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Diagnosis, Transmission, and Management of Phytoplasmas Infecting *Rubus* Species

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List of Abbreviations

%	percent
°C	degree Celsius
µl	microliter
µmol	micromole
bp	base pair
Ca.	*Candidatus*
CC	Creative Commons license
cf.	confer/conferatur, meaning compare
cm	centimeter
C_q	quantification cycle
CTAB	hexadecyl-trimethyl-ammonium bromide
Cy5	Cyanine 5
d	day
dd.mm.yyyy	date as two-digit day followed by two-digit month followed by four-digit year
DNA	deoxyribonucleic acid
E	East
EDTA	ethylenediaminetetraacetic acid
EPPO	European and Mediterranean Plant Protection Organization
FAM	fluorescein amidite
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
g	gram
ha	hectare
kb	kilo base pairs
kg	kilogram
l	liter
LAMP	loop-mediated isothermal amplification
L x W x H	length by width by height
m	meter
M	molar (moles per liter)

min	minute
ml	milliliter
MLOs	mycoplasma-like organisms
mM	millimolar
mm	millimeter
mol%	mole percent
N	North
NaCl	sodium chloride
ng	nanogram
NGS	Next-Generation Sequencing
nm	nanometer
No.	number(s)
NTC	no-template control
PCR	polymerase chain reaction
pH	decimal logarithm of the reciprocal of the hydrogen ion activity
qPCR	quantitative polymerase chain reaction
R^2	coefficient of determination
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
ROX	rhodamine X
rpm	revolutions per minute
s	second
SD	standard deviation
sec	second
sp.	species (singular)
spp.	species (plural)
Tris-HCl	2-Amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride
UV	ultraviolet
V	volt

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1 Summary

Phytoplasmas ('*Candidatus* Phytoplasma') are plant-pathogenic bacteria that colonize the phloem of their host plants and can cause diseases in more than 700 plant species world-wide, including many economically important crops, ornamentals, and forest trees. They are spread by phloem-feeding insect vectors, vegetative propagation (grafting or production of cuttings), or plant parasitic dodder species of the genus *Cuscuta*.

In *Rubus* species, phytoplasmas are associated with a disease referred to as Rubus stunt. Symptoms include stunting, witches' broom, small leaves, short internodes, enlarged sepals, phyllody, flower proliferation, and fruit malformations. As *Rubus* species are usually propagated vegetatively and the period of latency can be up to one year, the commercial production of planting material in plant nurseries can play a major role in the spread of Rubus stunt in addition to its only known insect vector, the leafhopper *Macropsis fuscula*. Furthermore, phytoplasmas usually occur in *Rubus* plants at concentrations too low for regular PCR to detect their DNA in plants and nested PCR is too time consuming for routine screening and prone to carry-over contamination. Hence, a fast, sensitive, and reliable molecular detection method for phytoplasmas in *Rubus* species is of major importance to stop the spread of this disease. Therefore, a multiplex TaqMan qPCR assay for the detection of Rubus stunt phytoplasmas was developed in this thesis, which provides an efficient tool for the screening of *Rubus* mother plants.

This qPCR assay was subsequently used in a screening of putative insect vectors of Rubus stunt phytoplasmas in raspberry plantations in southern and northern Germany. A total of 4868 hemipteran insects were sorted, identified to family, genus, or species level and 597 DNA extracts, including pooled samples representing all identified insects, sampling locations, and sampling dates, were analyzed for phytoplasma DNA. With only seven sampled individuals of *M. fuscula* the occurrence of the only known vector for Rubus stunt phytoplasmas was low. DNA extracts from 18 samples were positive for phytoplasma DNA, among them species from the genera *Euscelidius*, *Macrosteles*, *Euscelis*, *Anaceratagallia,* and *Psammotettix*.

Furthermore, the susceptibility of four raspberry cultivars to Rubus stunt after artificial graft inoculation was investigated. While graft inoculation was verified to be a practical tool for artificial phytoplasma inoculation in raspberries, assessing the disease severity of Rubus stunt proved to be difficult. All four raspberry cultivars used in this experiment ('Autumn Bliss', 'Glen Ample', 'Polka', and 'Tulameen') were successfully colonized by phytoplasmas.

The efficacy of heat therapy with subsequent tissue culture to eliminate phytoplasmas from infected raspberry and blackberry plants was also evaluated. All previously infected raspberry and blackberry plants were tested negative for the presence of phytoplasma DNA after this procedure. Therefore, this method is suitable as an important step during the production of healthy mother plants and nuclear stock material in order to secure the production of healthy planting material.

In conclusion, a fast and reliable molecular detection assay for routine diagnostics of phytoplasmas in *Rubus* species was developed and applied in experiments investigating putative insect vectors of Rubus stunt phytoplasmas, the susceptibility of different raspberry cultivars, and the efficacy of heat therapy coupled with tissue culture. The results of these trials can now be incorporated into disease management strategies for Rubus stunt.

2 Zusammenfassung

Phytoplasmen ('*Candidatus* Phytoplasma') sind phytopathogene Bakterien, die das Phloem ihrer Wirtspflanzen besiedeln und bei mehr als 700 Pflanzenarten weltweit, darunter viele wirtschaftlich wichtigen Kulturpflanzen, Zierpflanzen und Forstpflanzen, Krankheiten verursachen können. Verbreitetet werden können Phytoplasmen von phloemsaugenden Vektorinsekten, bei der vegetativen Vermehrung (Pfropfung oder Produktion von Stecklingen) oder über pflanzenparasitäre Seide-Arten der Gattung *Cuscuta*.

Bei Rubus-Arten erzeugen Phytoplasmen die sogenannte Rubus stunt, zu deren Symptome ein gestauchter Wuchs, Hexenbesenwuchs, kleine Blätter, kurze Internodien, vergrößerte Kelchblätter, Phyllodie, Blütenproliferation und Fruchtmissbildungen gehören. Das einzige beschriebene Vektorinsekt für Rubus stunt ist die Himbeermaskenzikade (*Macropsis fuscula*). Da Rubus-Arten normalerweise vegetativ vermehrt werden und die Latenzzeit von Rubus stunt bis zu einem Jahr betragen kann, spielt die kommerzielle Produktion von Pflanzgut eine wichtige Rolle bei der Verbreitung der Krankheit. Darüber hinaus treten Phytoplasmen in Rubus-Arten für gewöhnlich nur in sehr niedrigen Titern auf, die zu gering sind, um die Phytoplasmen DNA mit einer herkömmlichen PCR nachzuweisen. Die sensitivere nested PCR hingegen ist zu zeitintensiv für Routinescreenings und anfällig für Verunreinigungen. Deshalb wurde in dieser Arbeit ein schneller und zuverlässiger multiplex TaqMan qPCR Assay zum Nachweis von Rubus stunt Phytoplasmen entwickelt, der nun als effizientes Werkzeug für das Screening von Mutterpflanzen zur Verfügung steht.

Diese Nachweismethode wurde anschließend in einem Insektenscreening angewendet, für das potentielle Vektorinsekten für Rubus stunt Phytoplasmen in Himbeeranlagen in Süd- und Norddeutschland gefangen wurden. Es wurden insgesamt 4868 Insekten der Ordnung Hemiptera aussortiert und auf Familie, Gattung oder Art bestimmt. Davon wurden 597 DNA-Extrakte, die alle identifizierten Arten, Probenstandorte und Probenzeitpunkte repräsentieren, hinsichtlich des Vorkommens auf Phytoplasmen-DNA untersucht. Das Auftreten des einzigen beschriebenen Vektorinsekts für Rubus stunt Phytoplasmen, *M. fuscula*, war mit nur sieben Individuen gering. Insgesamt wurden 18 DNA-Extrakte positiv auf

Phytoplasmen-DNA getestet, darunter Arten der Gattungen *Euscelidius*, *Macrosteles*, *Euscelis*, *Anaceratagallia,* und *Psammotettix*.

Des Weiteren wurde die Anfälligkeit von vier Himbeersorten ('Autumn Bliss', 'Glen Ample', 'Polka' und 'Tulameen') auf Rubus stunt durch künstliche Infektion mittels Anplatten infizierter Pflanzen untersucht. Während sich das Anplatten als gute Methode für die künstliche Inokulation von Himbeeren erwies, stellte es sich als schwierig heraus, die Befallsstärke der Rubus stunt zu bonitieren. Alle vier Himbeersorten wurden erfolgreich von Phytoplasmen besiedelt.

Zusätzlich wurde die Wirksamkeit einer Wärmetherapie mit nachfolgender Gewebekultur zur Eliminierung von Phytoplasmen aus infizierten Himbeer- und Brombeerpflanzen untersucht. Alle zuvor infizierten Himbeer- und Brombeerpflanzen wurden nach dieser Behandlung negativ auf die Anwesenheit von Phytoplasma-DNA getestet. Diese Behandlung stellt daher ein geeignetes Mittel bei der Produktion von Mutterpflanzen dar, um die Produktion von gesundem Pflanzmaterial sicherzustellen.

Zusammenfassend wurde eine schnelle und zuverlässige molekulare Diagnosemethode, die sich für Routineuntersuchungen von Rubus-Arten auf Phytoplasmen eignet, entwickelt und in Versuchen zu potentiellen Rubus stunt Vektorinsekten, der Anfälligkeit verschiedener Himbeersorten und der Wirksamkeit von Wärmetherapie mit anschließender Gewebekultur angewendet. Die Ergebnisse dieser Studien können nun in Bekämpfungsstrategien gegen Rubus stunt integriert werden.

3 General Introduction

Parts of this chapter have been published as:

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3.1 Phytoplasmas

3.1.1 Morphology, Symptoms, Genomics, and Taxonomy

Phytoplasmas are cell wall-less bacteria that can colonize plants and insects. They were first discovered by Doi et al. (1967) who used an electron microscope to look at ultrathin sections of the phloem of mulberry trees with dwarf disease, potato and paulownia with witches' broom symptoms, and petunia infected with aster yellows. Because the morphology of the cell wall-less prokaryotes they saw resembled that of mycoplasmas, a genus of animal- and human-pathogenic bacteria, they named them mycoplasma-like organisms (MLOs). Afterwards, several hundred yellows diseases of plants, previously thought to be associated with virus infections, were found to be caused by MLOs (Bertaccini 2007). During the 1990s several independent phylogenetic analyses of their DNA sequences showed that all MLOs constitute a large monophyletic group within the class Mollicutes, and the trivial name "phytoplasma", followed by the designation of '*Candidatus* Phytoplasma', was adopted to denote this taxon of plant pathogens (The IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group 2004).

The genus '*Candidatus* Phytoplasma' was described by the IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group (2004) as follows:

- Morphology: cell wall-less, pleomorphic bodies with a mean diameter of 200 – 800 nm (Figure 1)
- Habitat: inhabit the phloem sieve elements of plants and the gut, haemolymph, salivary gland, and other organs of sap-sucking insects
- Antibiotic sensitivity: sensitive to tetracycline antibiotics, but not to penicillin
- Base composition of DNA: low content of guanine and cytosine (23 – 29 mol%)
- Chromosome size: 530 – 1350 kb
- Codon usage: UGA is used as a stop codon instead as a tryptophan codon as in several other mycoplasmas
- Sterols in cellular membrane: membranes are resistant to digitonin and sensitive to hypotonic salt solutions

– rRNA: specific nucleotide signatures that are characteristic of '*Ca*. Phytoplasma' are: adenine at position 242, thymine at position 286 and at position 1247 (in the sequence of *Oenothera* phytoplasma 86-7 with GenBank accession number M30790).

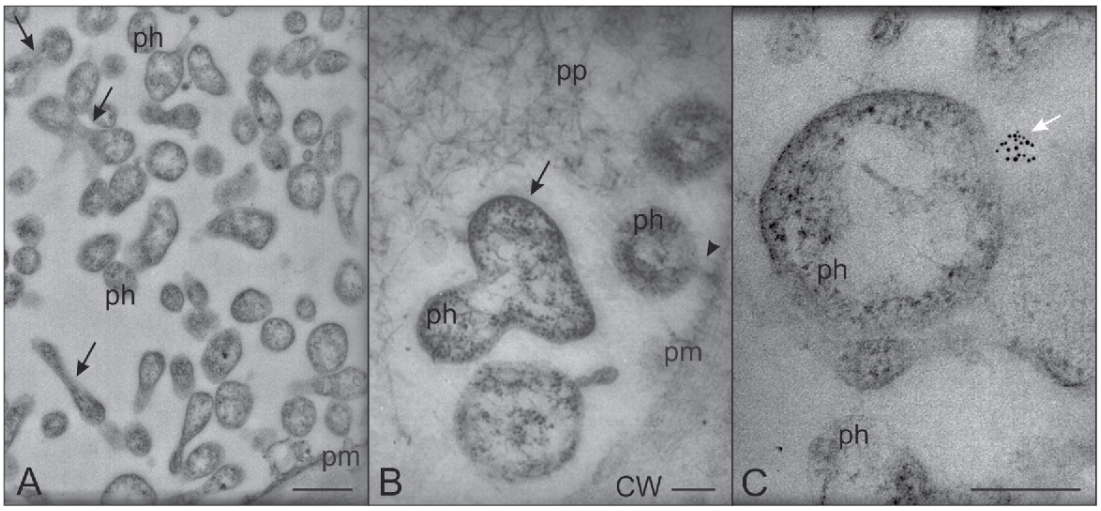

Figure 1: Transmission electron microscopy micrographs of phytoplasmas floating in the sieve element (SE) lumen. (A, B) Phytoplasmas are mostly roundish, sometimes elongated; a few are dividing (black arrows). (C) Aggregates of SE actin form unipolar fields on the phytoplasma surface in the SE lumen (white arrow). The arrowhead in (B) indicates the attachment of a phytoplasma to the SE plasma membrane. In (A), the bar corresponds to 500 nm; in (B) and (C) the bars correspond to 200 nm. CW: cell wall; ph: phytoplasma; pm: plasma membrane; pp: phloem protein. (From Musetti et al. 2016)

The *Candidatus* species classification system is build up on the foundation that isolates within a species share at least 97.5% sequence identity within their 16S rRNA gene. But biological differences like different insect hosts or geographic occurrence are also used in the *Candidatus* determination when sequence identity is not sufficient. In addition, there is the 16Sr group classification system. This system is based on restriction fragment length polymorphism (RFLP) profiles of a specified region of the 16S rDNA that is amplified with phytoplasma universal primers (Lee et al. 1998) and currently comprises 33 groups which are denoted by Roman numerals and are divided into subgroups by addition of Roman alphabetic characters. Both systems have been shown to be consistent with each other (Table 1), however, due to the high conservation of the 16S rRNA gene, many phytoplasma strains that are biologically or ecologically distinct might justify reassignment as new taxa following updated requirements based on additional genetic markers for '*Ca.* Phytoplasma' or subgroup identification (Dermastia et al. 2017).

Table 1: Phytoplasmas classified according to RFLP profiles (16Sr group) and 16S rDNA sequence (*Candidatus* species). (Modified from Dickinson et al. 2013).

16Sr group	*Candidatus* species	Description
I-A	*Ca*. Phytoplasma asteris	Aster yellows
I-B	*Ca*. Phytoplasma asteris	Onion yellows
I-C	*Ca*. Phytoplasma asteris	Clover phyllody
I-D	*Ca*. Phytoplasma asteris	Paulownia witches' broom
I-E	*Ca*. Phytoplasma asteris	Blueberry stunt
I-F	*Ca*. Phytoplasma asteris	Apricot chlorotic leaf roll
II-A	*Ca*. Phytoplasma aurantifolia	Peanut witches' broom
II-B	*Ca*. Phytoplasma aurantifolia	Lime witches' broom
II-C	*Ca*. Phytoplasma aurantifolia	Cactus witches' broom
II-D	*Ca*. Phytoplasma australasia	Papaya mosaic
III-A	*Ca*. Phytoplasma pruni	Western X disease
III-B	*Ca*. Phytoplasma pruni	Clover yellow edge
IV-A	*Ca*. Phytoplasma palmae	Coconut lethal yellowing
IV-B	*Ca*. Phytoplasma palmae	Yucatan coconut lethal decline
IV-C	*Ca*. Phytoplasma palmae	Coconut lethal disease
IV-D	*Ca*. Phytoplasma palmae	*Carludovica palmata* yellows
V-A	*Ca*. Phytoplasma ulmi	Elm yellows
V-B	*Ca*. Phytoplasma ziziphi	Jujube witches' broom
V-C	*Ca*. Phytoplasma vitis	Alder yellows / Flavescence dorée C
V-D	*Ca*. Phytoplasma vitis	Flavescence dorée D
V-E	*Ca*. Phytoplasma rubi	Rubus stunt
V-F	*Ca*. Phytoplasma balanitae	Balanites witches' broom
VI-A	*Ca*. Phytoplasma trifolii	Clover proliferation
VI-I	*Ca*. Phytoplasma sudamericanum	Passionfruit disease
VII-A	*Ca*. Phytoplasma fraxini	Ash yellows
VIII-A	*Ca*. Phytoplasma luffae	Loofah witches' broom
IX-A	N/A	Pigeon-pea witches' broom
IX-B	*Ca*. Phytoplasma phoenicium	Almond witches' broom
IX-C	*Ca*. Phytoplasma phoenicium	Picris echioides yellows
X-A	*Ca*. Phytoplasma mali	Apple proliferation
X-B	*Ca*. Phytoplasma prunorum	European stone fruit yellows
X-C	*Ca*. Phytoplasma pyri	Pear decline
X-D	*Ca*. Phytoplasma spartii	Spartium witches' broom

Table 1: (continued)

16Sr group	*Candidatus* species	Description
XI-A	*Ca*. Phytoplasma oryzae	Rice yellow dwarf
XII-A	*Ca*. Phytoplasma solani	Stolbur
XII-B	*Ca*. Phytoplasma australiense	Australian grapevine yellows
XII-C	N/A	Strawberry lethal yellows
XII-D	*Ca*. Phytoplasma japonicum	Japanese hydrangea phyllody
XII-E	*Ca*. Phytoplasma fragariae	Strawberry yellows
XIII-A	*Ca*. Phytoplasma hispanicum	Mexican periwinkle virescence
XIII-G	*Ca*. Phytoplasma meliae	Melia azedarach yellows
XIV-A	*Ca*. Phytoplasma cynodontis	Bermudagrass white leaf
XV-A	*Ca*. Phytoplasma brasiliense	Hibiscus witches' broom
XVI-A	*Ca*. Phytoplasma graminis	Sugarcane yellow leaf
XVII-A	*Ca*. Phytoplasma caricae	Papaya bunchy top
XVIII-A	*Ca*. Phytoplasma americanum	Potato purple top wilt
XIX-A	*Ca*. Phytoplasma castanae	Chestnut witches' broom
XX-A	*Ca*. Phytoplasma rhamni	Buckthorn witches' broom
XXI-A	*Ca*. Phytoplasma pini	Pine shoot proliferation
XXII-A	*Ca*. Phytoplasma palmicola	Mozambique coconut yellows
XXIII-A	N/A	Buckland valley grapevine yellows
XXIV-A	N/A	Sorghum bunchy shoot
XXV-A	N/A	Weeping tea witches' broom
XXVI-A	N/A	Sugar cane phytoplasma
XXVII-A	N/A	Sugar cane phytoplasma
XXVIII-A	N/A	Derbid phytoplasma
XXIX-A	*Ca*. Phytoplasma omanense	Cassia witches' broom
XXX-A	*Ca*. Phytoplasma tamaricis	Salt cedar witches' broom
XXXI-A	*Ca*. Phytoplasma costaricanum	Soybean stunt
XXXII-A	*Ca*. Phytoplasma malaysianum	Malaysian periwinkle virescence
XXXIII-A	*Ca*. Phytoplasma allocasuarinae	Allocasuarina phytoplasma

N/A: not available

Although there is preliminary evidence that phytoplasmas can be grown independently from their hosts in axenic media (Contaldo et al. 2012), further research to optimize culture systems is needed in order to achieve routine cultivation. Therefore, phytoplasma research still relies heavily on maintaining reference strains in host plants such as the Madagascar periwinkle (*Catharanthus roseus* (L.) G.DON), which has been found to be very susceptible to infections by phytoplasmas (Hodgetts et al. 2013).

Phytoplasmas have been found to be associated with diseases in more than 700 plant species, including many economically important crops, ornamentals, and forest trees (Hoshi et al. 2007; Bertaccini et al. 2014). They cause a wide range of symptoms that vary depending on the phytoplasma strain, their host, and environmental factors, and commonly include yellowing of leaves (Figure 2), virescence (greening of petals), phyllody (conversion of floral organs into leaf-like structures) (Figure 3), proliferation of shoots, witches' broom (Figure 4), stunting, general decline, and sometimes plant death (Dickinson et al. 2013). While phytoplasmas that are not distinguishable on their 16S rDNA can cause different symptoms and/or infect different hosts, different phytoplasma strains can be associated with similar symptoms in the same or different hosts.

Figure 2: Yellowing and necrosis of palm leaves due to coconut lethal yellowing. (By USDA Forest Service, CC-BY-3.0, https://commons.wikimedia.org/w/index.php?curid=3762486)

Figure 3: Purple coneflower (*Echinacea purpura* (L.) MOENCH) showing phyllody due to aster yellows phytoplasma infection. Normal, non-infected coneflowers can be seen in the background. (By Estreya, CC-BY-SA-3.0, https://commons.wikimedia.org/w/index.php?curid=4608429)

Figure 4: **On the left: showing branch of *Tephrosia purpurea* (L.) PERS. with witches' broom symptoms due to infection with '*Candidatus* Phytoplasma aurantifolia'. On the right: unsymptomatic branch of *Tephrosia purpurea.* (By Amityadav8 , CC-BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=31911848)**

Phytoplasma diseases are of economic importance worldwide, in developing countries as well as in countries where agriculture is highly industrialized. Some of the economically most important phytoplasma diseases in tropical areas encompass coconut lethal yellowing, sandal spike disease, pawlownia witches' broom, corn stunt, and rice yellow dwarf disease (Bertaccini 2007). In North America and Europe major economic losses are caused by aster yellows phytoplasmas in several vegetable crops and ornamentals, while peach yellows, X-disease, grapevine yellows, pear decline, apple proliferation, European stone fruit yellows, and other fruit declines associated with phytoplasmas lower yields and impede production of fruit crops. For example, a single phytoplasma outbreak in apple trees in 2001 caused losses of about 25 million Euro in Germany and about 100 million Euro in Italy (Strauss 2009). In the Middle East, phytoplasmas are severely affecting citrus production and in Asia they also cause considerable losses in legumes (Bertaccini and Duduk 2009). In addition to crops and ornamentals, forest trees are often severely damaged by phytoplasma epidemics. For example, elm yellows or witches' broom almost eliminated historical as well as new elm plantations in Europe and North America (Bertaccini 2007). Lethal yellowing of palm has killed millions of plants in the Caribbean alone over the past 40 years (Brown et al. 2006), destroying the livelihoods of many people who depend on palms for nourishment, building materials, and income (Strauss 2009). The European and Mediterranean Plant Protection Organization (EPPO) currently has six phytoplasmas on its A1 and four

phytoplasmas on its A2 list of pests recommended for regulation as quarantine pests (EPPO 2016).

