (2004). Effects of phytoplasma infections on pigments, chlorophyll-protein complex and photosynthetic activities in field grown apple leaves. *Biologia Plantarum,* 47, 237–242.

Bolker B. & R Development Core Team. (2017). bbmle: Tools for General Maximum Likelihood Estimation.

Carraro L., Ermacora P., Loi N. & Osler R. (2004). The recovery phenomenon in apple proliferation-infected apple trees. *Journal of Plant Pathology,* 86, 141–146.

Chen X.-Y. & Kim J.-Y. (2009). Callose synthesis in higher plants.*Plant Signaling & Behavior,* 4, 489–492.

Chisholm S.T., Coaker G., Day B. & Staskawicz B. (2006). Host-microbe interactions: shaping the evolution of the plant immune response.*Cell,* 124, 803–814.

Christensen N.M., Nicolaisen M., Hansen M. & Schulz A. (2004). Distribution of phytoplasmas in infected plants as revealed by real-time PCR and bioimaging. *Molecular Plant-Microbe Interactions* ,17, 1175–1184.

Curković Perica M. (2008). Auxin-treatment induces recovery of phytoplasma-infected periwinkle. *Journal of Applied Microbiology,*105, 1826–1834.

Dempsey D.A. & Klessig D.F. (2012). SOS – too many signals for systemic acquired resistance? *Trends in Plant Science,* 17, 538- 545.

Dermastia M. (2019). Plant Hormones in Phytoplasma Infected Plants.*Frontiers in Plant Science,* 10, 1–15.

Doyle J.J. & Doyle J.L. (1990). Isolation of plant DNA from fresh tissue.*Focus,* 12, 13–15.

Eurostat Jahrbuch der Regionen. (2009). *Amt für Veröffentlichungen der Europäischen Union* . ISBN 978-92-79-11695-7.

Fiore N., Bertaccini A., Bianco P.A., Cieslinska M., Ferretti L., Hoat T.X. & Quaglino F. (2019). Fruit Crop Phytoplasmas. In: Phytoplasmas: Plant Pathogenic Bacteria - I. Characterisation and Epidemiology of Phytoplasma-Associated Diseases (eds A. Bertaccini, P.G. Weintraub, G.P. Ra, N. Mori), pp. 153–190. Singapore: Springer Singapore.

Flors V., Ton J., Jakab G. & Mauch-Mani B. (2005). Abscisic Acid and callose: Team players in defence against pathogens? *Journal of Phytopathology,* 153, 377–383.

Fox J. & Weisberg S. (2019). *An R Companion to Applied Regression,*third. Thousand Oaks, California: Sage.

Furch A.C.U., Zimmermann M.R., Kogel K.-H., Reichelt M. & Mithöfer A. (2014). Direct and individual analysis of stress-related phytohormone dispersion in the vascular system of *Cucurbita maxima* after flagellin 22 treatment. *New Phytologist,* 201, 1176-1182.

Gallinger J. & Gross J. (2018). Unraveling the host plant alternation of Cacopsylla pruni – adults but not nymphs can survive on conifers due to phloem/xylem composition. *Frontiers in Plant Science,* 9, 686.

Gallinger J., Dippel C. & Gross J. (2019). Interfering host location of*Cacopsylla pruni* with repellent plant volatiles. *IOBC WPRS Bulletin,* 146, 10-12.

Gallinger J. & Gross J. (2020). Phloem metabolites of *Prunus* sp. rather than infection with *Candidatus* Phytoplasma prunorum influence feeding behavior of *Cacopsylla pruni* nymphs.*Journal of Chemical Ecology,* 8, e64938.

Gallinger J., Jarausch B., Jarausch W. & Gross J. (2020). Host plant preferences and detection of host plant volatiles of the migrating psyllid species*Cacopsylla pruni* , the vector of European Stone Fruit Yellows.*Journal of Pest Science,* 93, 461.

Gross J. (2016). Chemical communication between phytopathogens, their host plants and vector insects and eavesdropping by natural enemies.*Frontiers in Ecology and Evolution,* 4, 271.

Gross J. & Mekonen N. (2005). Plant odours influence the host finding behaviour of apple psyllids (*Cacopsylla picta* ; *C. melanoneura*). *IOBC WPRS Bulletin,* 28, 351–355.