There is, however, one example of pathogenic phytoplasmas as the causal agent of a desirable and economically important trait: the induction of free-branching in commercial poinsettia (*Euphorbia pulcherrima* WILLD. ex KLOTZSCH) cultivars (Lee et al. 1997). There are two morphotypes of poinsettia cultivars that are grown commercially. One is restricted-branching with strong apical dominance, few axillary shoots, and few bracts and the other one is free-branching with weak apical dominance, many axillary shoots, and many bracts (Figure 5 and Figure 6). The free-branching form is of commercial importance for large scale production of potted plants. To obtain free-branching without phytoplasma infections it would be necessary to treat the plants six to seven times with chemicals (Pondrelli et al. 2002).

Figure 5: Restricted-branching phytoplasma-free poinsettia derived from a somatic embryo (left) and free-branching poinsettia morphotype (right). (Figure courtesy of Mike Klopmeyer of Darwin Perennials)

Figure 6: Branching morphotypes of poinsettia with removed leaves. Restricted-branching (left) and free-branching (right). (Figure courtesy of Mike Klopmeyer of Darwin Perennials.)

Compared to other plant pathogens, little is known about the molecular mechanisms behind the pathogenicity and virulence of phytoplasmas. Due to their host-dependent life cycles, they have reduced genomes, missing important metabolic genes (Oshima et al. 2013). Therefore, it has been conjectured that they assimilate a wide range of metabolites from their host, leading to an adverse development. There is a strain of onion yellows phytoplasma causing mild symptoms and a strain causing severe symptoms which occurs in higher titers. The strain causing severe symptoms was

found to have a bigger chromosome size because glycolytic genes were duplicated, indicating that higher glycolysis activities and therefore a higher consumption of the carbon source may affect growth rate of phytoplasmas and cause more severe symptoms (Oshima et al. 2007). The energy metabolism is considered to play a major role in the understanding of phytoplasma pathogenesis (Bertaccini et al. 2014). It was shown that concentrations of carbohydrates in different plant parts differ in infected and healthy plants (Lepka et al. 1999; Maust et al. 2003). In addition, plants infected with phytoplasmas were found to have reduced concentrations of photosynthetic pigments and soluble proteins, as well as changes in hormone balance and amino acid transport (Lepka et al. 1999; Bertamini and Nedunchezhian 2001; Jagoueix-Eveillard et al. 2001; Bertamini et al. 2002; Maust et al. 2003; Musetti et al. 2005).

No homologs of known virulence genes of other phytopathogenic bacteria were identified in phytoplasma genomes (Maejima et al. 2014). The first phytoplasma virulence factor, tengu-su inducer (TENGU), was identified from onion yellows phytoplasma by Hoshi et al. (2009). When transgenic *Nicotiana benthamiana* DOMIN and *Arabidopsis thaliana* (L.) HEYNH. plants expressed *tengu*, they showed symptoms of witches' broom and dwarfism. Furthermore, Hoshi et al. (2009) could show by immunohistochemical analysis, that even though phytoplasmas are restricted to the phloem, the TENGU protein was transported to apical buds and also by microarray analysis of transgenic plants, that TENGU inhibits auxin-related pathways. Since then, several effector proteins causing classical phytoplasma symptoms were found (Bai et al. 2009; Himeno et al. 2011; MacLean et al. 2011).

3.1.2 Transmission and Control

Phytoplasmas are transferred between plants by phloem-feeding insect vectors of the order Hemiptera, mainly by leafhoppers (Cicadellidae), planthoppers (Fulgoroidea), and psyllids (Psylloidea) (Weintraub and Beanland 2006). Therefore, the host range of phytoplasmas is directly coupled to the feeding preference of their insect vector. The insect vectors take up the phytoplasmas during feeding (Figure 7). To be transmitted to an uninfected plant, however, the phytoplasmas need to cross the insect midgut membrane into the hemocoel and then penetrate the salivary glands (Hogenhout et al. 2008). It has also been shown, that phytoplasmas can infect the reproductive organs of the insect vectors and that transovarial transmission to

progeny is possible (Dickinson et al. 2013). Both reduced and enhanced fitness of infected insect vectors have been reported (Christensen et al. 2005). In addition to insect transmission, phytoplasmas can also be spread by vegetative propagation (grafting or production of cuttings) or via plant parasitic dodder species (*Cuscuta* L. spp.) and there are reports that suggest seed transmission (Dickinson et al. 2013).

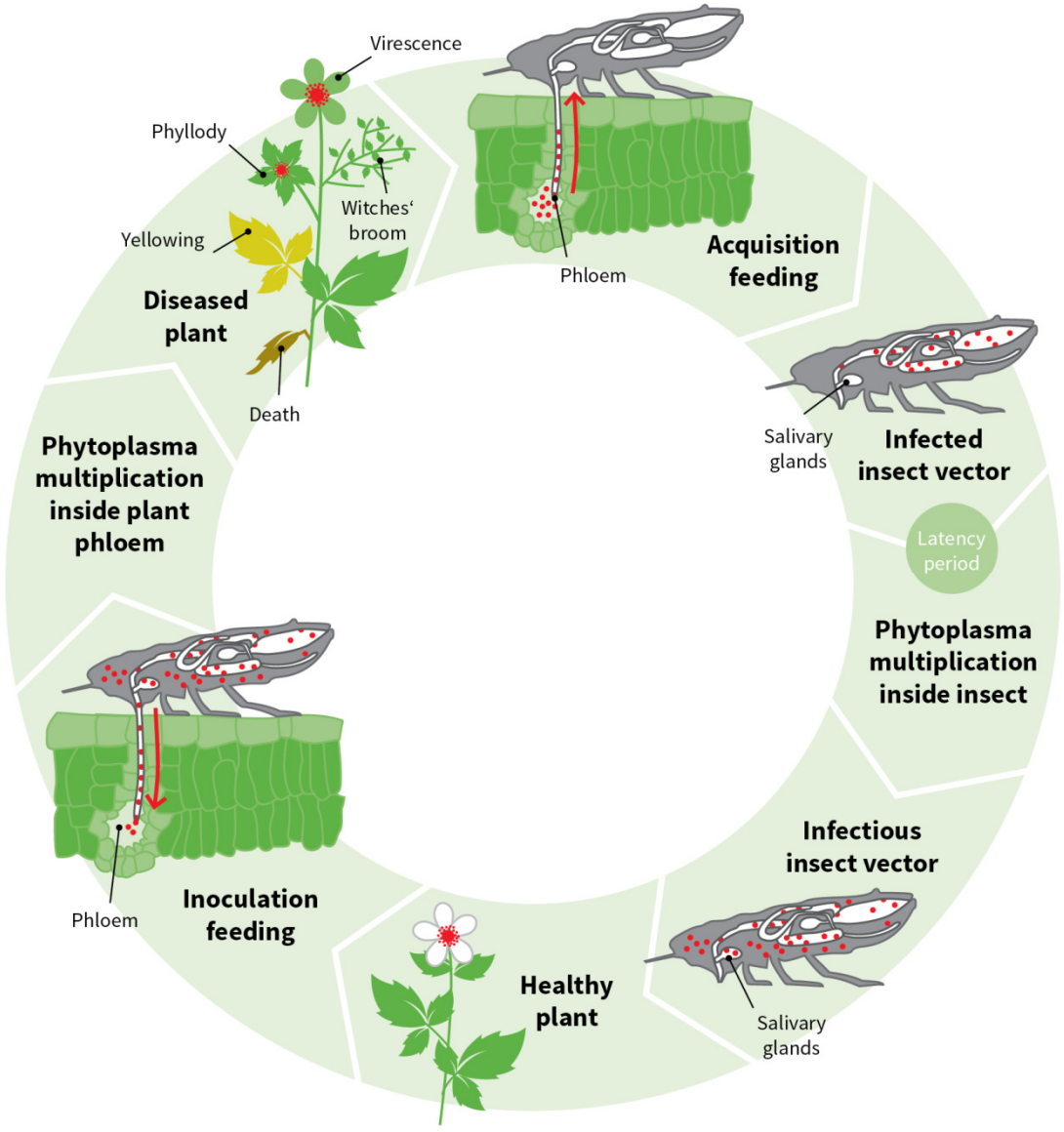

Figure 7: Generalized phytoplasma disease cycle. Phytoplasmas are represented by red dots. (Own illustration)

Once phytoplasmas are introduced into the phloem of their host plant they spread systemically through the plant. Even though phytoplasmas are small enough to pass freely through sieve pores and might be carried passively by the phloem stream from source to sink organs, high titers in source leaves and low titers in sink tissue can be

found (Christensen et al. 2004). Furthermore, studies using localized inoculation by insect transmission (Wei et al. 2004) and analyzing seasonal colonization of pear trees (Garcia-Chapa et al. 2003) provide evidence that translocation of phytoplasmas cannot be explained only based on assimilate flow. Active movement of phytoplasmas, however, seems highly unlikely considering the lack of any genes coding for cytoskeleton elements or flagella (Christensen et al. 2005).

Control strategies of phytoplasma diseases consist of controlling the insect vectors, eliminating the pathogens from infected plants via tetracycline antibiotics, use of phytoplasma resistant varieties, eliminating infected plants, and production of clean planting material. Controlling the insect vectors of phytoplasmas, which is currently the tool of choice for limiting outbreaks of phytoplasma diseases, relies heavily on the use of insecticides (Firrao et al. 2007). However, the results of this chemical vector control are unsatisfying as it is impossible to eliminate all vectors from environments (Bertaccini 2007) and for a great number of phytoplasma diseases the vectors are still unknown (Strauss 2009). It was shown, that phytoplasmas can be eliminated by foliar application of the antibiotic oxytetracycline (Chung and Choi 2002). The use of tetracycline antibiotics is, however, not practical because it is restricted in many countries, quite expensive, and is not always effective (Bertaccini et al. 2014). Breeding of phytoplasma resistant host plants remains challenging as the mechanisms of phytoplasma resistance are not yet completely understood (Bertaccini and Duduk 2009). As a result of this insufficient repertory of control measures, the most efficient measure to date remains the production of clean planting material and removal of infected plants. Therefore, an early detection of infections is of utmost importance.

Phytoplasma diseases are expected to increase in the future as most insect vectors known to date are rather adapted to warmer climates, thus global warming will facilitate their worldwide spread, making further research efforts into control strategies for these poorly characterized phytopathogens even more important (Maejima et al. 2014).

3.2 Rubus stunt

3.2.1 History, Geographic Distribution, and Phytoplasma Agents

In wild and cultivated red raspberry (*Rubus ideaeus* L.), blackberry (*Rubus* subgenus *Rubus* (Stace 2010)), black raspberry (*Rubus occidentalis* L.), loganberry (*Rubus* x *loganobaccus* L.H. BAILEY), dewberry (*Rubus caesius* L.), and other species of the genus *Rubus*, phytoplasmas cause a disease referred to as Rubus stunt (Davies 2000; Davis et al. 2001; Cieslinska 2011). According to van der Meer (1987) Rubus stunt was probably first mentioned by de Vries (1896) who discovered an epidemic of virescence in 27 plant species in his garden in Amsterdam. Since then, there are multiple reports of Rubus stunt epidemics and incidental occurrences throughout Europe, the former Soviet Union, the United States of America, Pakistan, and Turkey (van der Meer 1987; Mäurer and Seemüller 1994; Sertkaya et al. 2001; Valiūnas et al. 2007; Fahmeed et al. 2009; Cieslinska 2011; Ramkat et al. 2014). Like other phytoplasma diseases, Rubus stunt was initially thought to be caused by a virus (Prentice 1951), but was eventually shown to be caused by phytoplasmas via electron microscopy by Murant and Roberts (1971) and by Marani et al. (1977). Later on, studies using molecular techniques showed that Rubus stunt was caused by a phytoplasma belonging to the elm yellows group (16SrV) (Marcone et al. 1997) which was subsequently classified into new 16Sr subgroup V-E (Davis and Dally 2001). Eventually, the Rubus stunt phytoplasma was classified as a novel candidate taxon '*Candidatus* Phytoplasma rubi' by Malembic-Maher et al. (Malembic-Maher et al. 2011) due to specific 16S rRNA gene sequences.

Although Rubus stunt is usually associated with '*Ca*. Phytoplasma rubi' (16SrV-E), aster yellows phytoplasma (16SrI-B) has been reported in raspberry and blackberry (Borroto Fernández et al. 2007; Fahmeed et al. 2009; Reeder et al. 2010), X disease phytoplasma (16SrIII) in loganberry and black raspberry (Davies 2000; Davis et al. 2001), and stolbur phytoplasma (16SrXII-A) in raspberry (Borroto Fernández et al. 2007). Furthermore, more recently, a new *Ca*. Phytoplasma rubi-related strain causing Rubus stunt in blackberry plants in Portugal was identified and molecularly characterized as a new ribosomal subgroup (16SrV-I) and named blackPort phytoplasma (Fránová et al. 2016).

3.2.2 Symptoms and Transmission

Symptoms of Rubus stunt include typical phytoplasma disease symptoms like stunting, witches' broom, small leaves, short internodes, enlarged sepals, phyllody, flower proliferation, and fruit malformations (Figure 8) (van der Meer 1987; Mäurer and Seemüller 1994).

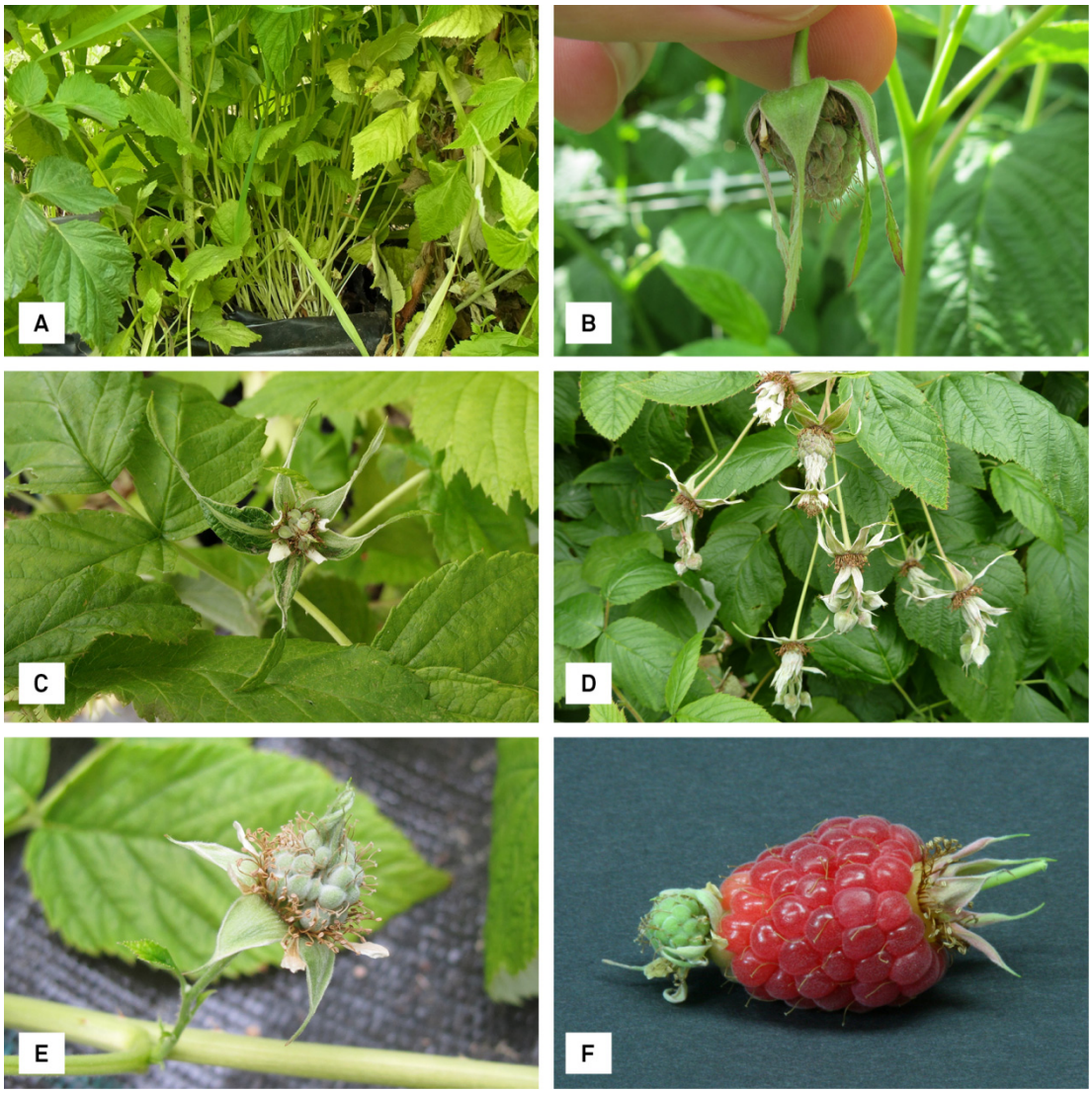

Figure 8: **Rubus stunt disease symptoms on red raspberry (*Rubus ideaeus*). (A) Witches' broom, (B) enlarged sepals, (C) phyllody, (D) flower proliferation, (E) initial fruit malformation, (F) advanced fruit malformation.**

These symptoms are similar in all affected *Rubus* species (Mäurer and Seemüller 1994). However, Davies (2000) found that infected plants, although stunted in their growth, lacked the more typical proliferation symptoms of phytoplasma diseases that are ascribed to Rubus stunt, indicating that infections in *Rubus* might be more common than thought, because they occur without the expression of overt symptoms under certain circumstances. There are no *Rubus* cultivars known in which

phytoplasma infections remain fully latent, but infected *Rubus* plants usually remain symptomless until one year after infection (Converse 1991). In contrast, plants that are already affected by the raspberry mosaic complex, a disease which can be caused by multiple different viruses, are much more sensitive to Rubus stunt and often die within a year after infection (van der Meer 1987).

In most literature about Rubus stunt, the only named insect vector transmitting the disease is the leafhopper *Macropsis fuscula* (ZETTERSTEDT) (Hemiptera: Cicadellidae) (Figure 9 and Figure 10) (Marcone et al. 1997; Davies 2000; Jarausch et al. 2001; Arnaud et al. 2007; Malembic-Maher et al. 2011), as it was able to transmit the Rubus stunt agent from raspberry to strawberry in transmission experiments (van der Meer and de Fluiter 1970). However, Jenser et al. (1981) showed that the froghopper *Philaenus spumarius* (LINNAEUS) (Hemiptera: Aphrophoridae) and the leafhopper *Allygus mayri* (KIRSCHBAUM) (Hemiptera: Cicadellidae) transmitted the Rubus stunt agent to celery and Lehmann (1973) showed that the leafhopper *Euscelis plebeja* (FALLÉN) (= *incisus* KIRSCHBAUM) (Hemiptera: Cicadellidae) transmitted virescence from blackberry to white clover (*Trifolium repens* L.) and tricolor daisy (*Chrysanthemum carinatum* SCHOUSB.). Yet, none of the latter three insects do specifically live on *Rubus* and therefore probably do not play an important role in the natural spread of Rubus stunt (Converse 1991).

Figure 9: ***Macropsis fuscula*** **(By Ian Boyd, CC BY-NC 2.0, https://flic.kr/p/cL5zCj)**

Figure 10: ***Macropsis fuscula*** **(By Ian Boyd, CC BY-NC 2.0, https://flic.kr/p/aewHEU)**

Furthermore, van der Meer (1987) states that it is probable that *Macropsis brabantica* WAGNER and *Macropsis scotti* EDWARDS are able to transmit Rubus stunt, however, this has not been proven in transmission experiments. All three *Macropsis* species have very few morphological differences between them (Wagner 1964) and can easily be misidentified without expert knowledge (Figure 11 and Figure 12).

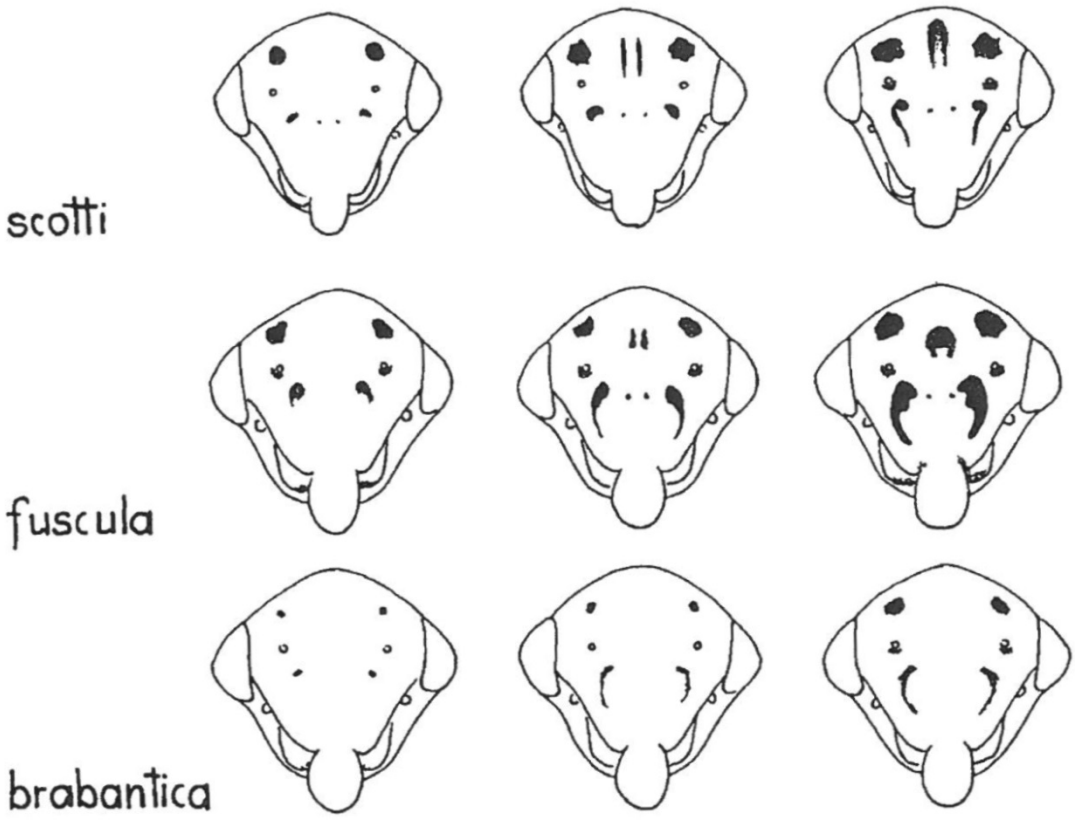

Figure 11: Variability of facial patterns of female *Macropsis scotti*, *fuscula*, and *brabantica*. (From Wagner 1964)

In *M. fuscula* males the face, pronotum, and scutellum are pale-yellow with dark patterns. The forewings are translucent, slightly hazy, with all or main veins strongly darkened. Females are similar to males, but patterns are less developed and with a body length between 4.5 – 5.0 mm they are slightly larger than males with 4.0 – 4.5 mm (Tishechkin 2002).