Hafke J.B., van Amerongen J.-K., Kelling F., Furch A.C.U., Gaupels F. & van Bel A.J.E. (2005). Thermodynamic battle for photosynthate acquisition between sieve tubes and adjoining parenchyma in transport phloem.*Plant Physiology,* 138, 1527-1537.

Hao P., Liu C., Wang Y., Chen R., Tang M., Du B., Zhu L. & He G. (2008). Herbivore-induced callose deposition on the sieve plates of rice: an important mechanism for host resistance. *Plant Physiology,* 146, 1810–1820.

Heil M. & Ton J. (2008). Long-distance signalling in plant defence. *Trends in Plant Science,* 13, 264–272.

Heyer M., Reichelt M. & Mithöfer A. (2018). A holistic approach to analyze systemic jasmonate accumulation in individual leaves of Arabidopsis rosettes upon wounding. *Frontiers in Plant Science,*9, 1569.

Hijaz F. & Killiny N. (2014). Collection and chemical composition of phloem sap from *Citrus sinensis* L. Osbeck (sweet orange). *PloS one,* 9, 1–11.

Janik K., Mithöfer A., Raffeiner M., Stellmach H., Hause B. & Schlink K. (2017). An effector of apple proliferation phytoplasma targets TCP transcription factors-a generalized virulence strategy of phytoplasma?*Molecular Plant Pathology,* 18, 435–442.

Jarausch B., Tedeschi R., Sauvion N., Gross J. & Jarausch W. (2019a). Psyllid Vectors. In *Phytoplasmas: Plant Pathogenic Bacteria - II .Transmission and Management of Phytoplasma - Associated Diseases.*(eds. A. Bertaccini, P. G. Weintraub, G. P. Rao, N. Mori), pp. 53–78.Singapore: Springer Singapore.

Jarausch W., Jarausch B., Fritz M., Runne M., Etropolska A. & Pfeilstetter E. (2019b). Epidemiology of European stone fruit yellows in Germany: the role of wild *Prunus spinosa* . *European Journal of Plant Pathology,* 50, 185.

Jung H.W., Tschaplinski T.J., Wang L., Glazebrook J. & Greenberg J.T. (2009). Priming in systemic plant immunity. *Science,* 324, 89-91.

Kison H. & Seemüller E. (2001). Differences in strain virulence of the European stone fruit yellows phytoplasma and susceptibility of stone fruit trees on various rootstocks to this pathogen. *Journal of Phytopathology* , 149, 533–541.

Koncz L., Petróczy M., Ladányi M., Maitz M. & Nagy G. (2017). Severity of symptoms of European stone fruit yellows on different apricot varieties. *Review on Agriculture and Rural Development* , 6, 63–70.

Kube M., Schneider B., Kuhl H., Dandekar T., Heitmann K., Migdoll A.M., Reinhardt R. & Seemüller E. (2008). The linear chromosome of the plant-pathogenic mycoplasma '*Candidatus* Phytoplasma mali'.*BMC Genomics,* 9, 306.

Kuznetsova A., Brockhoff P.B. & Christensen R.H.B. (2017). lmerTest Package: Tests in Linear Mixed Effects Models. *Journal of Statistical Software,*82,1-26.

Lenth R. (2019). emmeans: Estimated Marginal Means, aka Least-Squares Means.

Ma K.-W. & Ma W. (2016). Phytohormone pathways as targets of pathogens to facilitate infection. *Plant Molecular Biology,* 91, 713–725.Marcone C., Jarausch B. & Jarausch W. (2010).*Candidatus* Phytoplasma prunorum, the causal agent of European stone fruit yellows: an overview. *Journal of Plant Pathology,* 92, 19-34.

Marcone C. & Rao G.P. (2019). Control of Phytoplasma Diseases Through Resistant Plants. In Phytoplasmas: Plant Pathogenic Bacteria - II. Transmission and Management of Phytoplasma - Associated Diseases. (eds A. Bertaccini, P. G. Weintraub, G.P. Rao, N. Mori), pp. 165–184. Singapore: Springer Singapore.

Martini X., Coy M., Kuhns E. & Stelinski L.L. (2018). Temporal decline in pathogen-mediated release of methyl salicylate associated with decreasing vector preference for infected Over Uninfected Plants.*Frontiers in Ecology and Evolution,* 6, 78.