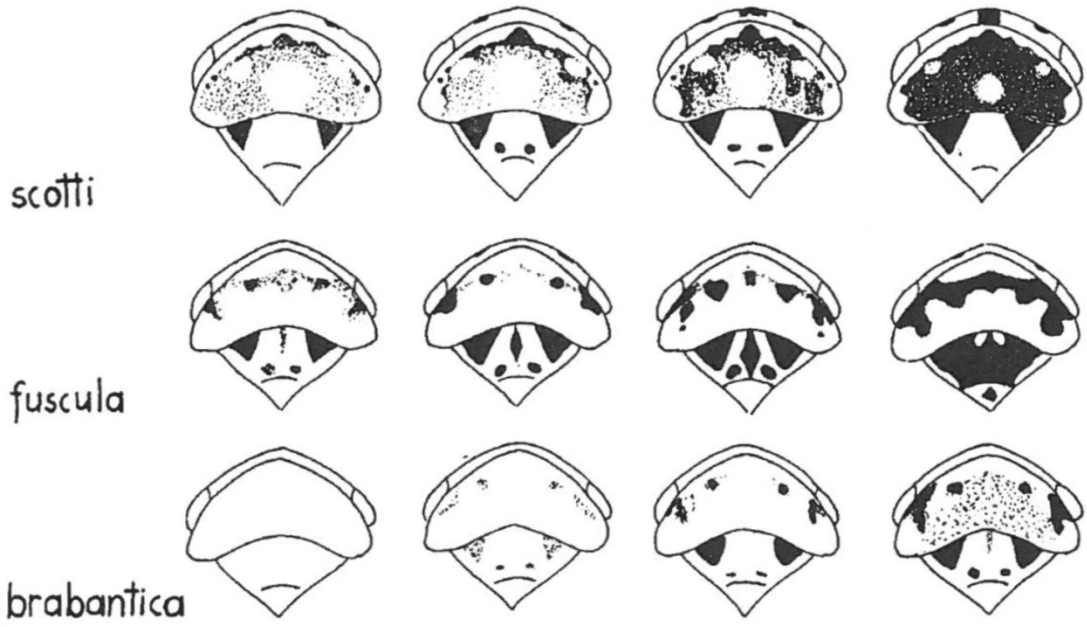

Figure 12: Variability of patterns on the forebody of *Macropsis scotti*, *fuscula*, and *brabantica* (From Wagner 1964).

M. fuscula can be found in Western Europe, Ukraine, European and Far East Russia, the Caucasus, Kazakhstan, Middle Asia, Japan, Western Canada, and the United States (van der Meer 1987; Tishechkin 2002). It produces only one generation per year and overwinters in the egg stage on bark of young canes of *Rubus* species (Brčák 1979). In a moderate maritime climate, the first larvae appear in the middle of May and the first adults appear at the end of June and can be observed until the beginning of October (de Fluiter and van der Meer 1958). Phytoplasmas are not transovarially transmitted in *M. fuscula*, but larvae are able to acquire the Rubus stunt agent, however, cannot transmit it until an 8-week latent period has passed (Converse 1991).

Rubus stunt phytoplasmas were also detected in wild mallow (*Malva* sylvestris L.) with proliferation symptoms and symptomless wild dog rose (*Rosa canina* L.) in areas where *M. fuscula* was present (Jarausch et al. 2001), representing a wild reservoir for phytoplasmas. In addition to its natural transmission by insect vectors, Rubus stunt can be spread by vegetative propagation of infected *Rubus* mother plants during commercial production of planting material in plant nurseries.

3.2.3 Economic Importance and Control

The fruit of *Rubus* species have a high nutritious value with high levels of vitamins, minerals, soluble fiber, antioxidants, and potential health beneficial phytochemicals (Howard and Hager 2007). Therefore, there is a high and increasing demand for these fruits due to a rise in health and nutrition awareness and growing interest in yogurt and juice blends, such as smoothies (Bushway 2008). Raspberry and blackberry are the most economically important cultivated crops derived from the genus *Rubus* worldwide (Hummer et al. 2009). The Food and Agriculture Organization Corporate Statistical Database (FAOSTAT) states an estimated worldwide production area of 93,229 ha and an annual production of 612,570 tons for raspberries for the year 2014 (FAOSTAT 2017). FAOSTAT does not separately evaluate blackberry production, but merges it together with other berry fruits, making it difficult to get an up to date estimate of the global blackberry production. However, Strik et al. (2008) estimated a commercially cultivated blackberry production area of 20,035 ha worldwide for 2005 with an annual production of 140,292 tons. Furthermore, in the same study, they projected an increase in production area to 27,032 ha for the year 2015.

Rubus species are high input crops with high initial investments needed, their fruit, however, can be sold for a greater price per kg than just about any fruit (Bushway 2008). This is why Rubus stunt is of major economic importance, as its period of latency of up to one year is quite long and infected but seemingly healthy plants remain in the field for the disease to spread or even end up in plant propagation, leading to the production of multitudinous infected seedlings. Therefore, molecular testing of mother plants is of utmost importance in order to control Rubus stunt and protect investments of berry producers.

Other control measures against Rubus stunt include elimination of infected plants from production fields and removal of potential wild reservoir plants for phytoplasmas from adjacent areas (like wild mallow and wild dog rose). The insect vectors of Rubus stunt can also be controlled by use of insecticides, although, due to the facts that the full spectrum of putative vectors is not known and it is impossible to annihilate all potential insect vectors from a field with insecticides, this is not an advisable control measure for Rubus stunt, especially when considering the environmentally harmful aspects of insecticides.

3.3 Molecular Plant Disease Detection

To ensure a safe, sustainable, and economical agricultural production, it is of crucial importance to detect plant disease agents like fungi, viruses, or bacteria in plant material. There is a wide range of visual symptoms caused by plant disease infections that can be used for diagnosis. However, many of these visual symptoms can be caused by different pathogens and also by abiotic factors. Furthermore, this visual examination fails to detect latent and early infection stages, which is especially important for viruses and bacteria, as there is a lack of efficient chemical control products for them and preventive measures, like avoiding the planting of contaminated material or early removal of infected plants to prevent the spread of the pathogens, are of highest importance in an integrated approach to disease control (López et al. 2003).

Before the rise of molecular phytodiagnostics, plant pathologists had to rely on a combination of visual symptoms, microscopy, microbiological testing, and biological indexing, all of which are time consuming, often not specific enough and offer only low throughput. Molecular phytodiagnostics existed as early as the late 1970s, as plant viruses were detected by gel electrophoresis of double-stranded RNA and dot-blot hybridization (Dodds et al. 1984). However, even after the development of the polymerase chain reaction (PCR) (Saiki et al. 1985) and its first application to detect a plant disease agent in 1989 (Puchta and Sänger 1989), the utilization of PCR in routine phytodiagnostics was slow due to several drawbacks (e.g. cross-contamination, misinterpretation of results, labor intensity for large sample quantities) and it took until the late 1990s for the main breakthrough of routine molecular phytodiagnostics, when quantitative polymerase chain reaction (qPCR) methods were developed (Mumford et al. 2006).

In this breakthrough of molecular phytodiagnostics initiated by qPCR, the development of TaqMan chemistry by Holland et al. (1991) played a major role (Mumford et al. 2006). TaqMan chemistry employs oligonucleotides, so called TaqMan probes, which have a fluorophore on one end and a quencher at the other end in addition to forward and reverse primers. As long as the probe is intact and the fluorophore and the quencher are in close proximity there is no fluorescence signal of the fluorophore. During amplification, the TaqMan probe, which is located in between the two primers, is degraded due to the exonuclease activity of Taq

polymerase, breaking the close proximity of fluorophore and quencher leading to fluorescence of the fluorophore, which is proportional to the amount of product amplified (Figure 13). Today, qPCR is considered the gold standard or first-line method for the detection of plant pathogens (Palacio-Bielsa et al. 2009; Alemu 2014; Nagy et al. 2017).

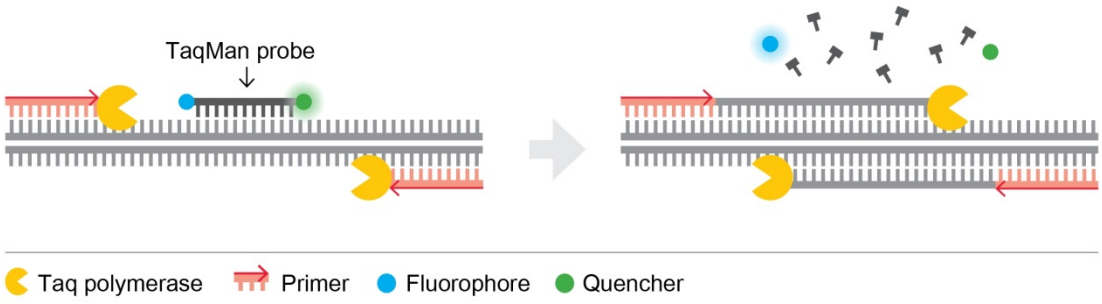

Figure 13: TaqMan probe chemistry mechanism: The primers and TaqMan probe anneal to the target sequence (left). While the TaqMan probe is intact, any excitation of the fluorophore gets transferred to the quencher. During amplification, the TaqMan probe is degraded due to the exonuclease activity of Taq polymerase, separating the fluorophore from the quencher resulting in an increase of fluorescence from the fluorophore (right). (Own illustration)

One major aim of plant pathologists developing new molecular detection methods today is the move from laboratory based methods to on-site testing in the field. The main DNA amplification technique with which this is currently being achieved is loop-mediated isothermal amplification (LAMP) (Le and Vu 2017). This method, which was developed by Notomi et al. (2000), utilizes the strand displacement activity of Bst DNA polymerase (isolated from *Bacillus stearothermophilus*) to amplify target DNA through two or three pairs of specific primers under isothermal conditions. In addition to the fact that LAMP assays can be carried out at a constant temperature, amplification products can be detected visually with the naked eye by adding indicators to the reaction that lead to a color change due to a shift in pH or an increase in turbidity due to magnesium pyrophosphate, making it a convenient method for on-site testing.

To complete the picture, it should be mentioned that microarrays and next-generation sequencing (NGS) are also powerful molecular methods to detect plant pathogens. They are the tools of choice when the causal agent of a plant disease is unknown. While DNA microarrays are able to detect hundreds of known pathogens with sequenced targets in a single test, next-generation sequencing makes it possible to identify new or highly distinct strains of pathogens without known targets by

generating sequences of the entire DNA in a sample and analyzing them with bioinformatics approaches to identify known or putative pathogen sequences (Mumford 2016).

After all, one important factor for successful testing with any molecular detection method is the technique of sample collection and sample preparation, as it plays a crucial role for the quantity and quality of pathogen targets in a DNA extract and the amount of inhibitory substances limiting the activity of enzymes during PCR.

3.4 Aim of this Thesis

The major aim of this PhD thesis was to develop a fast and sensitive molecular detection method for Rubus stunt phytoplasmas in order to have a potent tool for routine diagnostics in plant nurseries and an early detection in production fields (chapter 4). This detection method was subsequently used to investigate the range of potential insect vector species of Rubus stunt (chapter 5). In addition, it was used in comparing the susceptibility of different raspberry cultivars (chapter 6.1) and to examine how to improve and secure the production of healthy planting material by testing the suitability of heat therapy for phytoplasma elimination in mother plants (chapter 6.2).

4 A Multiplex TaqMan qPCR Assay for Sensitive and Rapid Detection of Phytoplasmas Causing Rubus Stunt

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Author Contributions

Conceptualization: HL AR EK.

Data curation: HL.

Formal analysis: HL.

Funding acquisition: AR EK.

Investigation: HL.

Methodology: HL AR EK.

Project administration: AR.

Resources: HL AR EK.

Supervision: HL AR EK.

Validation: HL.

Visualization: HL.

Writing – original draft: HL.

Writing – review & editing: HL AR EK.

4.1 Abstract

Rubus stunt is an economically important disease in the production of raspberries, blackberries, and loganberries. A fast, sensitive, and reliable diagnosis of phytoplasmas, the causal agent of the disease, is of prime importance to stop its spread by vegetative propagation and by insect vectors. Therefore, multiplex qPCR assays using TaqMan probes with different kinds of fluorophores in one reaction were developed, allowing the detection of phytoplasmas in general as well as a more specific detection of phytoplasmas belonging to group 16SrV and host DNA (either plant or insect). This assay now provides a practical tool for the screening of mother plants and monitoring the presence and distribution of phytoplasmas in *Rubus* plants of different geographic origins, cultivars, and cultivation systems, as well as in putative insect vectors like leafhoppers.

4.2 Introduction

Phytoplasmas are cell wall-less plant-pathogenic bacteria that inhabit the phloem of infected plants. They are transferred by phloem feeding insect vectors (Weintraub and Beanland 2006) or by vegetative propagation of infected plants, and can infect more than 700 plant species (Hoshi et al. 2007), including many economically important crops. In wild and cultivated *Rubus* species like raspberry (*Rubus ideaeus* L.), blackberry (*Rubus* subgenus *Rubus* (Stace 2010)), loganberry (*Rubus x loganobaccus* L.H. BAILEY), and European dewberry (*Rubus caesius* L.) they cause a disease referred to as Rubus stunt. Symptoms include stunted growth, shoot proliferation, small leaves, short internodes, enlarged sepals, phyllody, flower proliferation, as well as fruit malformations (van der Meer 1987; Mäurer and Seemüller 1994).

Rubus stunt is usually associated with '*Candidatus* Phytoplasma rubi', which belongs to the 16Sr group of elm yellows phytoplasmas (16SrV) (Mäurer and Seemüller 1994; Bertaccini et al. 1995; Lee et al. 1995; Marcone et al. 1997; Davies 2000; Malembic-Maher et al. 2011). However, phytoplasmas from the groups of X-disease (16SrIII), aster yellows (16SrI), and stolbur (16SrXII) have also been identified in Rubus stunt symptomatic *Rubus* spp. plants (Davies 2000; Borroto Fernández et al. 2007; Fahmeed et al. 2009; Reeder et al. 2010; Cieslinska 2011), and may cause similar symptoms like the ones described above. So far, little is known about the

presence and distribution of phytoplasmas in different *Rubus* species or cultivars, the impact of the respective cultivation system, their geographical spread as well as about the spectrum of putative insect vectors. The only vector of Rubus stunt in raspberries known to date, the leafhopper *Macropsis fuscula* (ZETTERSTEDT) (Hemiptera: Cicadellidae), has been identified in transmission experiments by de Fluiter and van der Meer (1953). As *Rubus* plants are produced by vegetative propagation and the time between plant infection and the development of phytoplasma disease symptoms varies from 4 to 11 months (de Fluiter and van der Meer 1953), an early detection of phytoplasmas in plant nurseries, using highly sensitive and rapid molecular methods, is of great importance to minimize their future spread.

Because phytoplasma titers in *Rubus* plants are generally very low, regular PCR is often not sensitive enough to detect phytoplasma DNA even in plants with clear proliferation symptoms (Jarausch et al. 2001). Therefore, the most utilized method to acquire diagnostic results is nested PCR (Delić 2012; Christensen et al. 2013). Nested PCR is very sensitive, but time consuming, requires post-amplification steps often with hazardous substances (Baric et al. 2006), and has an increased risk of carry-over contamination (Galetto et al. 2005; Angelini et al. 2007; Pelletier et al. 2009; Nikolić et al. 2010; Delić 2012). For the screening of Rubus stunt, qPCR, with its direct and sensitive detection of the amplification product, offers a major advantage compared to nested PCR, due to significant time savings and a reduced risk of false positive results. Furthermore, the possibility to employ TaqMan probes labeled with different fluorogenic dyes enables multiplex detection of different DNA targets in a single reaction tube. Here we present multiplex TaqMan qPCR assays that combine primers and TaqMan probes previously published in literature (Christensen et al. 2004; Marzachí and Bosco 2005; Oberhänsli et al. 2011) with a newly designed primer and probe pair, allowing a specific, rapid, and simultaneous diagnosis of phytoplasma infections in general as well as a more specific detection of elm yellows phytoplasmas (16SrV) infecting *Rubus* species. In addition, DNA of the host (plant or insect) is detected simultaneously in the same assay serving as an internal control.

4.3 Materials and Methods

4.3.1 Plant Material and Plant DNA Extraction

Healthy and Rubus stunt symptomatic raspberry and blackberry plant samples were obtained from different commercial plantings throughout Germany (Table 2). Plant DNA extraction was done according to a protocol modified from Daire et al. (Daire et al. 1997). Leaf or root tissue (1 g) was homogenized in a Bioreba extraction bag <Universal> (Bioreba AG, Switzerland) at room temperature in a mixture of 4 ml of CTAB buffer (3% CTAB, 0.1 M Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl) and 8 µl of 2-mercaptoethanol. The filtrate was incubated in a water bath at 65 °C for 20 min and was extracted with chloroform:isoamyl alcohol (24:1). Nucleic acids were obtained by isopropanol-precipitation. Extracted DNA was dissolved in deionized sterile water and stored at -20°C until use. All DNA extracts (including the insect samples) were measured for the concentration of nucleic acids and protein purity with a NanoDrop ND-1000 spectrophotometer (NanoDrop products, Wilmington, USA).

Table 2: Raspberry plant samples. Locations used for sampling as well as tissue types and observed symptoms are presented. The same DNA extracts were used for qPCR and nested PCR analysis. In case of discrepancies in the results between qPCR and nested PCR, reactions were repeated.

Sample ID	Sample Tissue	Symptoms	Location in Germany	Coordinates	qPCR [C_q-value]	nested PCR
H1	Leaf	stunted growth, fruit deformation	Telgte	51°59'27"N 7°49'23"E	33.09	positive
H2	Leaf	stunted growth, fruit deformation	Telgte	51°59'27"N 7°49'23"E	30.55	positive
H3	Bast	stunted growth, fruit deformation	Telgte	51°59'27"N 7°49'23"E	28.24	positive
H4	Leaf	fruit deformation	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H5	Leaf	fruit deformation	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H6	Bast	fruit deformation	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H7	Peduncle	fruit deformation	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H8	Peduncle	fruit deformation	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H9	Fruit	fruit deformation	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H10	Leaf	stunted growth	Apensen	53°28'16"N 9°35'40"E	31.34	positive
H11	Leaf	stunted growth	Apensen	53°28'16"N 9°35'40"E	33.85	positive
H12	Bast	stunted growth	Apensen	53°28'16"N 9°35'40"E	28.94	positive
H13	Leaf	crumbly fruit	Oberkirch	48°31'40"N 8°01'33"E	negative	negative
H14	Root	crumbly fruit	Oberkirch	48°31'40"N 8°01'33"E	negative	negative
H15	Leaf	fruit deformation	Oberkirch	48°31'40"N 8°01'33"E	negative	negative
H16	Root	fruit deformation	Oberkirch	48°31'40"N 8°01'33"E	negative	negative

Table 2: (continued)

Sample ID	Sample Tissue	Symptoms	Location in Germany	Coordinates	qPCR [Cq-value]	nested PCR
H17	Root	fruit deformation	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H18	Leaf	enlarged sepals	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H19	Root	enlarged sepals	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H20	Root	fruit deformation	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H21	Leaf	fruit deformation	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H22	Leaf	fruit deformation	Oberkirch	48°31'40"N 8°01'33"E	negative	negative
H23	Leaf	fruit deformation	Heuchlingen	49°15'12"N 9°13'40"E	negative	negative
H24	Leaf	fruit deformation	Heuchlingen	49°15'12"N 9°13'40"E	negative	negative
H25	Leaf	stunted growth	Kaichen	50°15'11"N 8°50'04"E	negative	negative
H26	Root	stunted growth	Kaichen	50°15'11"N 8°50'04"E	negative	negative
H27	Leaf	stunted growth	Kaichen	50°15'11"N 8°50'04"E	negative	negative
H28	Root	stunted growth	Kaichen	50°15'11"N 8°50'04"E	negative	negative
H29	Leaf	stunted growth	Kaichen	50°15'11"N 8°50'04"E	negative	negative
H30	Root	stunted growth	Kaichen	50°15'11"N 8°50'04"E	negative	negative
H31	Leaf	stunted growth	Telgte	51°59'27"N 7°49'23"E	33.24	positive
H32	Root	stunted growth	Telgte	51°59'27"N 7°49'23"E	33.87	positive
H33	Leaf	stunted growth	Telgte	51°59'27"N 7°49'23"E	negative	negative
H34	Root	stunted growth	Telgte	51°59'27"N 7°49'23"E	28.58	positive
H35	Leaf	long shoots	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H36	Root	long shoots	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H37	Leaf	crumbly fruit	Oberkirch	48°31'40"N 8°01'33"E	negative	negative
H38	Root	crumbly fruit	Oberkirch	48°31'40"N 8°01'33"E	negative	negative
H39	Leaf	stunted growth, fruit deformation, crumbly fruit	Oberkirch	48°31'40"N 8°01'33"E	negative	negative
H40	Root	stunted growth, fruit deformation, crumbly fruit	Oberkirch	48°31'40"N 8°01'33"E	negative	negative
H41	Leaf	stunted growth, fruit deformation, crumbly fruit	Oberkirch	48°31'40"N 8°01'33"E	negative	negative
H42	Leaf	stunted growth, shoot proliferation	Telgte	51°59'27"N 7°49'23"E	31.51	positive
H43	Leaf	fruit deformation	Geisenheim	49°58'53"N 7°57'01"E	negative	negative
H44	Leaf	enlarged sepals, fruit deformation	Heuchlingen	49°15'12"N 9°13'40"E	negative	negative
H45	Leaf	fruit deformation	Heuchlingen	49°15'12"N 9°13'40"E	negative	negative
H46	Leaf	fruit deformation	Heuchlingen	49°15'12"N 9°13'40"E	negative	negative
H47	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	34.24	positive
H48	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	31.63	positive
H49	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	negative	positive
H50	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	34.12	negative
H51	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	32.04	positive
H52	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	31.84	negative
H53	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	negative	positive
H54	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	negative	negative
H55	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	negative	negative
H56	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	negative	negative