Maust B.E., Espadas F., Talavera C., Aguilar M., Santamaría J.M. & Oropeza C. (2003). Changes in carbohydrate metabolism in coconut palms infected with the lethal yellowing phytoplasma. *Phytopathology,* 93, 976–981.

Mayer C.J., Vilcinskas A. & Gross J. (2008a). Pathogen-induced release of plant allomone manipulates vector insect behavior. *Journal of Chemical Ecology,* 34, 1518–1522.

Mayer C.J., Vilcinskas A. & Gross J. (2008b). Phytopathogen lures its insect vector by altering host plant odor. *Journal of Chemical Ecology,*34, 1045–1049.

Mayer C.J., Jarausch B., Jarausch W., Jelkmann W., Vilcinskas A. & Gross J. (2009). *Cacopsylla melanoneura* has no relevance as vector of apple proliferation in Germany. *Phytopathology,* 99, 729–738.

Mayer C.J., Vilcinskas A. & Gross J. (2011). Chemically mediated multitrophic interactions in a plant-insect vector-phytoplasma system compared with a partially nonvector species. *Agricultural and Forest Entomology,*13, 25–35.

Menzel T.R., Weldegergis B.T., David A., Boland W., Gols R., van Loon J.J.A. & Dicke M. (2014). Synergism in the effect of prior jasmonic acid application on herbivore-induced volatile emission. *Journal of Experimental Botany,* 65, 4821-4831.

Mittler T.E. & Dadd R.H. (1963). Studies on the artificial feeding of the aphid*Myzus persicae* (Sulzer): I. relative uptake of water and sucrose solutions. *Journal of Insect Physiology,* 9, 623–645.

Musetti R., Farhan K., De Marco F., Polittotto R., Paolacci A., Ciaffi M., Ermacora P., Grisan S., Santi S. & Osler R. (2013). Differentially-regulated defence genes in *Malus domestica* during phytoplasma infection and recovery. *European Journal of Plant Pathology,* 136, 13-19.

Nehela Y., Hijaz F., Elzaawely A.A., El-Zahaby H.M. & Killiny N. (2018). Citrus phytohormonal response to *Candidatus* Liberibacter asiaticus and its vector *Diaphorina citri* . *Physiological and Molecular Plant Pathology,* 102, 24–35.

Oshima K., Maejima K. & Namba S. (2013). Genomic and evolutionary aspects of phytoplasmas. *Frontiers in Microbiology,* 4, 230.

Park S.-W., Kaiyomo E., Kumar D., Mosher S.L. & Klessig D.F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science,* 318, 113-116.

Pinheiro J., Bates D., DebRoy S., Deepayan S. & R Core Team. (2019). nlme: Linear and Nonlinear Mixed Effects Models.

Potter, Eriksson T., Evans R.C., Oh S., Smedmark J.E.E., Morgan D.R., Kerr M., Robertson K.R., Arsenault M., Dickinson T.A. & Campbell C.S. (2007). Phylogeny and classification of Rosaceae. *Plant Systematics and Evolution,* 266, 5–43.

R Core Team. (2019). R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Richter S. (2002). Susceptibility of Austrian apricot and peach cultivars to ESFY. *Plant Protection Science* , 38, 281–284

Robert-Seilaniantz A., Grant M. & Jones J.D.G. (2011). Hormone crosstalk in plant disease and defense: more than just jasmonate-salicylate antagonism.*Annual Review of Phytopathology,* 49, 317–343.

Seemüller E., Kunze L. & Schaper U. (1984). Colonization behavior of MLO, and symptom expression of proliferation-diseased apple trees and decline-diseased pear trees over a period of several years.*Journal of Plant Diseases and Protection,* 91, 525-532.

Seemüller E., Schaper U. & Kunze L. (1986). Effect of pear decline on pear trees on 'Quince A' and *Pyrus* communis seedling rootstocks/Auswirkung des Birnenverfalls auf Birnbäume mit 'Quitte A' und Bir-

nensämling als Unterlage. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz/Journal of Plant Diseases and Protection* , 44-50.

Seemüller E., Garnier M. & Schneider B. (2002). Mycoplasmas of plants and insects. In Molecular biology and pathogenicity of mycoplasmas (pp. 91-115). Springer, Boston, MA.