Table 2: (continued)

Sample ID	Sample Tissue	Symptoms	Location in Germany	Coordinates	qPCR [Cq-value]	nested PCR
H57	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	36.29	positive
H58	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	37.15	positive
H59	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	34.62	positive
H60	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	negative	negative
H61	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	negative	negative
H62	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	negative	positive
H63	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	35.70	positive
H64	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	33.17	positive
H65	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	negative	negative
H66	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	37.56	negative
H67	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	35.27	positive
H68	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	32.02	positive
H69	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	28.23	positive
H70	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	negative	negative
H71	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	negative	negative
H72	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	37.59	positive
H73	Leaf	crumbly fruit	Oberkirch	48°31'40"N 8°01'33"E	negative	negative
H74	Root	crumbly fruit	Oberkirch	48°31'40"N 8°01'33"E	negative	negative
H75	Leaf	stunted growth, fruit deformation, crumbly fruit	Oberkirch	48°31'40"N 8°01'33"E	negative	negative
H76	Root	stunted growth, fruit deformation, crumbly fruit	Oberkirch	48°31'40"N 8°01'33"E	negative	negative
H77	Leaf	crumbly fruit	Oberkirch	54°17'29"N 8°47'56"E	negative	negative
H78	Leaf	crumbly fruit	Vollerwiek	54°22'19"N 10°19'17"E	negative	negative
H79	Leaf	crumbly fruit	Blekendorf	54°17'22"N 10°38'06"E	negative	negative
H80	Leaf	stunted growth	Oberkirch	48°31'40"N 8°01'33"E	29.62	positive
H81	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	26.44	positive
H82	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	32.21	positive
H83	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	24.90	positive
H84	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	28.91	positive
H85	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	29.26	positive
H86	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	33.84	positive
H87	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	32.52	positive
H88	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	30.47	positive
H89	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	28.95	positive
H90	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	30.54	positive
H91	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	32.82	positive
H92	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	30.38	positive
H93	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	27.39	positive
H94	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	28.95	positive
H95	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	26.94	positive
H96	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	34.20	negative
H97	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	33.61	positive
H98	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	negative	negative
H99	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	32.06	positive

Table 2: (continued)

Sample ID	Sample Tissue	Symptoms	Location in Germany	Coordinates	qPCR [Cq-value]	nested PCR
H100	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	27.96	positive
H101	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	28.97	positive
H102	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	28.90	positive
H103	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	30.05	negative
H104	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	24.38	positive
H105	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	24.65	positive
H106	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	24.16	positive
H107	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	22.77	positive
H108	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	23.70	positive
H109	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	22.58	positive
H110	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	23.58	positive
H111	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	23.45	positive
H112	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	21.58	positive
H113	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	23.25	positive
H114	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	23.06	positive
H115	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	24.21	positive
H116	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	24.80	positive
H117	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	23.21	positive
H118	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	22.46	positive
H119	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	22.66	positive
H120	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	24.91	negative
H121	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	23.99	positive
H122	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	24.80	positive
H123	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	27.29	positive
H124	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	25.58	positive
H125	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	23.15	positive
H126	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	23.12	positive
H127	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	23.12	positive
H128	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	23.12	positive
H129	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	25.93	positive
H130	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	34.74	positive
H131	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	25.26	positive
H132	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	27.21	positive
H133	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	23.78	positive
H134	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	27.48	positive
H135	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	28.05	positive
H136	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	26.42	positive
H137	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	26.66	positive
H138	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	31.47	positive
H139	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	25.75	positive
H140	Leaf	stunted growth	Rielingshausen	48°57'31"N 9°20'08"E	25.00	positive

DNA from the following phytoplasmas was used to check for specificity of the developed assay: apple proliferation (16SrX-A), aster yellows (16SrI-B), ash yellows (16SrVII-A), and elm yellows (16SrV-A), kindly provided by E. Seemüller (Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Dossenheim, Germany), western X (16SrIII-A) and Rubus stunt (16SrV-E), kindly provided by A. Bertaccini (Università di Bologna, Bologna, Italy), and flavescence dorée strain FD70 and palatinate grape vine yellows strain EY17-49 (both 16SrV) kindly provided by M. Maixner (Julius Kühn-Institut, Federal Research Centre for Cultivated Plants, Siebeldingen, Germany). A sample from a symptomless raspberry plant was used as negative control, and deionized sterile water was used as no-template control (NTC).

4.3.2 Insect Samples and Insect DNA Extraction

DNA from an individual leafhopper (Hemiptera: Cicadellidae) which was apparently free of phytoplasmas after a PCR with primer pairs P1/P7 (see below) was used as a negative control. Deionized sterile water was used as no-template control.

4.3.3 Oligonucleotide Primers and Probes

Elm yellows group specific primers and TaqMan probes were designed for the *secY* gene of '*Ca*. Phytoplasma rubi' (GenBank accession number AM397299) (Figure 14) using PrimerQuest (Integrated DNA Technologies, Inc., Coralville, Iowa, USA). Therefore, we aligned 14 sequences of *secY* genes of different phytoplasmas using Geneious 6.1.7 (Biomatters Ltd., Auckland, New Zealand). Respective accession numbers and origins are provided in Table 3. Specificity was checked by using NCBI's Primer-BLAST for the primers and Nucleotide BLAST for the TaqMan probe (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

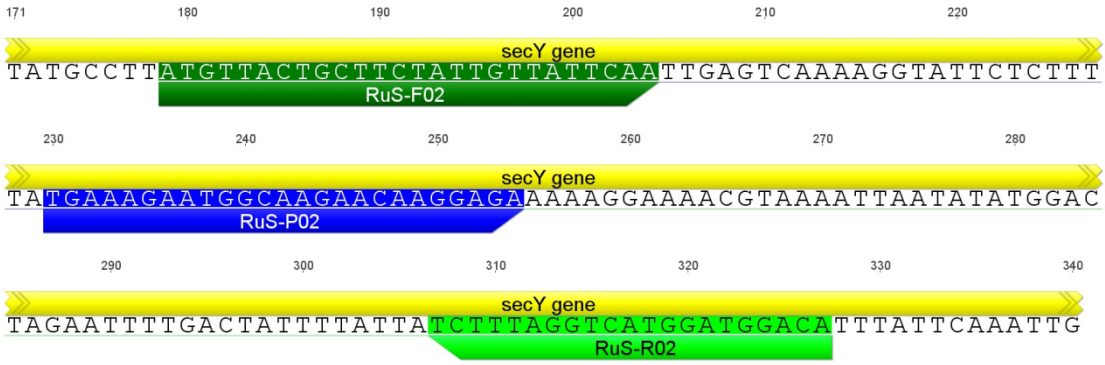

Figure 14: Sequence of the *secY* gene of *'Ca.* phytoplasma rubi' used for group specific amplification and detection of elm yellow phytoplasmas. Locations of forward primer (RuS-F02), probe (RuS-P02), and reverse primer (RuS-R02) are indicated. Numbering of nucleotide positions is according to GenBank accession number AM397299.

Table 3: Accession numbers used in sequence alignment of *secY* genes of different phytoplasmas

Accession Number	Description
AM397299	'*Candidatus* Phytoplasma rubi' partial *secY* gene for preprotein translocase SecY, isolate RuS
AM397300	'*Candidatus* Phytoplasma rubi' partial *secY* gene for preprotein translocase SecY, isolate RI04-2-6
JX857861	Blueberry stunt phytoplasma isolate BBS44secYc3-NJ protein translocase subunit secY (*secY*) gene, complete cds
JX857858	'*Candidatus* Phytoplasma phoenicium' isolate BBS41secYc8-NJ protein translocase subunit secY (*secY*) gene, complete cds
JX645768	'*Candidatus* Phytoplasma solani' strain 138/10 preprotein translocase subunit (*secY*) gene, complete cds; and adenylate kinase (*adk*) gene, partial cds
JQ268254	'*Candidatus* Phytoplasma pruni' strain PX11CT1 ribosomal protein L15 (*rpL15*) gene, partial cds; SecY protein translocase (*secY*) gene, complete cds; and methionine aminopeptidase (*map*) gene, partial cds
GU004330	'*Candidatus* Phytoplasma ulmi' strain EY1 ribosomal protein L15 (*rpl15*) gene, partial cds; protein translocase (*secY*) gene, complete cds; and methionine aminopeptidase (*map*) gene, partial cds
FJ648493	'*Candidatus* Phytoplasma vitis' isolate CL-NG98 ribosomal protein L15 (*rplO*) and preprotein translocase (*secY*) genes, partial cds
AY197694	Peach yellows phytoplasma strain PY-IN ribosomal protein L15 (*rpl15*) and translocation protein secY (*secY*) genes, partial cds
AY197693	Cherry lethal yellows phytoplasma strain CLY-5 ribosomal protein L15 (*rpl15*) and translocation protein secY (*secY*) genes, partial cds
AY197689	Spartium witches' broom phytoplasma strain SpaWB 229 ribosomal protein L15 (*rpl15*) and translocation protein secY (*secY*) genes, partial cds

Table 3: (continued)

Accession Number	Description
AY197687	Hemp dogbane yellows phytoplasma strain HD1 ribosomal protein L15 (*rpl15*) and translocation protein secY (*secY*) genes, partial cds
AY197684	Alder yellows phytoplasma strain ALY ribosomal protein L15 (rpl15) and translocation protein secY (secY) genes, partial cds
AM397297	'*Candidatus* Phytoplasma ulmi' partial *secY* gene for preprotein translocase SecY, isolate E04-D714

Furthermore, Geneious 6.1.7 (Biomatters Ltd., Auckland, New Zealand) was used to create an alignment of phytoplasma strains used to test for specificity in the validation of the assay for plant material, showing primer binding sites (Figure 15). For the universal detection of phytoplasmas a primer and probe pair from Christensen et al. (Christensen et al. 2004) was used. In addition, a primer and probe set for detection of host plant DNA (Oberhänsli et al. 2011) and for host insect DNA (2005), both targeting the 18S rDNA, were used as an internal control. TaqMan probes were either labelled with FAM, ROX, or Cy5, allowing simultaneous detection of three targets in a single reaction. Sequences, expected size of the amplification product, specificity, final concentrations, and fluorogenic dyes used for each primer and probe combination are shown in Table 4. All oligonucleotide primers and probes were synthesized by Biolegio (Nijmegen, the Netherlands).

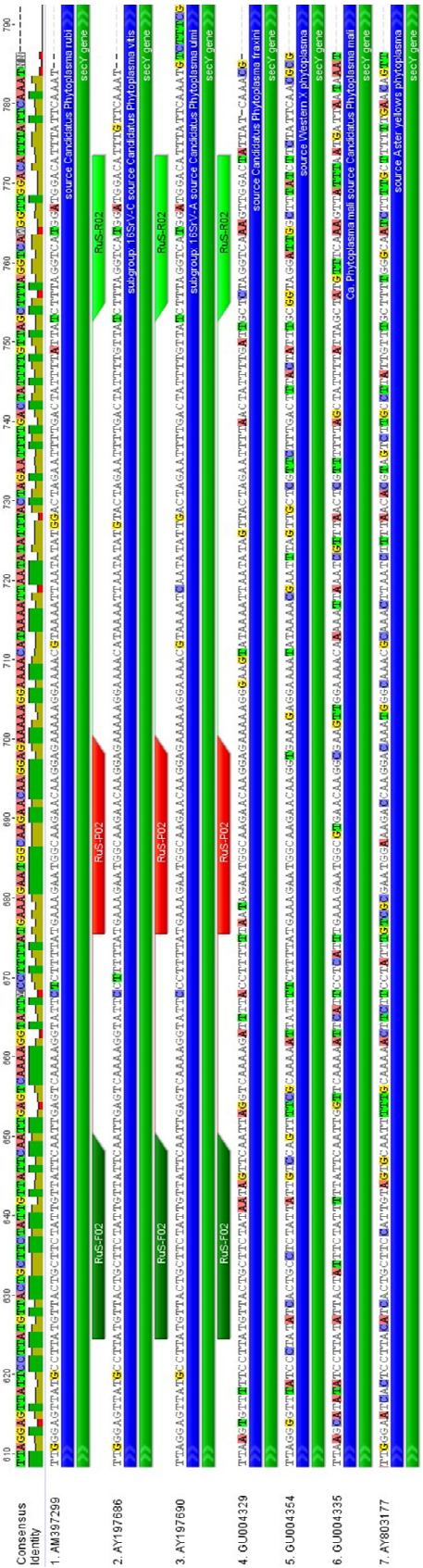

Figure 15: Sequence alignment of the phytoplasma strains used for testing specificity shown in Table 2 (except for palatinate grapevine yellows, as there was no sequence available), showing the binding sites of RuS-F02, RuS-P02, and RuS-R02. (AM397299) Rubus stunt 16SrV-E, (AY197686) flavescence dorée 16SrV, (AY197690) elm yellows 16SrV-A, (GU004329) ash yellows 16SrVII-A, (GU004354) western X 16SrIII-A, (GU004335) apple proliferation 16SrX-A, and (AY803177) aster yellows 16SrI-B.

Table 4: Sequence, size of the expected PCR product, specificity, final concentration, and attached fluorophores for primers and probes used in the Rubus stunt multiplex TaqMan qPCR assay.

			Final Concentration [nM]			
Primer /Probe	**Sequence [5' – 3']**	**Specificity**	**Plant Assay**	**Insect Assay**	**Fluorophore**	**Reference**
RuS-F02	ATGTTACTGCTTCTATTGTTATTCAA		400	200	-	
RuS-R02	TGTCCATCCATGACCTAAAGA	(16SrV)	400	200	-	This study
RuS-P02	TGAAAGAATGGCAAGAACAAGGAGA		400	200	FAM	
UPPFw*	CGTACGCAAGTATGAAACTTAAAGGA		100	100	-	
UPPRv*	TCTTCGAATTAAACAACATGATCCA	Phytoplasma universal	100	100	-	(Christensen et al. 2004)
UPPProbe*	TGACGGGACTCCGCACAAGCG		100	100	Cy5	
18SF*	AGAGGGAGCCTGAGAAACGG		100	-	-	
18SR*	CAGACTCATAGAGCCCGGTATTG	plant host DNA	100	-	-	(Oberhänsli et al. 2011)
18SP*	CCACATCCAAGGAAGGCAGCAGGCG		100	-	ROX	
MqFw	AACGGCTACCACATCCAAGG		-	100	-	
MqRv	GCCTCGGATGAGTCCCG	insect host DNA	-	100	-	(Marzachí and Bosco 2005)
MqProbe	AGGCAGCAGGCACGCAAATTACCC		-	100	ROX	

*as designated by the authors of the paper on hand

4.3.4 Standard Curve

To generate a qPCR standard curve for the elm yellows phytoplasma specific primers and probe a PCR with primer pairs RuS-F02 and RuS-R02 was carried out with '*Ca*. Phytoplasma rubi' DNA to get a 149 bp amplicon. The amplicon was purified with the Hi Yield Gel/PCR DNA Fragment Extraction Kit (Süd-Laborbedarf GmbH, Gauting, Germany) and cloned using the pGEM-T Easy Vector System II (Promega GmbH, Mannheim, Germany). The purified plasmids were quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop products, Wilmington, USA). The number of molecules in one µl of plasmid solution was calculated based on the molecular weight using the formula: number of copies = plasmid concentration/[(plasmid size + insert (bp) × 660)/(Avogadros's number)]. In this case, the purified cloned pGEM-T Easy Vector (3015 + 149 bp) had a concentration of

1.24×10^{-7} g/µl. Hence, the number of copies was 3.59×10^{10} molecules/µl. Ten-fold serial dilutions from 1×10^{9} to 1×10^{5} molecules/µl of the purified plasmids were used to generate the standard curve. As the *secY* gene is a single copy gene in phytoplasma genomes (Oshima et al. 2004) the copy number corresponds to the number of phytoplasma cells.

In addition, the same serial dilution of plasmid DNA was run with 1 µl of a DNA extract (at 100 ng/µl nucleic acid) from an uninfected raspberry plant on the same qPCR plate as the standard curve, to evaluate the degree of interference of plant DNA extracts in the quantification of phytoplasma copy numbers. All samples were run in triplicate.

4.3.5 TaqMan qPCR Assay

Oligonucleotides were combined in a qPCR assay to detect DNA of elm yellows phytoplasmas (RuS-F02, RuS-R02, RuS-P02), phytoplasma DNA in general (UPPFw, UPPRv, UPPProbe), and either plant (18SF, 18SR, 18SP) or insect (MqFw, MqRv, MqProbe) host DNA as an internal control. Assays were run in 25 µl reactions using the KAPA PROBE FAST Master Mix (2X) Universal (Kapa Biosystems, Cape Town, South Africa) on an iQ5 real-time thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA), with an initial denaturation step of 20 sec at 95 °C followed by 40 cycles with 3 sec denaturation at 95 °C and 30 sec annealing and elongation at 60 °C. Optimal final concentrations of primer and probe pairs were initially determined empirically and are shown in Table 4.

The multiplex assays were validated by comparing quantification cycle (C_q) values of samples that were run both in singleplex and multiplex assays on the same 96-well plate. One validation assay was run for plant samples and one for insects. For the plant assay DNA from a Rubus stunt symptomatic infected raspberry plant was adjusted to 250 ng/µl of total nucleic acid and then used in six 10-fold (from 1 to 10^{-5}) serial dilutions. Since there was no insect sample with DNA from '*Ca*. Phytoplasma rubi' available, an artificial reference sample was created by mixing DNA from a phytoplasma-free leafhopper (same sample as the one used as a negative control in the assays) with '*Ca*. Phytoplasma rubi' DNA from an infected periwinkle (*Catharanthus roseus* (L.) G.DON) plant in equal parts. This sample was adjusted to 200 ng/µl of total nucleic acid and was used in the same serial dilutions

as the plant DNA sample. All samples were run in triplicate in qPCR assays with 2 µl of DNA solution.

The assay for plant material was further validated by mixing fresh leaf material of infected and healthy raspberry plants in known weight ratios in a total of 1 g, to obtain proportions of 100, 75, 50, 33, 11, 6, 1, and 0% infected leaf material, respectively. Each mixture was extracted in three independent replicates. These samples were diluted to a concentration of 100 ng/µl of total nucleic acid for use in the qPCR assay and were run in triplicate as described above.

4.3.6 Nested PCR

Nested PCR was run with primer pairs P1/P7 (Deng and Hiruki 1991; Smart et al. 1996) for the direct PCR, followed by U5/U3 (Lorenz et al. 1995) for the nested PCR. DreamTaq DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) was used in 20 µl reactions on a MyCycler Thermal Cycler System (Bio-Rad Laboratories, Hercules, CA, USA). The product of the direct PCR was diluted 1:30 in deionized sterile water for use in the nested PCR. For comparability with the multiplex assay validation, the same serial dilutions as in the multiplex validation assays were run in nested PCRs. PCR products were visualized on 1% agarose gels stained with Invitrogen SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, MA, USA) and were run for 60 min at 80 V.

4.4 Results

4.4.1 Standard Curve

The efficiency of the multiplex TaqMan qPCR assay for the elm yellows specific primers and TaqMan probe presented by the standard curve using plasmid DNA containing the cloned phytoplasma amplicon was 99.9% (Figure 16). For target concentrations of at least 1×10^9 copy numbers, obtained C_q-values were similar for pure plasmid DNA and a mixture of plasmid DNA with plant DNA. Accordingly, 1×10^9 copy numbers can be regarded as a necessary threshold value for accurate quantification of phytoplasma DNA in infected plant tissues.