Seemüller E. & Schneider B. (2004). '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus*Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively.*International Journal of Systematic and Evolutionary Microbiology,* 54, 1217–1226.

Seemüller E. & Schneider B. (2007). Differences in virulence and genomic features of strains of '*Candidatus* Phytoplasma mali', the apple proliferation agent. *Phytopathology* , 97, 964–970.

Seemüller E., Kiss E., Sule S. & Schneider B. (2010). Multiple infection of apple trees by distinct strains of '*Candidatus* Phytoplasma mali' and its pathological relevance. *Phytopathology,* 100, 863–870.

Seemüller, E & Harries H. (2010). Plant resistance. Phytoplasmas: genomes, plant hosts and vectors. CAB International, Oxfordshire, UK, 147-169.

Seemüller E., Kampmann M., Kiss E. & Schneider B. (2011). HflB gene-based phytopathogenic classification of '*Candidatus*phytoplasma mali' strains and evidence that strain composition determines virulence in multiply infected apple trees. *Molecular Plant-Microbe Interactions,* 24, 1258–1266.

Seemüller E., Sule S., Kube M., Jelkmann W. & Schneider B. (2013). The AAA+ ATPases and HflB/FtsH proteases of '*Candidatus* Phytoplasma mali': phylogenetic diversity, membrane topology, and relationship to strain virulence. *Molecular Plant-Microbe Interactions,* 26, 367–376.

Seemüller E., Zikeli K., Furch A.C.U., Wensing A. & Jelkmann W. (2018). Virulence of '*Candidatus* Phytoplasma mali' strains is closely linked to conserved substitutions in AAA+ ATPase AP460 and their supposed effect on enzyme function. *European Journal of Plant Pathology,* 150, 701-711.

Siewert C., Luge T., Duduk B., Seemüller E., Büttner C., Sauer S. & Kube M. (2014). Analysis of expressed genes of the bacterium '*Candidatus*phytoplasma mali' highlights key features of virulence and metabolism.*PloS one,* 9, e94391.

Soroker V., Blumberg D., Haberman A., Hamburger-Rishard M., Reneh S., Talebaev S., Anshelevich L. & Harari A.R. (2005). Current status of red palm weevil infestation in date palm plantations in Israel.*Phytoparasitica; Israel Journal of Plant Protection Sciences,* 33, 97–106.

Strauss E. (2009). Phytoplasma research begins to bloom. *Science,*325, 388-390.

Sugio A., MacLean A.M., Kingdom H.N., Grieve V.M., Manimekalai R. & Hogenhout S.A. (2011a). Diverse targets of phytoplasma effectors: from plant development to defense against insects. *Annual Review of Phytopathology,* 49, 175–195.

Sugio A., Kingdom H.N., MacLean A.M., Grieve V.M. & Hogenhout S.A. (2011b). Phytoplasma protein effector SAP11 enhances insect vector reproduction by manipulating plant development and defense hormone biosynthesis. *Proceedings of the National Academy of Sciences of the United States of America,* 108, E1254-63.

Tauzin A.S. & Giardina T. (2014). Sucrose and invertases, a part of the plant defense response to the biotic stresses. *Frontiers in Plant Science* , 5, 293

van Bel A.J.E. (1996). Interaction between sieve element and companion cell and the consequences for photoassimilate distribution. Two structural hardware frames with associated physiological software packages in dicotyledons? *Journal of Experimental Botany,* 47, 1129-1140.

Walling L.L. (2000). The Myriad Plant Responses to Herbivores. *Journal of Plant Growth Regulation,* 19, 195–216.

Weintraub P.G. & Gross J. (2013). Capturing insect vectors of phytoplasmas. In Phytoplasma. Methods in Molecular Biology (Methods and Protocols). (ed Dickinson M. H. J.), pp. 61–72. Totowa, NJ: Humana Press.

Weintraub P.G. & Beanland L. (2006). Insect vectors of phytoplasmas. *Annual Review of Entomology,* 51, 91–111.

Wickham H. (2016). ggplot2: Elegant Graphics for Data Analysis. New York: Springer-Verlag.

Will T., Kornemann S.R., Furch A.C.U., Tjallingii W.F. & van Bel A.J.E. (2009). Aphid watery saliva counteracts sieve-tube occlusion: a universal phenomenon? *The Journal of Experimental Biology,* 212, 3305–3312.

Will T., Furch A.C.U. & Zimmermann M.R. (2013). How phloem-feeding insects face the challenge of phloem-located defenses. *Frontiers in Plant Science,* 4.

Zafari S., Niknam V., Musetti R. & Noorbakhsh S.N. (2012). Effect of phytoplasma infection on metabolite content and antioxidant enzyme activity in lime (*Citrus aurantifolia*). *Acta Physiologiae Plantarum,* 34, 561–568.

Zimmermann M.R., Schneider B., Mithöfer A., Reichelt M., Seemüller E. & Furch A.C.U. (2015). Implications of *Candidatus* Phytoplasma mali infection on phloem function of apple trees. *Journal of Endocytobiosis and Cell Research,* 26, 67–75.

Zuur A.F., Ieno E.N., Walker N., Saveliev A.A. & Smith G.M. (2009). Mixed effects models and extensions in ecology with R.

Legends

Fig. 1. Symptoms of phytoplasma infected apple, pear and peach trees. **(a)** The apple proliferation (AP) induced by '*Candidatus* Phytoplasma mali' led to typical disease symptoms like witches' broom, enlarged and highly serrated stipules. The leaf size ratio (length width^{-1}) and the midrib ratio (diameter midrib width^{-1}) did not show any effects.**(b)** The leaves of '*Candidatus* Phytoplasma pyri' affected trees inducing pear decline (PD) were characterized by premature foliar reddening and a significant decrease of the leaf size ratio but not by an impact on the midrib. **(c)** The leaves of '*Candidatus*Phytoplasma prunorum' infected trees inducing European stone fruit yellows (ESFY) showed chlorosis, and a significant increase of the leaf size and midrib ratio. Boxes represent the interquartile range (IQR) and whiskers extend to 1.5*IQR. Bars represent the 95% confidence intervals with the estimated marginal means obtained from mixed effect models as dots (both back transformed to the response scale). Letters indicate statistical differences between EMMs of groups at the 0.05 significance level.

Fig. 2. Analysis of morphological leaf parameters in healthy and phytoplasma infected apple trees. **(a)** The infection of apple trees with a virulent classified '*Candidatus* Phytoplasma mali' accession (3/6) was investigated with the morphology of the leaf main vein in the midrib. **(b)** The morphological analysis consisted of the vascular bundle area, the xylem area, the phloem area, the sieve element area, the xylem/phloem ratio and the sieve element/phloem ratio and showed a significant decrease for nearly all studied parameters in AP infected trees but not for the sieve element/phloem ratio. Box-whisker plots with median as lines and jittered raw values as closed circles (corresponding to each measurement). Boxes represent the interquartile range (IQR) and whiskers extend to 1.5*IQR. Bars represent the 95% confidence intervals with the estimated marginal means obtained from mixed effect models as dots (both back transformed to the response scale). Letters indicate statistical differences between EMMs of groups at the 0.05 significance level.

Fig. 3. Analysis of morphological leaf parameters in healthy and phytoplasma infected pear trees. **(a)** The infection of a pear tree with '*Candidatus* Phytoplasma pyri' inducing pear decline (PD) was studied by the morphology of the leaf main vein.**(b)** The morphological analysis consisted of the vascular bundle area, the

xylem area, the phloem area, the sieve element area, the xylem/phloem ratio and the sieve element/phloem ratio and showed no significant changes. Box-whisker plots with median as lines and jittered raw values as closed circles (corresponding to each measurement). Boxes represent the interquartile range (IQR) and whiskers extend to 1.5*IQR. Bars represent the 95% confidence intervals with the estimated marginal means obtained from mixed effect models as dots (both back transformed to the response scale). Letters indicate statistical differences between EMMs of groups at the 0.05 significance level.

Fig. 4. Analysis of morphological leaf parameters in healthy and phytoplasma infected peach trees. **(a)** The infection of a peach tree with '*Candidatus* Phytoplasma prunorum' inducing European stone fruit yellows (ESFY) was studied by the morphology of the leaf main vein. **(b)** The morphological analysis consisted of the vascular bundle area, the xylem area, the phloem area, the sieve element area, the xylem/phloem ratio and the sieve element/phloem ratio and showed significant decreases for the sieve element areas and ratios of sieve element to the phloem. Box-whisker plots with median as lines and jittered raw values as closed circles (corresponding to each measurement). Boxes represent the interquartile range (IQR) and whiskers extend to 1.5*IQR. Bars represent the 95% confidence intervals with the estimated marginal means obtained from mixed effect models as dots (both back transformed to the response scale). Letters indicate statistical differences between EMMs of groups at the 0.05 significance level.