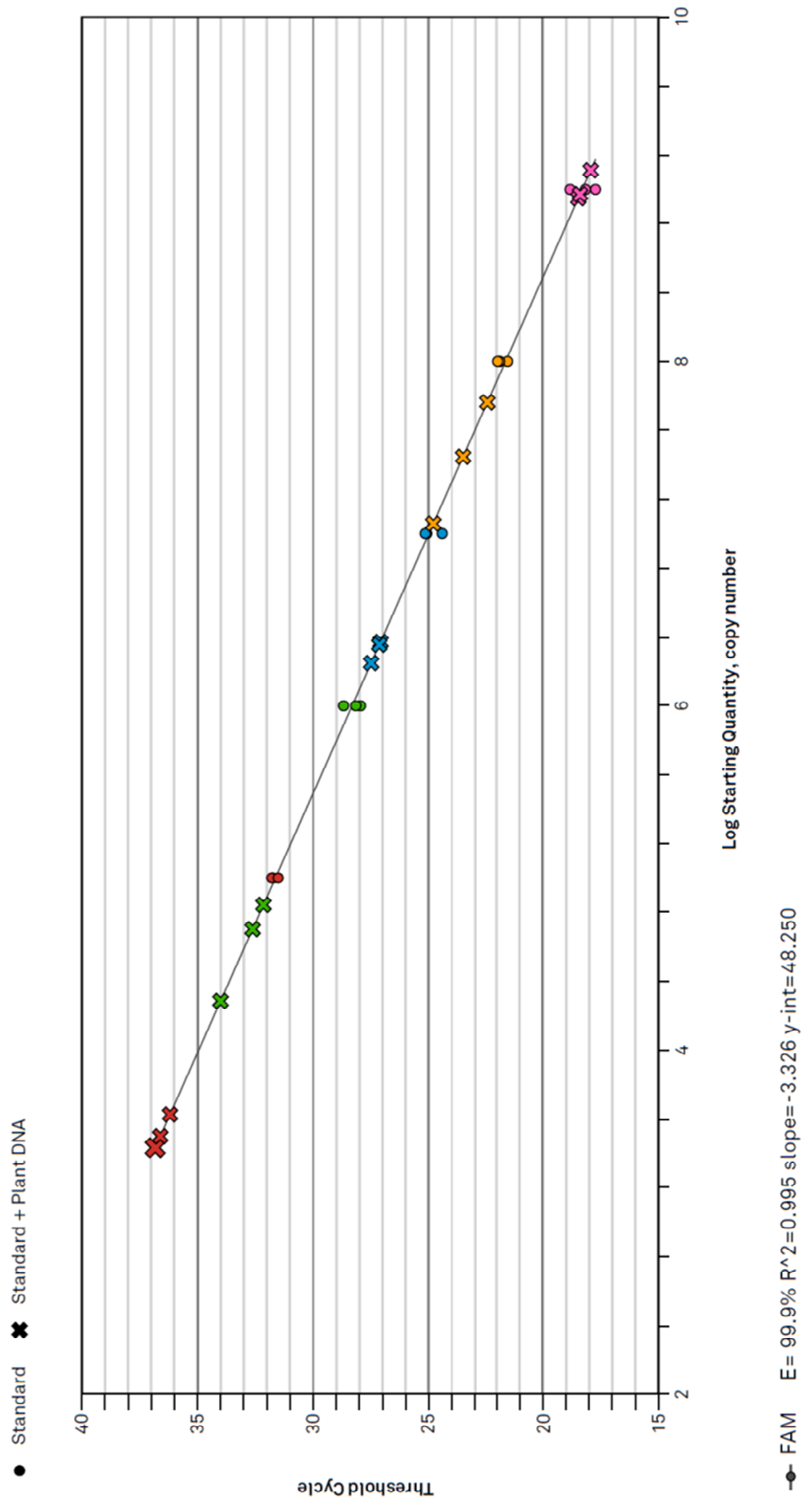

Figure 16: **Standard curve for the elm yellows specific primers and probe of the multiplex TaqMan qPCR assay. The standard curve was generated with 10-fold dilutions of plasmid DNA (Standard, indicated by a dot) containing an insert from the *secY* gene of '*Ca*. Phytoplasma rubi'. In addition, plasmid DNA was mixed with raspberry plant DNA (Standard + Plant DNA, indicated by a cross) to show interference of the DNA extract when quantifying phytoplasmas in plant tissues.**

4.4.2 Validation of the Multiplex TaqMan Assay for Plant Material

The results of the multiplex TaqMan qPCR assay validation on *Rubus* plants infected with phytoplasmas are shown in Figure 17. Graphs show C_q-values plotted against serial dilutions from a Rubus stunt infected raspberry DNA extract. Both primer/probe sets for the detection of phytoplasma DNA (elm yellows phytoplasmas and phytoplasma universal) were able to detect their respective targets along a 10-fold serial dilution gradient from undiluted DNA (500 ng of total nucleic acid) up to a dilution of 10^{-3}. The internal control for plant host DNA yielded positive results for all six 10-fold serial dilutions. There were no obvious differences in C_q-values between multiplex and singleplex assays (Figure 17). The negative control showed a signal only for the internal control (plant host DNA). No signals were obtained for the no-template controls (data not shown).

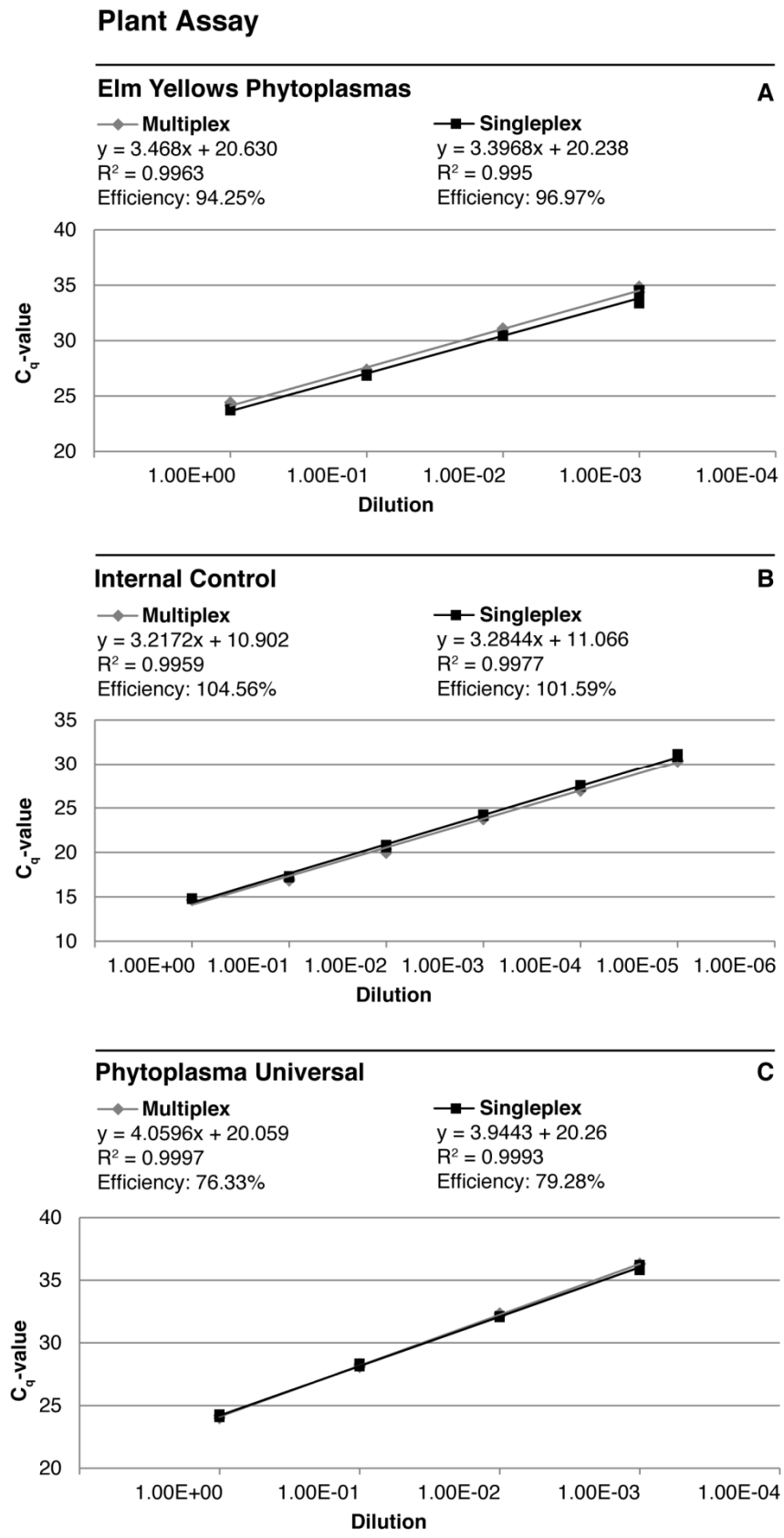

Figure 17: Standard curves obtained in the multiplex validation assay with Rubus plant samples. C_q-values of the singleplex and multiplex TaqMan qPCR are plotted against a 10-fold serial dilution from a Rubus stunt positive DNA extract with primers and probes for the detection of (A) elm yellows phytoplasmas, (B) plant host DNA as an internal control, and (C) phytoplasmas in general. Slopes, R^2 and efficiencies of the respective reactions are presented for each curve.

When the same samples were run in nested PCR with primer pairs P1/P7 (direct PCR) and U5/U3 (nested PCR), an amplification product of the expected size could be obtained up to a dilution of 10^{-4} (Figure 18). However, the nested PCR had to be carried out four times and had to be set up under an UV sterilization cabinet in order to achieve results without contaminations in the no-template control or the negative control.

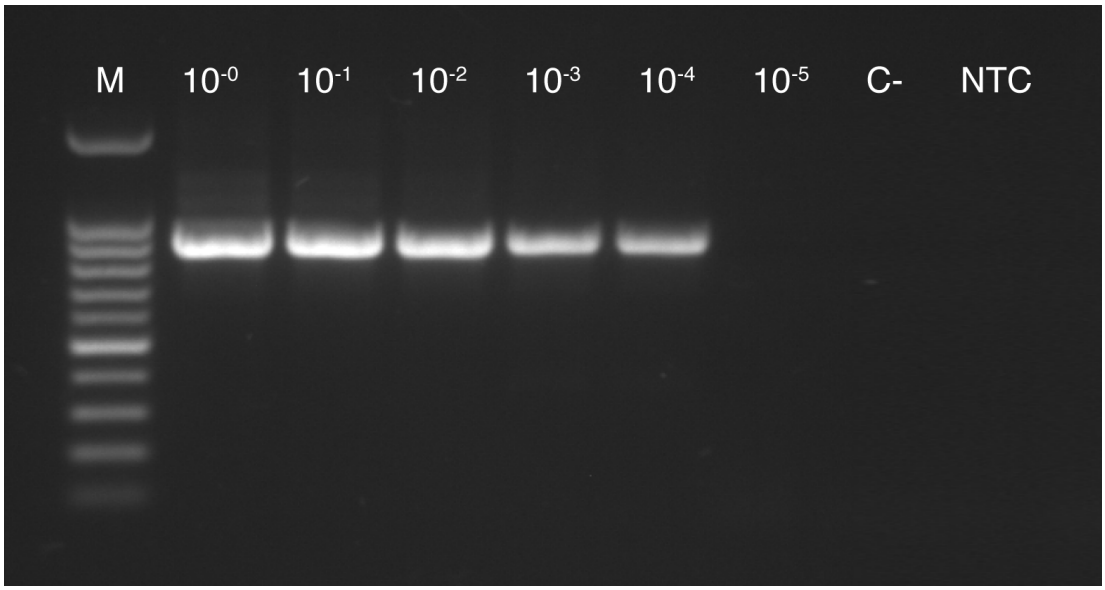

Figure 18: Phytoplasma specific nested PCR products of a 10-fold serial dilution (10^0 to 10^{-5}) from a Rubus stunt positive DNA extract using primer pairs P1/P7 and U5/U3. For nested PCR the same DNA extracts as in the validation assay for the multiplex qPCR for plant samples (Figure 17) were used. (M) Metabion mi-100 bp+ DNA Marker Go, (C-) negative control, (NTC) no template control.

For routine purposes in diagnostics of phytoplasmas, material of several asymptomatic plants (whether infected or not) is often mixed, resulting in different amounts of phytoplasma infected material. When the qPCR assay for plant samples was run with different proportions of infected and healthy leaf material (100, 75, 50, 33, 11, 6, 1, and 0% infected material), it was evident that it is possible to reliably detect phytoplasma DNA in samples containing only 1% of infected leaf material (Table 5).

Table 5: Mean C_q-values and standard deviations of the multiplex TaqMan qPCR assay for DNA samples obtained from different proportions of phytoplasma infected and healthy leaf material. Each leaf sample was extracted in three independent replicates (designated as 1-3) and each DNA extract was analyzed in triplicate in the qPCR.

Amount infected leaf material [%]	Replicate	Plant host DNA		16SrV Phytoplasmas		Phytoplasma universal	
		C_q Mean	C_q Std. Dev	C_q Mean	C_q Std. Dev	C_q Mean	C_q Std. Dev
100	1	14.28	0.035	24.12	0.038	25.32	0.163
100	2	14.54	0.046	24.66	0.046	26.00	0.042
100	3	14.06	0.031	24.41	0.156	25.78	0.074
75	1	14.06	0.069	24.75	0.104	26.00	0.113
75	2	16.26	0.063	27.06	0.151	29.09	0.190
75	3	14.23	0.126	24.31	0.542	26.56	0.131
50	1	14.12	0.017	23.56	0.353	25.33	0.101
50	2	14.30	0.040	24.57	0.249	26.11	0.140
50	3	14.25	0.122	24.78	0.090	26.22	0.081
33	1	14.33	0.099	26.08	0.021	27.97	0.125
33	2	14.43	0.037	25.33	0.102	26.76	0.102
33	3	14.29	0.071	24.90	0.105	26.25	0.090
11	1	14.66	0.149	28.13	0.058	30.56	0.051
11	2	14.33	0.018	26.75	0.190	28.35	0.139
11	3	18.17	0.069	31.43	0.042	33.67	0.319
6	1	14.54	0.035	27.76	0.278	29.85	0.095
6	2	14.51	0.099	28.41	0.182	30.45	0.120
6	3	14.36	0.037	26.84	0.243	28.71	0.029
1	1	14.41	0.032	25.91	0.082	27.41	0.053
1	2	14.39	0.039	27.15	0.086	29.21	0.057
1	3	14.63	0.078	27.43	0.383	28.26	0.130
0	1	14.86	0.030	00.00	N/A	00.00	N/A
0	2	14.84	0.076	00.00	N/A	00.00	N/A
0	3	14.86	0.028	00.00	N/A	00.00	N/A

When the assay was run with phytoplasma DNA from aster yellows (16SrI-B), ash yellows (16SrVII-A), western X (16SrIII-A), elm yellows (16SrV-A), palatinate grapevine yellows (16SrV), flavescence dorée (16SrV), Rubus stunt (16SrV-E), and apple proliferation (16SrX-A), all were positive for phytoplasmas in general. A

positive signal for the 16SrV specific primer-probe combination was only obtained for elm yellows, palatinate grapevine yellows, flavescence dorée, and Rubus stunt DNA (Table 6).

Table 6: Results from the developed multiplex TaqMan qPCR assay when run with strains from a variety of different phytoplasma groups. Mean Cq-values of three technical replicates are shown. Cq-values below 38 are regarded as positive values. (N/A) not applicable because no fluorescent signal above the background fluorescence could be detected.

Phytoplasma strain	Plant host DNA	16SrV Phytoplasmas	Phytoplasma universal
aster yellows (16SrI-B)	13.59	N/A	18.32
western X (16SrIII-A)	33.29	N/A	34.52
elm yellows (16SrV-A)	13.96	18.59	17.69
palatinate grapevine yellows strain EY17-49 (16SrV)	13.01	19.39	16.59
flavescence dorée strain FD70 (16SrV)	14.27	21.95	18.31
Rubus stunt (16SrV-E)	22.27	28.47	27.32
ash yellows (16SrVII-A)	13.68	N/A	15.85
apple proliferation (16SrX-A)	13.73	N/A	19.22

4.4.3 Validation of the Multiplex TaqMan Assay for Phytoplasmas Present in Insects

When the multiplex TaqMan assay was applied to insect DNA artificially mixed with phytoplasma DNA, all respective targets (elm yellow phytoplasma DNA, phytoplasma DNA in general, and insect host DNA) could be detected up to a serial DNA dilution of 10^{-4} (Figure 19). C_q-values did not differ from each other when comparing multiplex with singleplex assays. All no-template controls were negative, and the negative control was positive only for the internal control (data not shown).

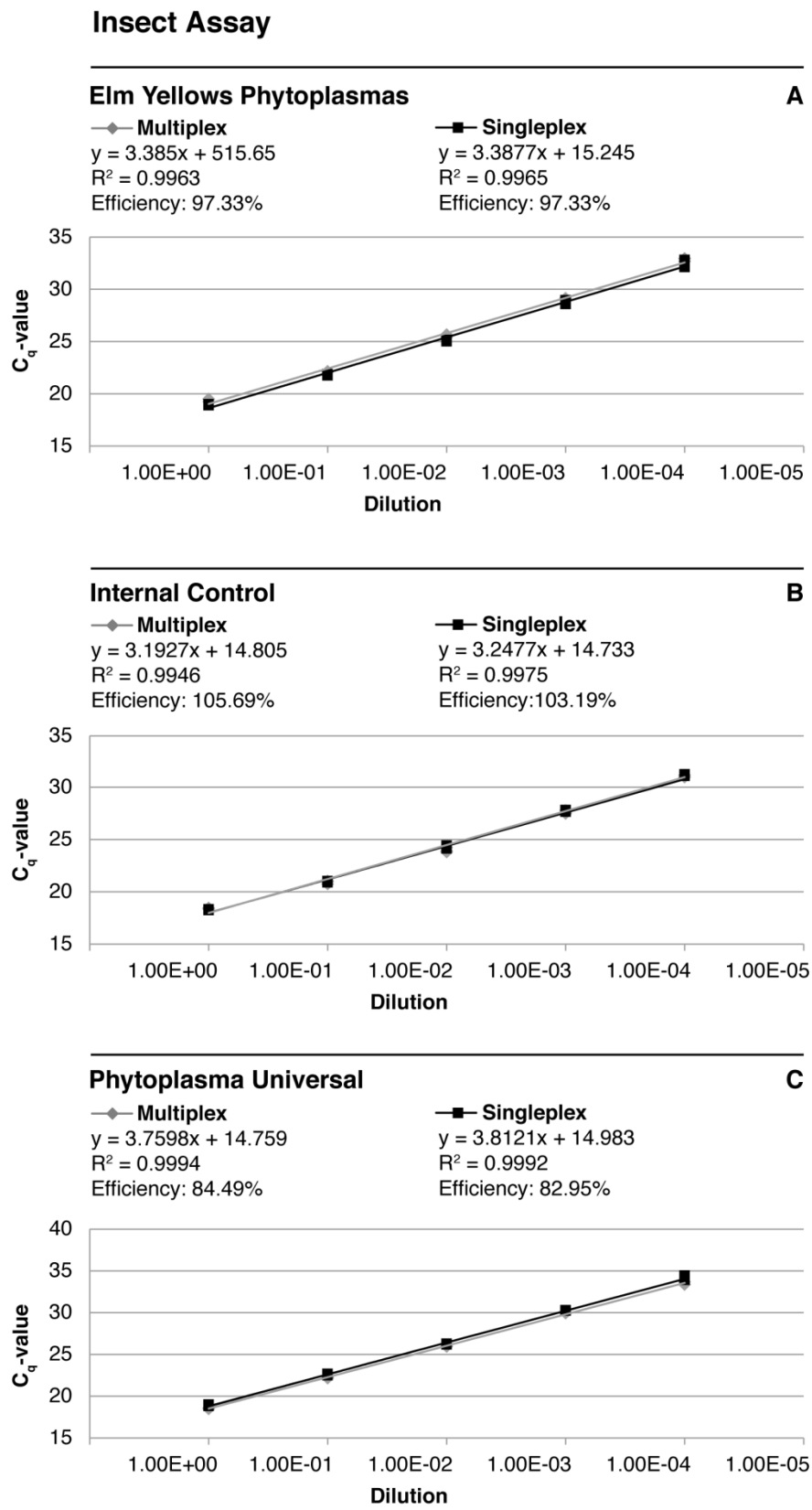

Figure 19: Standard curves obtained in the multiplex validation assay with insect samples. C_q-values of the singleplex and multiplex TaqMan qPCR are plotted against a 10-fold serial dilution of a DNA mixture from an uninfected leafhopper sample and *Catharanthus roseus* infected with '*Ca*. Phytoplasma rubi'. Primers and probes for detection of (A) elm yellows phytoplasmas, (B) insect host DNA as an internal control, and (C) phytoplasmas in general were used. Slopes, R^2 and efficiencies of the respective reactions are presented for each curve.

Nested PCR was able to detect phytoplasma DNA in insect DNA samples up to a dilution of 10^{-4}, the same dilution as the multiplex TaqMan qPCR assay (Figure 20).

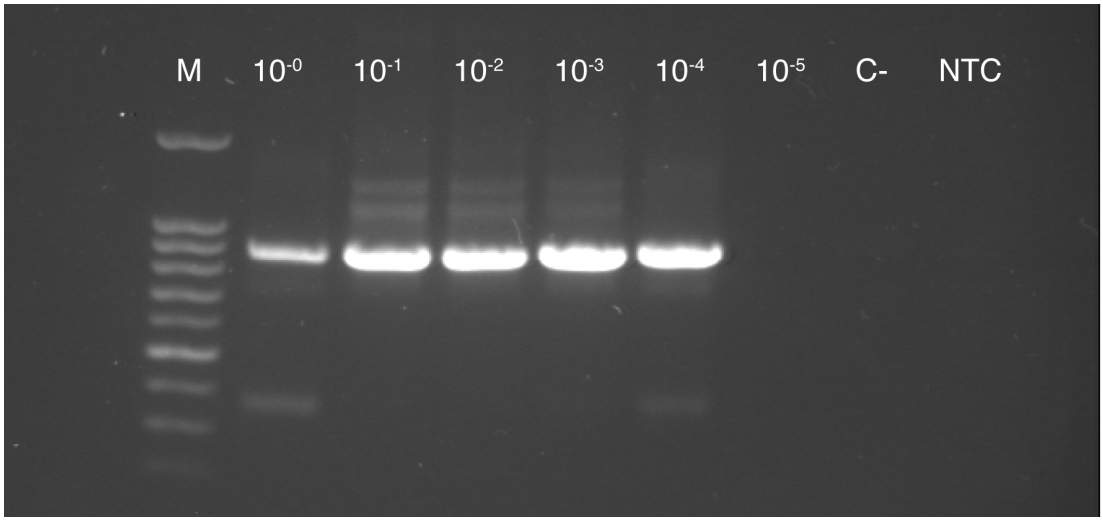

Figure 20: Phytoplasma specific nested PCR products of a 10-fold serial dilution (10^{0} to 10^{-5}) of a DNA mixture containing DNA of an uninfected leafhopper and *Catharanthus roseus* infected with '*Ca.* Phytoplasma rubi' using primer pairs P1/P7 and U5/U3. For nested PCRs the same DNA sample as in the validation assay for multiplex qPCR for insect samples (Figure 18) were used. (M) Metabion mi-100 bp+ DNA Marker Go, (C-) negative control, (NTC) no template control.

4.4.4 Field Validation of the Multiplex TaqMan Assay

A total of 140 raspberry and blackberry plant DNA samples (leaf, roots, bast, peduncle and fruit) obtained from nine different commercial plantings throughout Germany were tested for the presence of phytoplasma DNA using both the newly developed multiplex TaqMan qPCR assay and the standard nested PCR assay. Some of the plants were showing distinctive symptoms pointing to a phytoplasma infection, however, for the majority of plants, these symptoms were not clearly visible (Table 2). Of these samples, 85 were positive for phytoplasma DNA with the multiplex TaqMan qPCR assay whereas only 82 samples were positive with nested PCR. The qPCR assay was positive in six cases where nested PCR was negative and, in turn, nested PCR was positive in three cases where the qPCR was negative (Table 2). Again, nested PCRs had to be repeated several times and had to be set up under sterile conditions to obtain amplification-free no-template controls or negative controls.

4.5 Discussion

Phytoplasmas are known as serious pathogens of a variety of commercial crop plants, with their diagnosis often being challenging due to unspecific symptoms, a long period of latency and low concentrations in sample tissues because they are limited to the phloem. Therefore, a fast, sensitive, and reliable diagnostic method is of prime importance to minimize their spread by insect vectors and by vegetative propagation. Nested PCR has been the most commonly used tool for the detection of phytoplasmas since the early 1990s (Christensen et al. 2013; Monti et al. 2013). However, nested PCR has a high risk for cross-contaminations and needs time consuming post-amplification steps. We know of 28 publications to date in which qPCR assays for detection of phytoplasmas were developed (Table 7). Of these 28 papers, 8 employed DNA dyes (SYBR Green or EvaGreen), whereas 21 used TaqMan probes (one paper used both, SYBR Green and TaqMan chemistry, in separate assays). Despite of this high number of published TaqMan assays for phytoplasma detection only seven papers employed a multiplex approach. These seven assays combine the following specificities: apple proliferation and *Malus domestica* (Baric and Dalla-Via 2004); aster yellows (16SrI) or other group (Hodgetts et al. 2009); flavescence dorée (16SrV), bois noir (16SrXII-A), and grapevine (Pelletier et al. 2009); stolbur (16SrXII-A), '*Candidatus* Phlomobacter fragariae', and plant DNA (Danet et al. 2010); phytoplasmas in general and plant host DNA (Oberhänsli et al. 2011); phytoplasmas in general, pear blister canker viroid, and apple scar skin viroid (Malandraki et al. 2015); European stone fruit yellows (16SrX-B) and plant host DNA (Minguzzi et al. 2016).

Here, we present a multiplex phytoplasma TaqMan qPCR assay that allows for the first time a fast and simultaneous detection of phytoplasmas in general, a group specific detection of elm yellows phytoplasmas (16SrV), and the detection of either host plant DNA or insect vector DNA in one single reaction.

Table 7: Publications in which qPCR assays for the detection of phytoplasmas were developed. Hyphen bullets in the specificity column mark separate assays.

Specificity	Target gene	SYBR	TaqMan	Multiplex	Reference
apple proliferation (16SrX) and *Malus domestica*	16S rDNA		✓	✓	(Baric and Dalla-Via 2004)
flavescence dorée (16SrV-C (alder yellows) and 16SrV-D)	16S rDNA		✓		(Bianco et al. 2004)
-phytoplasma Universal -plant 18S rDNA	-16S rDNA -18S rDNA		✓		(Christensen et al. 2004)
apple proliferation	genomic fragment	✓			(Jarausch et al. 2004)
onion yellows phytoplasma	tuf		✓		(Wei et al. 2004)
-flavescence dorée (16Sr-V) -bois noir (16Sr-XII) -apple proliferation (16Sr-X) -universal	-16S rDNA -genomic fragment -nitroreductase -16S rDNA	✓	✓		(Galetto et al. 2005)
-universal -chrysanthemum carinatum -Insect DNA	-16S rDNA -ITS1 -18S rDNA		✓		(Marzachí and Bosco 2005)
apple proliferation (16SrX)	16S rDNA	✓			(Torres et al. 2005)
beet leafhopper-transmitted virescence agent (16SrVI) and aster yellows (16SrI) and pigeon pea witches' broom (16SrIX)	16S rDNA		✓		(Crosslin et al. 2006)
'*Candidatus* Phytoplasma mali' (16SrX)	16S rDNA		✓		(Aldaghi et al. 2007)
-flavescence dorée (16Sr-V) -bois noir (Sr-XII) -aster yellows (Sr-I) -grapevine	-16S rDNA -16S rDNA -16S rDNA -Chaperonin		✓		(Angelini et al. 2007)
-phytoplasma universal -flavescence dorée -bois noir	-16S rDNA -secY -genomic fragment		✓		(Hren et al. 2007)
'*Candidatus* Phytoplasma prunorum' (European stone fruit yellows) (16SrX)	ribosomal protein rplV	✓			(Martini et al. 2007)
apple proliferation	16S rDNA		✓		(Bisognin et al. 2008)
aster yellows (16SrI)	16S rDNA	✓			(Hollingsworth et al. 2008)
-Phytoplasma universal or -16SrI or other group -16SrII -16SrXII -16SrIV	23S rRNA		✓	✓	(Hodgetts et al. 2009)

Table 7: (continued)

Specificity	Target gene	SYBR	TaqMan	Multiplex	Reference
flavescence dorée (16SrV) and bois noir (16SrXII-A) and grapevine	-map -chloroplastic DNA		✓	✓	(Pelletier et al. 2009)
-'*Ca*. P. prunorum' (European stone fruit yellows) -*Cacopsylla pruni* (insect-host)	-16S-ITS rDNA -18S rDNA	✓			(Yvon et al. 2009)
stolbur phytoplasma (16SrXII-A) and '*Ca*. Phlomobacter fragariae' and plant DNA	-map -spoT -cox		✓	✓	(Danet et al. 2010)
-elm yellows (16SrV) -*Ulmus*	-secY -trnL		✓		(Herath et al. 2010)
-'*Ca*. P. mali' -'*Ca*. P. prunorum' -'*Ca*. P. pyri'	IGS between 16S and 23S rRNA		✓		(Nikolić et al. 2010)
-aster yellows -*Macrosteles quadrilineatus*	-tuf and lysS -chromosomal DNA	✓			(Frost et al. 2011)
coconut lethal yellows (16SrIV)	16S rRNA		✓		(Myrie et al. 2011)
phytoplasma universal (from Hodgetts 2009) and plant-host	-18S rRNA		✓	✓	(Oberhänsli et al. 2011)
-'*Ca*. P. mali' (apple proliferation) -insect-host (from Marzachi & Bosco 2005)	-rplV	✓			(Monti et al. 2013)
-'*Ca*. P. phoenicium' (almond witches' broom; 16SrIX) -*Prunus dulcis*	-56 rRNA (16S-ITS-23S) -18S rRNA		✓		(Jawhari et al. 2015)
pear blister canker viroid and apple scar skin viroid and phytoplasmas universal (from Christensen 2004)			✓	✓	(Malandraki et al. 2015)
'*Ca*. P. prunorum' and Plant 18S rRNA	-16S rRNA		✓	✓	(Minguzzi et al. 2016)

TaqMan assays for detection of phytoplasmas were shown to be at least as sensitive as nested PCR (Smart et al. 1996; Angelini et al. 2007; Herath et al. 2010), but less susceptible to inhibitory substances in the reaction mixture (Oberhänsli et al. 2011). Accordingly, DNA extracts can be used less diluted for TaqMan assays, usually resulting in higher detection sensitivities compared to nested PCR assays. However, this well-known advantage of TaqMan assays could not be proven in this study as both the multiplex TaqMan qPCR assay and nested PCR were able to detect the

highest concentration levels of the tested serial dilutions of target DNAs. When comparing the multiplex TaqMan qPCR assay for plant material with results from nested PCR, the nested PCR was able to show a clear PCR product for one dilution factor higher than qPCR. However, labor-intensive nested PCR is not suitable for routine analysis in plant nurseries where usually high numbers of samples need to be analyzed as quickly and accurate as possible. In addition, we needed to set up nested PCRs under an UV sterilization cabinet in order to achieve reproducible results with clean negative controls and/or no-template controls. Unspecific PCR products and false positive or negative results are a general problem of nested PCR due to its high sensitivity combined with high risks for contaminations (Delić 2012; Minguzzi et al. 2016). Moreover, we assume that the discrepancies in results obtained via qPCR and nested PCR with the same raspberry DNA sample are due to a limit of detection and/or the presence of inhibitory substances in the DNA extract.

The possibility of multiplexing by equipping TaqMan probes with different fluorogenic dyes allows detecting multiple targets in a single reaction. In the assays presented here, multiplexing was used not only to detect specifically elm yellows phytoplasmas and phytoplasmas in general at the same time, but also to include internal controls detecting either insect or plant host DNA. This enables the confirmation of a successful DNA extraction, and excludes false negative results resulting from a potential inhibition of the PCR. This, together with the lower risk of contamination due to detection of amplification products in a closed-tube system, makes the assays much more reliable than nested PCR. Furthermore, multiplex TaqMan assays are more time-saving than nested PCR because there is no need for post-PCR processing, and only one round of PCR has to be carried out. Using the KAPA PROBE FAST Master Mix as described here, once PCR reactions are fully set up on a 96-well plate, about 1 hour is needed until results can be obtained. In comparison, with nested PCR approximately about 6 – 8 hours are needed to get results from the same number of samples. In addition, the results obtained here using different proportions of phytoplasma infected and healthy leaf material showed that it can be a valid option for plant nurseries to pool multiple leaf samples of different plants while still being able to reliably detect phytoplasmas and thus saving even more time and resources. Accordingly, the assays developed in this study provide a rapid and practical tool for screening of *Rubus* mother plants for the presence of both

elm yellows phytoplasmas (to which Rubus stunt '*Ca.* Phytoplasma rubi' belongs) and phytoplasmas in general in nurseries or during plant propagation.

In addition, the multiplex qPCR TaqMan assay developed in the present study will now also allow a quick and reliable identification of phytoplasma insect vectors in orchards. Since measurements targeting insect vectors of phytoplasmas are the only options for managing phytoplasmas and particularly for preventing their spread within and among orchards, a detailed knowledge of respective vectors is a prerequisite for improvement of plant protection strategies against phytoplasma diseases.

5 Screening of Putative Insect Vectors of Rubus Stunt Phytoplasmas

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Author Contributions

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Data curation: HL.

Formal analysis: HL.

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Investigation: HL.

Methodology: HL AR.

Project administration: AR.

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Supervision: HL AR.

Validation: HL.

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Writing – original draft: HL.

Writing – review & editing: HL AR.

5.1 Introduction

Phytoplasmas are cell wall-less bacteria which colonize the phloem of their host plants as obligate parasites and are transferred between plants by phloem-sucking insect vectors of the order Hemiptera, mainly by leafhoppers (Cicadellidae), planthoppers (Fulgoroidea), and psyllids (Psylloidea) (Weintraub and Beanland 2006). Phytoplasma diseases affect more than 700 plant species worldwide, including many economically important crops, ornamentals, and forest trees (Hoshi et al. 2007; Bertaccini et al. 2014). As most phytoplasma insect vectors known to date are rather thermophilic species, climate change will facilitate their worldwide spread and phytoplasma diseases are expected to increase in the future (Maejima et al. 2014).

In *Rubus* species like raspberry (*Rubus ideaeus* L.) and blackberry (*Rubus* subgenus *Rubus* (Stace 2010)) phytoplasmas cause a disease referred to as Rubus stunt. Symptoms of Rubus stunt include stunting, witches' broom, small leaves, short internodes, enlarged sepals, phyllody, flower proliferation, and fruit malformations (van der Meer 1987; Mäurer and Seemüller 1994). Usually, Rubus stunt is associated with '*Candidatus* Phytoplasma rubi', which is a phytoplasma belonging to the elm yellows group of phytoplasmas (16SrV). However, phytoplasmas from other groups have also been reported from symptomatic *Rubus* plants, like aster yellows phytoplasmas (16SrI-B) (Borroto Fernández et al. 2007; Fahmeed et al. 2009; Reeder et al. 2010), X disease phytoplasmas (16SrIII) (Davies 2000; Davis et al. 2001), and stolbur phytoplasmas (16SrXII-A) (Borroto Fernández et al. 2007). More recently, a new phytoplasma was identified in blackberry plants showing witches' broom symptoms in Portugal that was named blackPort phytoplasma (Fránová et al. 2016).

The main control method to avoid the spread of phytoplasma diseases relies in the use of insecticides in order to eliminate insect vectors (Firrao et al. 2007). The efficiencies of this chemical vector control, however, are unsatisfying because it is impossible to eliminate all potential insect vectors from an environment by spraying insecticides (Bertaccini 2007) and applying insecticides has adverse environmental effects. Moreover, and in the case of Rubus stunt, only little is known about the spectrum of Rubus stunt vector insects. The only insect vector which is usually associated with the transmission of Rubus stunt phytoplasmas is the raspberry leafhopper, or sometimes called brambleberry leafhopper, *Macropsis fuscula*

(ZETTERSTEDT) (Hemiptera: Cicadellidae) (Marcone et al. 1997; Davies 2000; Jarausch et al. 2001; Arnaud et al. 2007; Malembic-Maher et al. 2011). Van der Meer and de Fluiter (1970) reported the successful transmission of Rubus stunt from raspberry to strawberry by *M. fuscula*. In some literature, *Macropsis brabantica* WAGNER and *Macropsis scotti* EDWARDS are also named as insect vectors of Rubus stunt (Weintraub and Beanland 2006; Bosshard et al. [date unknown]), however, this has never been proven in transmission experiments. Van der Meer (1987) stated that it is probable that these species are insect vectors of Rubus stunt as they were found on European dewberry (*Rubus caesius* L.) and blackberry (*Rubus* subgenus *Rubus*) during severe outbreaks of this disease. Nonetheless, other species outside the genus *Macropsis* have been shown to be able to transmit the Rubus stunt agent like *Philaenus spumarius* (LINNAEUS) (Hemiptera: Aphrophoridae), *Allygus mayri* (KIRSCHBAUM) (Hemiptera: Cicadellidae), and *Euscelis plebeja* (FALLÉN) (= *incisus* KIRSCHBAUM) (Hemiptera: Cicadellidae) (Lehmann 1973; Jenser et al. 1981), but they are unlikely to play an important role in the natural spread of Rubus stunt as they do not specifically live on *Rubus* spp. (Converse 1991).

In this study, an extensive screening of putative insect vectors of Rubus stunt in raspberry and blackberry plantations spanning an area from southern to northern Germany was carried out during two successive years (2014 and 2015) with multiple sampling dates throughout the growing seasons in order to investigate the spectrum and abundance of Rubus stunt vector insects. This data will form the basis for choosing and timing appropriate control measures against Rubus stunt and also for potential insect vector transmission experiments.

5.2 Materials and Methods

5.2.1 Sampling of Insects

Insects present in the canopy of raspberry and blackberry plants were sampled using a G-Vac suction sampler (modified Stihl SH 56, Waiblingen, Germany) (Figure 21) in commercial plantings as well as in wild blackberry plants. The G-Vac suction sampler is a vacuum shredder equipped with a net collection bag inserted into the inlet tube and secured around the nozzle.

Figure 21: G-Vac suction sampler (modified Stihl SH 56).

Insects were sampled at different times throughout the growing season in 2014 and 2015 in locations spreading from southern to northern Germany (Figure 22). Sampling dates and geographic coordinates are shown in Table 8. After G-Vac sampling in the canopy of plants for several minutes, the net collection bag was removed from the G-Vac with the engine still running in order to prevent insects from escaping and then sealed by hand until the whole net collection bag was immersed in 70% ethanol to kill and preserve the insects (Figure 23). Hemipteran insects were sorted, identified according to family, genus, or species if possible, and stored at room temperature in 70% ethanol.

Figure 22: Geographical distribution of sampling locations (green) across Germany.

Table 8: Locations and dates for the sampling of putative insect vectors of Rubus stunt.

Sampling Location in Germany	Geographic Coordinates	Sampling Date [dd.mm.yyyy]
Oberkirch	48°31'40"N 8°01'33"E	15.07.2014
		11.09.2014
Heuchlingen	49°15'12"N 9°13'40"E	22.05.2014
		14.07.2014
		15.09.2014
		06.07.2015
		08.10.2015
Geisenheim	49°58'52"N 7°56'55"E	15.05.2014
		02.07.2014
		29.09.2014
		06.07.2015
Telgte	51°59'27"N 7°49'23"E	13.05.2014
		30.07.2014
		16.09.2014
		30.06.2015
		06.10.2015
Ostbevern	52°04'47"N 7°48'53"E	13.05.2014
		30.07.2014
		16.09.2014
Ladbergen	52°06'07"N 7°48'20"E	30.06.2015
		06.10.2015
Wedel	53°35'25"N 9°40'10"E	14.05.2014
		29.07.2014
		17.09.2014
		01.07.2015
		07.10.2015

Figure 23: Sampling of putative insect vectors of Rubus stunt. (A) Sampling of insects in the canopy of raspberry plants using a G-Vac suction sampler. (B) Removal of the net collection bag from the G-Vac while the engine is still running. (C) Insects caught in the net collection bag. (D) Immersion of net collection bag in 70% ethanol to kill the insects.

5.2.2 Insect DNA Extraction and Phytoplasma DNA Detection

DNA was extracted from a subset of collected insects after their taxonomic determination, representing all hemipteran insects sorted and grouped by sampling location, sampling date, and species. Depending on the quantity of individual insects one to 27 DNA extracts were produced per group. DNA extraction was conducted according to a protocol modified from that of Marzachi et al. (1998). In order to represent the best possible coverage of potential vectors, depending on size, one to ten insects of the same species, sampling date, and sampling location were pooled together and DNA was extracted from this pool. Insects were ground using a micro pestle in a mixture of 500 µl CTAB buffer (2% CTAB, 0.1 M Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl) and 1 µl 2-mercaptoethanol. After vortexing, the suspension was incubated at 60 °C for 30 min and centrifuged for 10 min at 13,000 rpm. The supernatant was transferred to a new tube, extracted with chloroform:isoamyl alcohol (24:1), and DNA was precipitated by adding cold isopropanol. After centrifugation the pellet was washed with 70% ethanol, and dissolved in 20 µl of deionized sterile water. DNA samples were tested with the multiplex TaqMan qPCR assay for insect samples developed in chapter 4.

5.3 Results

In the course of monitoring putative Rubus stunt insect vectors in different commercial raspberry and blackberry fields throughout Germany 1293 individual hemipteran insects were caught in 2014 (Table 9) and 3575 in 2015 (Table 10), respectively. After sorting and identification, a total of 597 DNA samples (379 from the 2014 samples and 218 from the 2015 samples) of putative Rubus stunt insect vectors, representing the full range of sampled locations, dates, and hemipteran insects, were extracted and analysed for presence of phytoplasma DNA with the multiplex TaqMan qPCR assay developed in chapter 4.

From the 2014 samples, nine DNA samples were tested positive for the presence of phytoplasmas in general, but negative for elm yellows phytoplasmas. Three of these phytoplasma positive samples were from leafhoppers of the genus *Euscelidius* and six from leafhopper species identified as *Macrosteles* spp.. From the 2015 samples, also nine DNA samples were tested positive for phytoplasma DNA, but in consistence with the results from 2014, all nine were negative for elm yellows phytoplasmas as well. Of these nine phytoplasma positive samples, four where from *Macrosteles* spp., two from *Euscelis* spp., two from *Anaceratagallia* cf. *ribauti*, and one from *Psammotettix* spp. (Table 9 and Table 10).

5.4 Discussion

In the screening of putative insect vectors of Rubus stunt in southern and northern Germany phytoplasma positive insects from five genera were found, namely *Euscelidius*, *Macrosteles*, *Euscelis*, *Anaceratagallia*, and *Psammotettix*. Leafhoppers of all of these five genera are known to transmit phytoplasmas, however, it has only been reported for *Euscelis incisus* (Kirschbaum) (= *plebeja* Fallén) to transmit a yellows agent from a *Rubus* sp. to white clover (*Trifolium repens* L.) and to tricolor daisy (*Chrysanthemum carinatum* SCHOUSB.) (Lehmann 1973). *Euscelis* spp. are known to be insect vectors for stolbur (16SrXII-A) (Brčák 1979), clover witches' broom (16SrVI) (Posnette and Ellenberger 1963), chrysanthemum yellows (16SrI-B) (Alma et al. 2001), clover phyllody (16SrI-C) (Savio and Conti 1983), and green petal disease (16SrI-C) (Frazier and Posnette 1956). From the genus *Euscelidius*, *Euscelidius variegatus* (KIRSCHBAUM) is known to transmit a variety of phytoplasmas such as aster yellows (Severin 1947), western X-disease (16SrIII-A)

(Jensen 1969), or flavescense dorée (16SrV-C) (Boudon-Padieu et al. 1989). With at least nine different known species reported to transmit numerous phytoplasma diseases, the genus *Macrosteles* also comprises a large number of insect vectors (Weintraub and Beanland 2006). *Psammotettix alienus* (DAHLBOM) is a known vector of '*Ca*. Phytoplasma asteris' (16SrI-B and 16SrI-C) (Mitrović et al. 2012; Landi et al. 2013), but has also been found positive for elm yellows (16SV-A) and stolbur (16SrXII-A) phytoplasmas (Prota et al. 2006). Furthermore, *Psammotetix striatus* LINNAEUS was reported to transmit an aster yellows phytoplasma (16SrI-C) causing wheat blue dwarf disease (Wu et al. 2010). *Anaceratagallia ribauti* OSSIANNILSSON has been shown to transmit stolbur (16SrXII-A) phytoplasmas (Riedle-Bauer et al. 2008).

It has to be noted, that the screening of potential phytoplasma vectors, including phytoplasma DNA detection in field-collected insects, provides indications on a possible role of the respective species in transmitting a certain phytoplasma disease. However, as phytoplasmas can be acquired by insect feeding without being transmitted to a new plant species, transmission assays using living insects are necessary to provide final proof for an insect species to be an efficient vector of phytoplasmas (Bosco and Tedeschi 2013).

Out of a total of 4868 caught and identified hemipteran insects from the canopies of raspberry and blackberry plants across Germany, only seven individuals were identified as *M. fuscula* (two in 2014 and five in 2015; all seven individuals sampled in July), the only insect vector of Rubus stunt so far described in the literature. All seven *M. fuscula* individuals were tested negative for phytoplasma DNA with the multiplex TaqMan qPCR. This rare occurrence of *M. fuscula* is consistent with the findings of Vindimian et al. (2004), who did not capture any *Macropsis* spp. in blackberry orchards in the Trento province in Italy using chromotropic traps, even though a disease incidence of 43% of Rubus stunt infected plants was recorded in the same plantations. Vindimian et al. (2004) also caught *Macrosteles* spp., but all tested insects were negative for phytoplasma DNA in nested PCRs.

The low occurrence of *M. fuscula* and other potential insect vectors of Rubus stunt suggests that its spread by insect vectors only plays a minor role in commercial raspberry and blackberry production compared to its spread by vegetative propagation during plant production. Nonetheless, insect vectors play an important

role for wild reservoirs of Rubus stunt phytoplasmas in plants like wild raspberry and blackberry, as well as mallow (*Malva sylvestris* L.) and dog rose (*Rosa canina* L.) (Jarausch et al. 2001; Vindimian et al. 2004; Borroto Fernández et al. 2007; Cieslinska 2011). In the future, surveys on plants present in the surroundings of commercial raspberry and blackberry fields might be useful to better understand the epidemiology of Rubus stunt. Control measures of phytoplasma diseases like Rubus stunt therefore have to focus not just on the chemical control of potential insect vectors in *Rubus* plantation, but also on the application of phytosanitary measures during plant production and in plantation adjoining environments, like routine diagnostics in plant nurseries and selective removal of non-crop phytoplasma or insect vector host plants.