Fig. 5. Analysis of translocation in phloem sieve elements of healthy and phytoplasma infected apple, pear and peach trees. The translocation was examined with the determination of the velocity of the phloem mass flow (cm h^{-1}) using fluorescence and with the calculation of volumetric flow rates (cm^3 h^{-1}) in mean single sieve elements. Both parameters were individually determined for **(a)** apple, **(b)** pear and **(c)** peach trees. Apple trees were infected with '*Candidatus* Phytoplasma mali' inducing apple proliferation (AP). Pear trees were infected with '*Candidatus* Phytoplasma pyri' causing pear decline (PD) and peach trees were infected with '*Candidatus* Phytoplasma prunorum' inducing European stone fruit yellows (ESFY). Infected apple trees (AP) showed a significant decrease of phloem mass flow velocity and volumetric flow rates in contrast to infected pear trees (PD) where a significant rise was observed. In infected peach trees (ESFY) the phloem mass flow velocity was not affected but the volumetric flow rate decreased significantly. Box-whisker plots with median as lines and jittered raw values as closed circles (corresponding to each measurement). Boxes represent the interquartile range (IQR) and whiskers extend to 1.5*IQR. Bars represent the 95% confidence intervals with the estimated marginal means obtained from mixed effect models as dots (both back transformed to the response scale). Letters indicate statistical differences between EMMs of groups at the 0.05 significance level.

Fig. 6. Analysis of callose deposition in the leaf phloem tissue of healthy and phytoplasma infected apple, pear and peach trees. At cross sections of the leaf mid rip, the callose deposition in sieve elements was stained with aniline blue and detected via fluorescence microscopy (see panels on the left side). The callose fluorescence was quantified after subtracting auto-fluorescence (see panels on the right side). **(a)** In apple trees, an infection with the virulent accession (3/6) inducing apple proliferation (AP) did not show any differences in the callose deposition in comparison to healthy plants.**(b+c)** The phytoplasma infection of pear trees (PD) and peach trees (ESFY) induced a significant ($p<0.05$) increase of callose deposition in sieve elements. Box-whisker plots with median as lines and jittered raw values as closed circles (corresponding to each measurement). Boxes represent the interquartile range (IQR) and whiskers extend to 1.5*IQR. Bars represent the 95% confidence intervals with the estimated marginal means obtained from generalized least square models as dots (both back transformed to the response scale). Letters indicate statistical differences between EMMs of groups at the 0.05 significance level.

Fig. 7. The phytohormone balance in healthy and phytoplasma infected apple, pear and peach trees. The concentrations (ng g_{FM}^{-1}) of several phytohormones – salicylic acid (SA), jasmonic acid-iso leucine (JA-Ile), jasmonic acid (JA) and abscisic acid (ABA) – were measured in the leaves of healthy and phytoplasma infected **(a)** apple, **(b)** pear and**(c)** peach. For apple, a virulent accession (3/6) was considered inducing apple proliferation (AP). Pear trees showed pear decline (PD) and peach trees showed the European stone fruit yellows (ESFY). Box-whisker plots with median as lines and jittered raw values as closed circles

(corresponding to each measurement). Boxes represent the interquartile range (IQR) and whiskers extend to 1.5*IQR. Bars represent the 95% confidence intervals with the estimated marginal means obtained from linear models as dots (both back transformed to the response scale). Letters indicate statistical differences between EMMs of groups at the 0.05 significance level.