Table 9: Sampled putative insect vectors in 2014 and their qPCR results for the presence of phytoplasma DNA. Phytoplasma positive species are shaded in grey.

Species	No. of Caught Individuals	Sampling Date [DD.MM.YYYY]	Location	No. of DNA Samples	No. of qPCR Positive Samples
Aphidoidea	12	14.05.2014	Wedel	3	0
Aphidoidea	12	29.07.2014	Wedel	2	0
Aphidoidea	35	17.09.2014	Wedel	5	0
Arthaldeus pascuellus	1	17.09.2014	Wedel	1	0
Arthaldeus spp.	2	17.09.2014	Wedel	1	0
Cicadula cf. *persimilis*	1	17.09.2014	Wedel	1	0
Eupteryx spp.	4	17.09.2014	Wedel	1	0
Euscelidius spp.	6	29.07.2014	Wedel	6	0
Euscelidius spp.	1	17.09.2014	Wedel	1	0
Euscelis incisus	3	17.09.2014	Wedel	3	0
Heteroptera	5	17.09.2014	Wedel	4	0
Javesella cf. *dubia*	2	14.05.2014	Wedel	1	0
Javesella cf. *dubia*	3	29.07.2014	Wedel	1	0
Javesella cf. *dubia*	5	17.09.2014	Wedel	3	0
Laodelphax spp.	4	14.05.2014	Wedel	2	0
Laodelphax spp.	4	17.09.2014	Wedel	1	0
Lygaeidae	1	14.05.2014	Wedel	1	0
Macrosteles spp.	1	29.07.2014	Wedel	1	0
Macrosteles spp.	53	17.09.2014	Wedel	27	6
Mirinae	2	17.09.2014	Wedel	1	0
Nabidae	10	17.09.2014	Wedel	3	0
Nabis cf. *ferus*	4	17.09.2014	Wedel	4	0
Scolopostethus affinis	6	17.09.2014	Wedel	2	0
Stenocranus cf. *major*	3	17.09.2014	Wedel	3	0
Typhlocybinae	98	17.09.2014	Wedel	14	0
Zygina spp.	2	17.09.2014	Wedel	2	0

Table 9: (continued)

Species	No. of Caught Individuals	Sampling Date [DD.MM.YYYY]	Location	No. of DNA Samples	No. of qPCR Positive Samples
Aphidoidea	1	30.07.2014	Ostbevern	1	0
Aphidoidea	10	13.05.2014	Ostbevern	2	0
Aphidoidea	10	16.09.2014	Ostbevern	2	0
Heteroptera	10	13.05.2014	Ostbevern	3	0
Heteroptera	1	16.09.2014	Ostbevern	1	0
Lygus spp.	1	30.07.2014	Ostbevern	1	0
Macrosteles spp.	3	16.09.2014	Ostbevern	2	0
Nabis cf. *ferus*	1	13.05.2014	Ostbevern	1	0
Tingidae	1	13.05.2014	Ostbevern	1	0
Typhlocybinae	4	13.05.2014	Ostbevern	2	0
Typhlocybinae	5	16.09.2014	Ostbevern	3	0
Zygina spp.	1	13.05.2014	Ostbevern	1	0
Aphidoidea	1	30.07.2014	Telgte	1	0
Aphidoidea	1	13.05.2014	Telgte	1	0
Aphidoidea	17	16.09.2014	Telgte	4	0
Heteroptera	10	13.05.2014	Telgte	6	0
Javesella cf. *dubia*	4	13.05.2014	Telgte	2	0
Laodelphax spp.	1	30.07.2014	Telgte	1	0
Lygus spp.	1	16.09.2014	Telgte	1	0
Macrosteles spp.	3	30.07.2014	Telgte	1	0
Macrosteles spp.	2	16.09.2014	Telgte	1	0
Nabis cf. *ferus*	1	13.05.2014	Telgte	1	0
Nabis cf. *mirmicoides*	1	16.09.2014	Telgte	1	0
Nabis spp.	1	30.07.2014	Telgte	1	0
Typhlocybinae	3	13.05.2014	Telgte	1	0
Typhlocybinae	3	30.07.2014	Telgte	2	0
Typhlocybinae	24	16.09.2014	Telgte	8	0
Zygina cf. *rosincola*	1	30.07.2014	Telgte	1	0
Anaceratagallia cf. *ribauti*	1	02.07.2014	Geisenheim	1	0
Anaceratagallia cf. *ribauti*	2	29.09.2014	Geisenheim	1	0
Anaceratagallia ribauti	1	02.07.2014	Geisenheim	1	0
Anaceratagallia ribauti	1	29.09.2014	Geisenheim	1	0
Aphidoidea	1	15.05.2014	Geisenheim	1	0
Aphidoidea	30	02.07.2014	Geisenheim	9	0
Aphidoidea	45	29.09.2014	Geisenheim	3	0
Eupteryx cf. *vittata*	1	02.07.2014	Geisenheim	1	0
Euscelidius spp.	2	02.07.2014	Geisenheim	2	1
Fruticidia spp.	1	29.09.2014	Geisenheim	1	0
Heteroptera	1	02.07.2014	Geisenheim	1	0
Javesella cf. *dubia*	2	02.07.2014	Geisenheim	1	0
Laodelphax spp.	2	02.07.2014	Geisenheim	2	0
Lygus spp.	3	29.09.2014	Geisenheim	3	0

Table 9: (continued)

Species	No. of Caught Individuals	Sampling Date [DD.MM.YYYY]	Location	No. of DNA Samples	No. of qPCR Positive Samples
Macropsis cf. *fuscula*	1	02.07.2014	Geisenheim	1	0
Megadelphax spp.	1	15.05.2014	Geisenheim	1	0
Nabis cf. *ferus*	2	29.09.2014	Geisenheim	2	0
Nabis cf. *ferus*	2	02.07.2014	Geisenheim	2	0
Neoaliturus fenestratus	1	29.09.2014	Geisenheim	1	0
Psammotettix cf. *confinis*	2	29.09.2014	Geisenheim	1	0
Psammotettix cf. *confinis*	1	15.05.2014	Geisenheim	1	0
Stenocranus cf. *major*	1	29.09.2014	Geisenheim	1	0
Typhlocybinae	6	15.05.2014	Geisenheim	3	0
Typhlocybinae	53	02.07.2014	Geisenheim	9	0
Typhlocybinae	149	29.09.2014	Geisenheim	18	0
Zyginidia	2	02.07.2014	Geisenheim	1	0
Zyginidia	1	29.09.2014	Geisenheim	1	0
Agallia cf. *consobrina*	3	14.07.2014	Heuchlingen	2	0
Alydidae	1	14.07.2014	Heuchlingen	1	0
Anaceratagallia cf. *ribauti*	1	15.09.2014	Heuchlingen	1	0
Aphrophora spp.	1	14.07.2014	Heuchlingen	1	0
Delphacidae	5	14.07.2014	Heuchlingen	2	0
Deltocephalus pulicaris	1	14.07.2014	Heuchlingen	1	0
Dolycoris baccarum	1	22.05.2014	Heuchlingen	1	0
Dolycoris baccarum	2	14.07.2014	Heuchlingen	2	0
Errastunus ocellaris	1	15.09.2014	Heuchlingen	1	0
Eupteryx cf. *melissae*	1	14.07.2014	Heuchlingen	1	0
Eupteryx cf. *melissae*	1	15.09.2014	Heuchlingen	1	0
Euscelidius spp.	4	14.07.2014	Heuchlingen	4	0
Euscelidius spp.	14	15.09.2014	Heuchlingen	14	2
Euscelis incisus	5	14.07.2014	Heuchlingen	3	0
Fieberiella spp.	1	14.07.2014	Heuchlingen	1	0
Heteroptera	1	22.05.2014	Heuchlingen	1	0
Heteroptera	7	14.07.2014	Heuchlingen	3	0
Heteroptera	4	15.09.2014	Heuchlingen	4	0
Heterotoma planicornis	1	14.07.2014	Heuchlingen	1	0
Javesella cf. *dubia*	7	14.07.2014	Heuchlingen	4	0
Laodelphax spp.	11	14.07.2014	Heuchlingen	6	0
Laodelphax spp.	1	15.09.2014	Heuchlingen	1	0
Lygaeidae	1	14.07.2014	Heuchlingen	1	0
Lygus spp.	1	14.07.2014	Heuchlingen	1	0
Lygus spp.	3	15.09.2014	Heuchlingen	2	0
Macropsis fuscula	1	14.07.2014	Heuchlingen	1	0
Macrosteles spp.	3	14.07.2014	Heuchlingen	3	0
Nabidae	1	14.07.2014	Heuchlingen	1	0
Nabis cf. *ferus*	7	15.09.2014	Heuchlingen	6	0

Table 9: (continued)

Species	No. of Caught Individuals	Sampling Date [DD.MM.YYYY]	Location	No. of DNA Samples	No. of qPCR Positive Samples
Nabis cf. *mirmicoides*	3	15.09.2014	Heuchlingen	3	0
Neoaliturus fenestratus	1	15.09.2014	Heuchlingen	1	0
Orientus ishidae	1	14.07.2014	Heuchlingen	1	0
Palomina prasina	1	22.05.2014	Heuchlingen	1	0
Palomina prasina	1	14.07.2014	Heuchlingen	1	0
Psammotettix cf. *confinis*	1	14.07.2014	Heuchlingen	1	0
Pyrrhocoridae	1	14.07.2014	Heuchlingen	2	0
Pyrrhocoridae	1	15.09.2014	Heuchlingen	1	0
Ribautiana cf. *tenerinna*	1	14.07.2014	Heuchlingen	1	0
Ribautiana cf. *tenerinna*	2	15.09.2014	Heuchlingen	1	0
Scolopostethus affinis	3	15.09.2014	Heuchlingen	2	0
Typhlocybinae	1	22.05.2014	Heuchlingen	1	0
Typhlocybinae	181	14.07.2014	Heuchlingen	16	0
Typhlocybinae	152	15.09.2014	Heuchlingen	19	0
Zygina spp.	4	15.09.2014	Heuchlingen	2	0
Zyginidia spp.	4	14.07.2014	Heuchlingen	2	0
Coreus marginatus	1	11.09.2014	Oberkirch	1	0
Fieberiella spp.	5	15.07.2014	Oberkirch	4	0
Fieberiella spp.	5	11.09.2014	Oberkirch	4	0
Heteroptera	20	15.07.2014	Oberkirch	4	0
Heteroptera	1	11.09.2014	Oberkirch	1	0
Liocoris spp.	11	15.07.2014	Oberkirch	7	0
Liocoris spp.	2	11.09.2014	Oberkirch	2	0
Lygus spp.	2	15.07.2014	Oberkirch	1	0
Nabidae	1	15.07.2014	Oberkirch	1	0
Palomina prasina	2	11.09.2014	Oberkirch	2	0
Rhyparochromus cf. *vulgaris*	1	15.07.2014	Oberkirch	1	0
Scolopostethus affinis	4	11.09.2014	Oberkirch	3	0
Typhlocybinae	9	15.07.2014	Oberkirch	4	0
Typhlocybinae	1	11.09.2014	Oberkirch	1	0

Table 10: Sampled putative insect vectors in 2015 and their qPCR results for the presence of phytoplasma DNA. Phytoplasma positive species are shaded in grey.

Species	No. of Caught Individuals	Sampling Date [DD.MM.YYYY]	Location	No. of DNA Samples	No. of qPCR Positive Samples
Aphidoidea	150	01.07.2015	Wedel	3	0
Aphidoidea	19	07.10.2015	Wedel	2	0
Cicadula cf. *persimilis*	3	01.07.2015	Wedel	1	0
Cicadula cf. *persimilis*	2	07.10.2015	Wedel	1	0
Emelyanoviana spp.	19	01.07.2015	Wedel	1	0
Emelyanoviana spp.	4	07.10.2015	Wedel	1	0
Eupteryx cf. *urticae*	49	01.07.2015	Wedel	2	0
Eupteryx cf. *urticae*	17	07.10.2015	Wedel	3	0
Eupteryx vittata	5	01.07.2015	Wedel	1	0
Euscelidius spp.	2	07.10.2015	Wedel	2	0
Macrosteles spp.	19	01.07.2015	Wedel	3	2
Macrosteles spp.	8	07.10.2015	Wedel	1	1
Ribautiana debilis	16	01.07.2015	Wedel	3	0
Ribautiana debilis	6	07.10.2015	Wedel	1	0
Stenocranus cf. major	3	07.10.2015	Wedel	1	0
Typhlocybinae	67	01.07.2015	Wedel	5	0
Typhlocybinae	32	07.10.2015	Wedel	3	0
Zyginidia cf. scutelaris	11	01.07.2015	Wedel	2	0
Zyginidia cf. scutelaris	26	07.10.2015	Wedel	2	0
Aphidoidea	454	30.06.2015	Ladbergen	7	0
Aphidoidea	6	06.10.2015	Ladbergen	1	0
Calocoris spp.	2	30.06.2015	Ladbergen	1	0
Emelyanoviana spp.	9	30.06.2015	Ladbergen	2	0
Emelyanoviana spp.	46	06.10.2015	Ladbergen	1	0
Errastunus ocellaris	4	30.06.2015	Ladbergen	1	0
Eupteryx cf. cyclops	5	30.06.2015	Ladbergen	3	0
Eupteryx cf. urticae	11	30.06.2015	Ladbergen	1	0
Macrosteles spp.	15	30.06.2015	Ladbergen	3	0
Macrosteles spp.	1	06.10.2015	Ladbergen	1	0
Miridae	8	30.06.2015	Ladbergen	2	0
Notostira erratica	5	30.06.2015	Ladbergen	1	0
Orius minutus	6	30.06.2015	Ladbergen	3	0
Orius minutus	4	06.10.2015	Ladbergen	1	0
Ribautiana debilis	4	30.06.2015	Ladbergen	1	0
Ribautiana debilis	3	06.10.2015	Ladbergen	1	0
Stenodema spp.	4	30.06.2015	Ladbergen	1	0
Typhlocybinae	8	30.06.2015	Ladbergen	2	0
Typhlocybinae	35	06.10.2015	Ladbergen	1	0
Zyginidia cf. scutelaris	26	30.06.2015	Ladbergen	1	0
Zyginidia cf. scutelaris	3	06.10.2015	Ladbergen	1	0
Aphidoidea	626	30.06.2015	Telgte	6	0
Aphidoidea	12	06.10.2015	Telgte	3	0
Eupteryx cf.urticae	2	30.06.2015	Telgte	1	0

Table 10: (continued)

Species	No. of Caught Individuals	Sampling Date [DD.MM.YYYY]	Location	No. of DNA Samples	No. of qPCR Positive Samples
Eupteryx spp.	1	30.06.2015	Telgte	1	0
Euscelidius spp.	10	30.06.2015	Telgte	6	0
Euscelis spp.	5	30.06.2015	Telgte	3	0
Lygus spp.	15	30.06.2015	Telgte	5	0
Macrosteles cf. sexnotatus	1	06.10.2015	Telgte	1	0
Macrosteles spp.	21	30.06.2015	Telgte	4	0
Miridae	1	30.06.2015	Telgte	1	0
Miridae	1	06.10.2015	Telgte	1	0
Nabis spp.	2	30.06.2015	Telgte	1	0
Orius minutus	3	06.10.2015	Telgte	3	0
Ribautiana debilis	27	30.06.2015	Telgte	3	0
Ribautiana debilis	2	06.10.2015	Telgte	2	0
Typhlocybinae	58	30.06.2015	Telgte	8	0
Typhlocybinae	587	06.10.2015	Telgte	10	0
Zygina	2	06.10.2015	Telgte	1	0
Zyginidia cf. scutelaris	1	30.06.2015	Telgte	1	0
Zyginidia cf. scutelaris	1	06.10.2015	Telgte	1	0
Agallia cf. consobrina	2	06.07.2015	Geisenheim	2	0
Aphidoidea	1	06.07.2015	Geisenheim	1	0
Euscelis spp.	3	06.07.2015	Geisenheim	1	0
Javesella cf. obscurella	8	06.07.2015	Geisenheim	1	0
Javesella cf. pellucida	20	06.07.2015	Geisenheim	1	0
Laodelphax spp.	7	06.07.2015	Geisenheim	1	0
Macropsis cf. Fuscula	5	06.07.2015	Geisenheim	2	0
Nabis spp.	4	06.07.2015	Geisenheim	1	0
Neophilaneus cf. lineatus	2	06.07.2015	Geisenheim	1	0
Typhlocybinae	162	06.07.2015	Geisenheim	7	0
Zyginidia cf. scutelaris	3	06.07.2015	Geisenheim	2	0
Agallia consobrina	1	06.07.2015	Heuchlingen	1	0
Anaceratagallia cf. ribauti	2	06.07.2015	Heuchlingen	1	1
Anaceratagallia cf. ribauti	5	08.10.2015	Heuchlingen	1	1
Aphidoidea	2	06.07.2015	Heuchlingen	1	0
Aphidoidea	40	08.10.2015	Heuchlingen	3	0
Deltocephalidae	12	08.10.2015	Heuchlingen	2	0
Deltocephalus pulicaris	1	06.07.2015	Heuchlingen	1	0
Euscelidius spp.	1	08.10.2015	Heuchlingen	1	0
Euscelis spp.	9	06.07.2015	Heuchlingen	4	2
Euscelis spp.	4	08.10.2015	Heuchlingen	1	0
Halticus cf. luteicollis	5	06.07.2015	Heuchlingen	1	0
Javesella cf. dubia	285	06.07.2015	Heuchlingen	2	0
Javesella cf. pellucida	38	06.07.2015	Heuchlingen	2	0
Lygus spp.	2	06.07.2015	Heuchlingen	1	0
Macrosteles spp.	17	06.07.2015	Heuchlingen	4	0
Macrosteles spp.	32	08.10.2015	Heuchlingen	5	1

Table 10: (continued)

Species	No. of Caught Individuals	Sampling Date [DD.MM.YYYY]	Location	No. of DNA Samples	No. of qPCR Positive Samples
Nabis spp.	5	08.10.2015	Heuchlingen	2	0
Neoaliturus fenestratus	4	08.10.2015	Heuchlingen	2	0
Psammotettix spp.	9	08.10.2015	Heuchlingen	2	1
Pyrrhocoris apterus	2	08.10.2015	Heuchlingen	1	0
Reptalus spp.	2	06.07.2015	Heuchlingen	1	0
Scolopostethus cf. affinis	3	08.10.2015	Heuchlingen	1	0
Trisonotylus caelestialium	3	08.10.2015	Heuchlingen	1	0
Typhlocybinae	133	06.07.2015	Heuchlingen	9	0
Typhlocybinae	241	08.10.2015	Heuchlingen	10	0
Zygina spp.	5	08.10.2015	Heuchlingen	2	0

6 Propagation of Healthy Planting Material

6.1 Susceptibility of Different Raspberry Cultivars to Rubus stunt after Artificial Graft Inoculation

6.1.1 Introduction

Rubus stunt is an economically important disease in *Rubus* species like raspberry (*Rubus ideaeus* L.) and blackberry (*Rubus* subgenus *Rubus* (Stace 2010)). It is caused by phytoplasmas which are non-cultivated wall-less bacteria belonging to the class Mollicutes and are responsible for hundreds of diseases in ornamentals, cultivated plants, and weeds worldwide (Eveillard et al. 2016). Phytoplasmas colonize the phloem of their host plants and are spread by phloem feeding insect vectors, through grafting, or by vegetative propagation (Weintraub and Beanland 2006). Disease symptoms of Rubus stunt include stunting, formation of numerous weak and erect root shoots (witches' broom), small leaves, short internodes, enlarged sepals, phyllody, flower proliferation, and fruit malformations (Mäurer and Seemüller 1994).

Control strategies for phytoplasma diseases only consist of preventive measures like the use of healthy planting material, removal of infected plants in order to reduce infection sources, and chemical control of insect vectors as there is no direct treatment of infected plants (Seemüller et al. 2018). Therefore, Rubus stunt resistant raspberry cultivars would be a promising approach for the control of this disease. However, breeding of phytoplasma resistant host plants remains challenging as the mechanisms of phytoplasma resistance are not yet completely understood (Bertaccini and Duduk 2009).

Taxa with varying susceptibilities to phytoplasma diseases have been reported for apple, pear, *Prunus* spp., date palms, and rice among others, however, a correlation between phytoplasma titers and severity of symptom expression has not been established (Firrao et al. 2007). Moreover, it has been shown that symptom expression can be related to differences in the virulence of different phytoplasma strains, for example for '*Candidatus* Phytoplasma prunorum', the causal agent of European stone fruit yellows (Kison and Seemüller 2001).

There are several reports of differences in susceptibility to Rubus stunt of different *Rubus* cultivars. Nyerges et al. (2001) report that some cultivars appear to be affected much more readily than others, suggesting that in some cultivars either there is some resistance to the causal agent or to the insect vector, but do not provide further details on the topic. Converse (1991) states that infected raspberry cultivar 'Malling Promise' rarely exhibits flower proliferation symptoms, while raspberry cultivar 'Malling Landmark' and blackberry cultivar 'Thornless Evergreen' are very sensitive. However, Converse (1991) does not further describe how these conclusions were drawn. Vindimian et al. (2004) found a high susceptibility of the blackberry cultivar 'Lochness' in epidemiological studies on Rubus stunt in blackberry orchards in northern Italy. There are no *Rubus* cultivars known in which phytoplasma infections remain fully latent, but infected *Rubus* plants usually remain symptomless until one year after infection (Converse 1991).

In order to assess the susceptibility of a plant to a phytoplasma infection, healthy plants need to be deliberately inoculated. Their natural transmission by insect vector is not suitable in this case as the only known vector of Rubus stunt, *Macropsis fuscula* (ZETTERSTEDT), can only be sampled in low numbers (chapter 5) and produces only one generation per year (Brčák 1979). Furthermore, there is no way to control that each plant will get infected by the vectors. Experimentally, phytoplasmas can also be transmitted by grafting or by using plant parasitic dodders (*Cuscuta* spp.) (Dickinson et al. 2013). This has, however, not been demonstrated in the literature for *Rubus* species previously.