Tables

Table 1 – Mean (±SD) leaf morphology parameters. Different letters indicate significant differences between phytoplasma infected and uninfected trees compared within each species. AP = apple proliferation; PD = pear decline; ESFY = European stone fruit yellowing

	Apple	Apple	Pear	Pear	Peach
parameter	Healthy	AP	Healthy	PD	Healthy
leaf length [cm]	11.35 (±1.94)^{a}	9.43 (±1.79)^{b}	6.13 (±0.52)^{a}	6.17 (±0.48)^{a}	12.44 (±1.08)^{a}
leaf width [cm]	4.95 (±0.95)^{a}	3.84 (±0.76)^{b}	3.98 (±0.48)^{a}	4.32 (±0.42)^{b}	4.06 (±0.53)^{a}
diameter midrib [µm]	916.40 (±124.40)^{a}	667.75 (±161.49)^{b}	821.10 (±141.85)^{a}	740.64 (±69.42)^{a}	799.37 (±95.06)^{a}

Table 2 – Mean (±SD) functional/physiological parameters. Various physicochemical parameters (refractive index, dynamic viscosity and density) were determined for the centrifugates of the apple, pear and peach bark. Different letters indicate significant differences between phytoplasma infected and uninfected trees compared within each species. AP = apple proliferation; PD = pear decline; ESFY = European stone fruit yellows; mPa = milli Pascal; s = second; g = gram; L = litre

	Apple	Apple	Pear	Pear	Peach
parameter	Healthy	AP	Healthy	PD	Healthy
refractive index [°Brix]	9.643 (±1.200)^{a}	9.423 (±1.018)^{a}	7.250 (±1.909)^{a}	14.333 (±6.280)^{b}	11.727 (±2.494)^{a}
dynamic viscosity [mPa s]	4.078 (±0.652)^{a}	3.916 (±1.093)^{a}	1.957 (±0.368)^{a}	3.999 (±2.035)^{b}	n.d.
density [g L^{-1}]	996.5 (±94.9) ^{a}	1003.6 (±133.4) ^{a}	1001.4 (±50.8)^{a}	1058.1 (±55.7)^{a}	n.d.

Table 3 – Comparison of all morphological and physiological results of phytoplasma infected apple, pear and peach trees. All studied parameters are listed in the first column and significant changes in comparison to healthy plants are shown for the considered plant species – increase (+), decrease (-), no change (=) and not determined (n.d.).

	Apple	**Pear**	**Peach**
leaf size ratio [length/width]	=	-	+
midrib ratio [diameter midrib/leaf width]	=	=	+
diameter midrib [µm]	-	=	=
leaf length [cm]	-	=	=
leaf width [cm]	-	+	=
vascular bundle [μm^2]	-	=	=
xylem [μm^2]	-	=	=
phloem [μm^2]	-	=	=
sieve elements [μm^2]	-	=	-
ratio xylem/phloem	-	=	=
ratio sieve element/phloem	=	=	-
mass flow [cm h^{-1}]	-	+	=
volumetric flow rate [cm^3 h^{-1}]	-	+	-
dynamic viscosity [mPa s]	=	+	n.d.

	Apple	Pear	Peach
refractive index [°Brix]	=	+	=
absolute density [g L^{-1}]	=	=	n.d.
callose intensity	=	+	+
salicylic acid [ng g_{FM}^{-1}]	+	=	+
jasmonic acid-iso leucine [ng g_{FM}^{-1}]	+	=	+
jasmonic acid [ng g_{FM}^{-1}]	=	=	=
abscisic acid [ng g_{FM}^{-1}]	+	=	=
12-oxo-phytodienoic acid [ng g_{FM}^{-1}]	-	=	=
Indole-3-acetic acid [ng g_{FM}^{-1}]	=	=	-

Supporting Information

The following Supporting Information is available for this article:

Figure S1 *Phytohormone concentrations of cis-12-oxo-phytodienoic acid (cis-OPDA) and indole-3-acetic acid (IAA) in healthy and phytoplasma infected (**a**) apple, (**b**) pear and (**c**) peach trees.*

Table S1

Table S2 *Details of analysis of phytohormones by LC-MS/MS [HPLC 1260 (Agilent Technologies)-QTRAP6500 (SCIEX)] in negative ionisation mode.*

Table S3 *Specification and results of statistical models used for analysis of morphology parameters.*

Table S4 *Specification and results of statistical models used for analysis of vascular morphology.*

Table S5 *Specification and results of statistical models used for analysis of the translocation in phloem sieve elements and physicochemical parameters.*

Table S6 *Specification and results of generalized least square models analysing the maximum callose fluorescence.*

Table S7 *Specification and results of linear models used for analysis of phytohormone concentrations.*

Table S8 *Overview of mean phytohormone concentrations (ng g_{FM}^{-1}).*