The aim of this study is to examine the disease susceptibility of four raspberry cultivars that are commonly grown by raspberry producers in Germany, to evaluate the suitability of graft inoculation for artificial infection of raspberries with phytoplasmas, to investigate the pathogenesis of Rubus stunt in raspberries and to evaluate the optimal time and age of leaf tissue for reliable phytoplasma DNA detection.

6.1.2 Materials and Methods

Plant Material

Four different raspberry (*Rubus ideaeus*) cultivars were used as test plants: 'Autumn Bliss' (AB), 'Glen Ample' (GA), 'Polka' (PO), and 'Tulameen' (TM). All test plants were acquired from Kraege Beerenpflanzen GmbH & Co. KG (Telgte, Germany) and potted in 7.5 l pots (TEKU MCI 26, Pöppelmann GmbH & Co. KG, Lohne, Germany) using Klasmann container substrate "BBB" (Klasmann-Deilmann GmbH, Geeste, Germany). During the growing season, plants were randomized and kept in two insect proof outdoor tents (Agro Quick, Rovero, Raamsdonksveer, NL) with dimensions of 5 x 3 x 2 m (L x W x H) and a net mesh-size of 0.69 x 0.69 mm (Figure 24). From December to April, plants were kept in a cold chamber at 2 – 4 °C in order to protect the potted plants from frost injury caused by low winter temperatures. Plants were watered by a drip irrigation system and fertilized manually with liquid fertilizer (Ferty EcoPhos 3, Planta Düngemittel GmbH, Regenstauf, Germany) on a weekly basis throughout the growing season.

Figure 24: Insect proof outdoor tents that were used for the experiment.

Rubus stunt phytoplasma infected raspberry (*Rubus ideaeus*) plants of the cultivar 'Tulameen' were used as inoculum source plants and were kindly provided by Michael Petruschke (Agricultural Technology Centre (LTZ) Augustenberg, Karlsruhe, Germany). Infected plants were originally sampled from a commercial fruit farm in Rielingshausen in Southern Germany (48°57'31"N 9°20'08"E). The

plants were multiplied by splitting their root balls in order to have a sufficient number of inoculum source plants for the artificial infection of the test plants. The inoculum source plants were cultivated the same as the test plants.

Artificial Phytoplasma Inoculation

From each of the four raspberry (*Rubus ideaeus*) cultivars, 13 test plants were artificially inoculated by grafting one shoot of an inoculum source plant onto one shoot of a test plant. In addition, two plants per cultivar were kept as control without artificial inoculation. This way, 21 inoculum source plants were grafted onto 52 test plants in the course of three days (see Table 11 for a chronological overview of procedures) with single inoculum source plants being grafted on one up to five test plants simultaneously. Grafting was performed by cutting away a single slice of the outer stem layers of a shoot from both, an inoculum source plant and a test plant, with an approximate size of 2 – 3 cm in length and 1 mm in depth, bringing the cut surfaces into contact and binding them together with Parafilm M All-Purpose Laboratory Film (Bemis Company, Inc., Oshkosh, WI, USA) (Figure 25). All plants remained grafted for at least 4 months until the inoculum source plants were removed from the insect proof outdoor tents.

Table 11: Chronological overview of major procedures in the susceptibility trial and type of sampled leaf material (young leaf = second fully developed leaf of a shoot; old leaf = leaf from the lower quarter of a shoot).

Procedure	Sampled Leaf Material	Date [dd.mm.yyyy]
Initial sampling of inoculation sources and test plants	young and old mixed	15.07.2015
Graft inoculation	—	05.08.2015 – 07.08.2015
Sampling date 1	young and old separate	04.09.2015
Sampling date 2	young and old mixed	02.11.2015
Dormancy in cold chamber	—	15.12.2015 – 07.04.2016
Sampling date 3	young and old separate	09.05.2016
Sampling date 4	young and old separate	20.06.2016
Sampling date 5	young and old separate	08.08.2016

Figure 25: **The stages of grafting raspberry shoots for artificial phytoplasma inoculation. (A) Cutting outer stem layer from healthy shoot. (B) Cutting outer stem layer of infected inoculum source plant shoot. (C) Finished cut on healthy shoot. (D) Fixing of healthy and infected cut shoot surfaces onto each other with Parafilm. (E) Finished grafting process of healthy and infected shoots. (F) Successful graft of healthy and infected shoots. (All pictures courtesy of Winfried Schönbach)**

Sampling of Plant Material and Assessment of Rubus Stunt Disease Symptoms

Young (second fully developed leaf of a shoot) and old (leaf from the lower quarter of a shoot) leaves of inoculum source plants and test plants were sampled once before the graft inoculation. After the graft inoculation the test plants were sampled five times designated as "sampling date 1" to "sampling date 5" in the same manner with periods of four to eight weeks in between throughout the growing season. Exact sampling dates are shown in Table 11. Sampled leafs were stored at –20° C in sealed plastic bags until used for DNA extraction.

In order to assess Rubus stunt disease symptoms in this experimental set-up, the development of witches' broom symptoms was evaluated as number of root shoots per plant on each sampling date.

DNA Extraction and Phytoplasma DNA Detection

For the samples taken before the graft inoculation and sampling date 2, leaf tissue from old and young leaves was mixed to produce one DNA extract for each plant. For all other sampling dates, two DNA extracts were produced for each plant: one from young and one from old leaf tissue in order to evaluate the suitability of each tissue at different sampling times for reliable phytoplasma DNA detection. DNA extractions were performed according to the CTAB extraction described in chapter 4.3.1.

DNA extracts were analyzed for the presence of phytoplasma DNA with the multiplex TaqMan qPCR assay as described in chapter 4.3.5. All samples were run in triplicate, including positive, negative, and no-template controls.

6.1.3 Results

Phytoplasma DNA Detection

All 21 inoculum source plants were tested positive for the presence of phytoplasma DNA with the multiplex TaqMan qPCR assay. Unfortunately, 7 out of 15 test plants from the cultivar 'Autumn Bliss' and one plant from the cultivar 'Polka' were tested positive before graft inoculation with the infected plants was carried out (Table 12). All other test plants were tested negative for phytoplasma DNA before the graft inoculation.

Table 12: Results of performed qPCRs for phytoplasma DNA detection and number of root shoots for all sampling dates and each plant. The initial sampling took place before the graft inoculation was carried out (see Table 11). (AB01 – AB13) 'Autumn Bliss', (GA01 – GA13) 'Glen Ample', (PO01 – PO13) 'Polka', and (TM01 – TM13) 'Tulameen' test plants. (+) positive qPCR, shaded in red. (-) negative qPCR. Blue bars indicate the amount of root shoots for each cell. Non-inoculated control plants are shaded in grey.

		Initial Sampling 15.07.2015	Sampling No. 1 04.09.2015		Sampling No. 2 02.11.2015		Sampling No. 3 09.05.2016		Sampling No. 4 20.06.2016		Sampling No. 5 08.08.2016	
	Sample	qPCR	qPCR	No. of Root Shoots	qPCR	No. of Root Shoots	qPCR	No. of Root Shoots	qPCR	No. of Root Shoots	qPCR	No. of Root Shoots
'Autumn Bliss'	AB01	+	+	0	+	0	-	7	+	9	+	16
	AB02	+	+	7	-	7	-	45	-	47	+	45
	AB03	-	-	0	-	0	-	6	+	11	+	38
	AB04	+	+	0	+	0	-	5	+	7	+	33
	AB05	+	+	9	-	11	-	25	+	31	+	39
	AB06	-	-	0	-	0	-	5	+	9	+	27
	AB07	-	-	0	-	0	-	1	+	2	+	14
	AB08	+	-	4	+	7	-	25	+	32	+	33
	AB09	-	-	0	-	0	-	11	+	15	+	41
	AB10	-	-	0	+	0	-	14	+	23	+	40
	AB11	-	-	0	-	0	-	4	+	6	+	23
	AB12	+	+	7	-	10	-	43	+	43	+	46
	AB13	-	-	0	-	0	-	3	-	4	+	18
	ABC1	+	+	0	+	0	-	22	+	41	+	54
	ABC2	-	-	0	-	0	-	4	-	5	-	6
'Glen Ample'	GA01	-	-	0	-	0	-	14	-	23	+	25
	GA02	-	-	0	-	0	-	10	+	33	+	54
	GA03	-	-	0	-	0	-	13	-	20	-	22
	GA04	-	-	0	-	0	-	4	+	20	+	21
	GA05	-	-	0	-	0	-	5	+	17	-	16
	GA06	-	-	0	-	0	-	9	+	32	+	41
	GA07	-	-	0	-	0	-	10	+	20	-	20
	GA08	-	-	0	-	0	-	7	+	31	+	32
	GA09	-	-	0	-	0	-	16	+	48	+	53
	GA10	-	-	0	-	0	-	8	-	35	+	49
	GA11	-	-	0	-	0	-	11	+	26	-	32
	GA12	-	-	0	-	0	-	6	-	36	+	36
	GA13	-	-	0	-	0	-	3	+	28	+	42
	GAC1	-	-	0	-	0	-	7	-	17	-	21
	GAC2	-	-	0	-	0	-	2	-	10	-	11
'Polka'	PO01	-	-	0	-	0	-	4	+	5	-	9
	PO02	-	-	0	-	0	-	13	+	13	+	10
	PO03	-	-	0	-	0	-	6	+	13	+	13
	PO04	-	-	0	-	0	-	12	+	11	+	10
	PO05	-	-	0	+	0	-	4	+	7	+	5
	PO06	+	+	0	+	0	-	3	-	3	+	3
	PO07	-	-	0	-	0	-	7	-	12	+	11
	PO08	-	-	0	-	0	-	11	+	14	+	16
	PO09	-	-	0	-	0	-	14	-	18	+	15
	PO10	-	-	0	-	0	-	8	-	11	+	10
	PO11	-	-	0	-	0	-	9	-	9	+	8
	PO12	-	-	0	-	0	-	12	-	10	+	9
	PO13	-	-	0	-	0	-	8	+	12	+	23
	POC1	-	-	0	-	0	-	7	-	11	-	12
	POC2	-	-	0	-	0	-	7	-	11	-	13
'Tulameen'	TM01	-	-	0	-	0	-	3	+	15	+	16
	TM02	-	-	0	-	0	+	1	-	13	+	16
	TM03	-	-	0	-	0	+	2	+	15	+	19
	TM04	-	-	0	-	0	+	5	+	9	+	13
	TM05	-	+	0	-	0	-	2	-	13	-	13
	TM06	-	-	0	-	0	-	2	-	8	-	7
	TM07	-	-	0	-	0	+	3	+	18	+	20
	TM08	-	-	0	-	0	+	4	+	19	+	28
	TM09	-	-	0	-	0	-	2	+	25	+	34
	TM10	-	-	0	-	0	+	5	+	6	+	9
	TM11	-	+	0	-	0	-	1	+	10	+	15
	TM12	-	-	0	-	0	-	6	+	26	+	25
	TM13	-	-	0	-	0	-	2	+	13	-	14
	TMC1	-	-	0	-	0	-	1	-	9	-	11
	TMC2	-	-	0	-	0	-	2	-	4	-	6

For sampling date 1, which took place in early September of 2015 four weeks after the graft inoculation, all plants that were previously tested positive were again tested positive except for one plant from the cultivar 'Autumn Bliss' (AB08). In addition, two plants of the cultivar 'Tulameen' were tested positive for the first time. However, out of nine plants that were tested positive, in five cases only the DNA extract produced from younger leaves was positive while the one produced from older leaves was negative.

For sampling date 2, which was carried out in early November of 2015 about eight weeks after sampling date 1, some plants that were previously tested positive were tested negative now, including both plants from the cultivar 'Tulameen' and three plants from the cultivar 'Autumn Bliss'. In contrast, one plant from the cultivar 'Polka' was tested positive for the first time.

For sampling date 3, which was performed in early May of 2016 approximately four weeks after the plants were taken out of the cold chamber, all plants were tested negative for phytoplasmas, except for the cultivar 'Tulameen' of which six plants were tested positive but in each case only in old leaves and not in young leaves.

The next sampling, sampling date 4, was in the middle of June of 2016 six weeks after sampling date 3. Out of 13 plants that were graft inoculated for each cultivar, 11 were now tested positive from 'Autumn Bliss', nine from 'Glen Ample', seven from 'Polka', and 10 from 'Tulameen'. However, from seven plants only the samples from old leaves were positive and in nine plants only the samples from young leaves were positive.

The last sampling that took place after the graft inoculation, sampling date 5, was in early August of 2016. All 13 'Autumn Bliss' test plants were now tested positive for phytoplasma DNA. Again, nine 'Glen Ample' test plants were positive, however, that includes three plants that were previously tested negative and, vice versa, three plants were negative that were tested positive in the previous sampling date 4. From 'Polka' 12 plants were tested positive for phytoplasma DNA in this sampling and 10 plants from 'Tulameen'. For 11 plants only the DNA extracts produced from young leaves were positive for phytoplasma DNA and in contrast also for 11 plants only the DNA extracts from the old leaves were positive.

Only two plants (GA03 and TM06) and all control plants remained negative for the presence of phytoplasma DNA throughout the experiment, except for the one control plant from the cultivar 'Autumn Bliss' (ABC1) that turned out to be infected already in the initial sampling before graft inoculation.

Rubus stunt Disease Symptoms

In the two samplings that were carried out in the same year as the graft inoculation, all plants had produced no root shoots except for the four plants (AB02, AB05, AB08, and AB12) which were already infected before the graft inoculation and had produced between four and 11 root shoots (Table 12).

In the first sampling in the following year (sampling date 3), approximately four weeks after plants were taken from dormancy in the cold chamber, it is noticeable that the only four plants which had root shoots in the year before, had high numbers of root shoots (between 25 and 45 root shoots) when compared with all other plants. The cultivar 'Tulameen' had a low number of root shoots compared to the other cultivars even though it was the only cultivar where phytoplasma DNA was successfully detected in this sampling.

In the last two samplings the numbers of root shoots increased especially for the cultivars 'Autumn Bliss' and 'Glen Ample', but the number of root shoots were variable within the same cultivars of all inoculated plants. In Figure 26, an infected plant from the cultivar 'Tulameen' is shown at the last sampling date with 34 root shoots as an example.

Figure 26: Root shoots of an infected plant from the cultivar 'Tulameen' (TM09) at the last sampling date (sampling date 5) with 34 root shoots.

6.1.4 Discussion

All four used raspberry cultivars in this study, namely 'Autumn Bliss', 'Glen Ample', 'Polka', and 'Tulameen', were successfully colonized by phytoplasmas via artificial graft inoculation. Unfortunately, seven out of 15 plants of the cultivar 'Autumn Bliss' were already infected with phytoplasmas before the graft inoculation. Due to work schedules and space requirements it was not possible to replace these plants with uninfected ones. Nonetheless, as the plants were received from a commercial plant propagator just as a regular producer would. This emphasizes the importance and the current relevance of Rubus stunt and the need for further efforts and research in controlling this phytoplasma disease in order to ensure healthy *Rubus* planting material.

Graft inoculation proved to be well suited for artificial phytoplasma inoculation in raspberries and can now be used as a validated inoculation method for further experiments regarding Rubus stunt in raspberry plants.

Even though there are reports of differences in susceptibility to Rubus stunt of different *Rubus* cultivars (Converse 1991; Davies 2000; Nyerges et al. 2001; Vindimian et al. 2004), it remains unclear how these assessments were made. The details of how the symptom expression and severity is assessed are especially

important as the expression of phytoplasma disease symptoms is influenced by environmental conditions, agronomical features, and disease progression (Ermacora and Osler 2019). Furthermore, mixed infections of phytoplasmas with viruses play an important role in disease progression and severity as it is for example known that plants affected by raspberry mosaic viruses and raspberry leaf mottle virus are much more sensitive to Rubus stunt (van der Meer 1987). In addition, also mixed infections of phytoplasma strains with differences in their virulence can play a role in disease severity (Kison and Seemüller 2001). And on top of that, phytoplasma disease symptoms generally have a high variability and are often equivocal. Therefore, strong field experience as a plant pathologist is needed for a reliable evaluation and as a general rule three different most typical known symptoms of the phytoplasma disease should be assessed for visual diagnosis (Ermacora and Osler 2019). These circumstances make it difficult to assess and compare the disease susceptibility of different raspberry cultivars to Rubus stunt and stress the importance of carrying out such experiments under controlled and insect proof conditions.

In an attempt to assess the severity of Rubus stunt disease symptoms, the number of produced root shoots was evaluated for each plant as van der Meer (1987) states that the first symptoms in red raspberry are numerous weak and erect shoots developing from the root buds, a symptom that is usually referred to as witches' broom. Unfortunately, this root shoot evaluation proved to be rather problematic in this study and yielded only inconclusive results for several reasons. It was sometimes difficult to count the exact number of all root shoots, especially when there were high numbers of shoots of up to 54 shoots in a single pot with a diameter of 26 cm. This explains why sometimes the number of root shoots was lower in a later sampling than before (Table 12). In addition, it is known that different raspberry cultivars produce different numbers of root shoots even when they are healthy. Therefore, an experiment would be needed to analyze the correlation between the infection with phytoplasmas and the number of root shoots. This was not possible to be analyzed in this experiment as the number of uninfected control plants was too low because of space restrictions.

Nonetheless, looking at the results of the numbers of root shoots, at least a trend is visible that the cultivars 'Autumn Bliss' and 'Glen Ample' produce more witches' broom symptoms than 'Polka' and 'Tulameen'. However, as long as there are no

cultivars that are fully resistant to Rubus stunt, the use of cultivars with less overt symptoms cannot be regarded as a tool in the control of this disease as a major problem in stopping its spread are the often hard to spot symptoms. This is also in line with the presumption made by Davies (2000) that it is possible that infections of *Rubus* species with phytoplasmas are more common than thought because infected plants may occur without the expression of overt symptoms.

Furthermore, this experiment showed the importance of taking a mixed sample from several parts of a single plant, as independent of the sampling date or cultivar sometimes only young and sometimes only old leaves were positive for phytoplasma DNA in the qPCR assay.

In conclusion, a recommendation for one of the tested raspberry cultivars concerning Rubus stunt tolerance cannot be given from this experiment. All four cultivars were successfully infected with phytoplasmas by graft inoculation. Further experiments are needed to identify a suitable method to assess Rubus stunt disease severity.

6.2 Elimination of Phytoplasmas in *Rubus* Mother Plants by Tissue Culture Coupled with Heat Therapy

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6.2.1 Abstract

Phytoplasmas are plant pathogenic bacteria that cause a disease in *Rubus* species which is referred to as Rubus stunt. As phytoplasmas can be spread by vegetative propagation and latency periods of Rubus stunt can be up to one year, the use of pathogen free *Rubus* propagation material in plant nurseries is important in order to stop the spread of this disease. Even though heat therapy has been commonly applied against viruses in many plants, its potential for phytoplasma eradication has been much less explored. Here, the efficacy of heat therapy with subsequent tissue culture to eliminate phytoplasmas from infected raspberry and blackberry plants is evaluated. Heat therapy was performed on 25 phytoplasma infected raspberry and 33 infected blackberry plants out of which 100 raspberry and 65 blackberry plants were regenerated via subsequent tissue culture. All plants were negative for the presence of phytoplasma DNA by qPCR at the end of cultivation periods between 481 to 565 days for the treated raspberry plants and 231 to 337 days for the treated blackberry plants. These results show the suitability of heat therapy combined with tissue culture as a routine tool to ensure the presence of phytoplasma free *Rubus* mother plants in nurseries.

6.2.2 Introduction

For the production of healthy planting material of any vegetatively propagated crop plant, the elimination of disease agents in propagation material (mother plants and nuclear stock material, respectively) is an important necessity. This is especially critical for phytoplasma and virus infections as they can spread inside the mother plant without showing any symptoms (latency period) (Mannini 2007; Martin et al. 2013). Heat therapy, which was used as early as 1869 when Scottish gardeners immersed bulbs in hot water before planting, is the oldest method used to remove viruses, viroids, and phytoplasmas from vegetatively propagated plants (Zandbergen 1964). Generally, when performing heat therapy nowadays, plants are incubated in a temperature cabinet at 30 – 40 °C for a period of 6 to 12 weeks with subsequent *in vitro* plant tissue cultures using meristem tips (Varveri et al. 2015). Doing so, more than 100 different pathogens may be eliminated from one plant simultaneously (Nienhaus 1985). Therefore, heat treatment is a valuable and already well-tried tool to eliminate pathogens from plants (Varveri et al. 2015) and is, for example,

integrated in the certification scheme for fruit trees in Germany (Lenz and Lankes 2006).

Phytoplasmas are cell wall-less plant pathogenic bacteria which colonize the phloem of their host plants and can be transferred by insect vectors, grafting, or vegetative propagation (Bertaccini 2007). In *Rubus* species they cause a disease that is called Rubus stunt and consists of symptoms like stunting, witches' broom, small leaves, short internodes, enlarged sepals, phyllody, flower proliferation, and fruit malformations (Mäurer and Seemüller 1994).

Heat therapy was reported to cure phytoplasma diseases for the first time in 1936 for peach yellows (Kunkel 1936), followed by aster yellows (Kunkel 1941), potato witches' broom (Kunkel 1943), and cranberry false blossom (Kunkel 1945). Today, however, even though heat therapy has been commonly applied for virus elimination in a wide range of host plants, its potential for phytoplasma eradication has been much less explored so far (Chalak et al. 2013). Hollings and Stone (1970) used heat therapy to treat chrysanthemum stunt in 'Mistletoe' chrysanthemums, but from 72 plants which were successfully regenerated from them via meristem-tips only two plants stayed without symptoms. More recently, it was reported that stem cutting culture coupled with heat therapy is effective for the elimination of '*Candidatus* Phytoplasma phoenicium' from Lebanese almond (*Prunus dulcis* (MILL.) D. A. WEBB) varieties (Chalak et al. 2005), as well as for elimination of bois noir phytoplasmas from grapevine (*Vitis vinifera* L.) (Chalak et al. 2013). For woody plant material like grapevine scions, hot water treatment has also been reported as an effective heat therapy against phytoplasmas (Bianco et al. 2000; Tassart-Subirats et al. 2003). In addition, tissue culture alone without any prior heat therapy but based on apical meristems and embryogenic callus was shown to eliminate phytoplasmas from sugarcane (Parmessur et al. 2002). Furthermore, heat therapy was found to eliminate '*Candidatus* Liberibacter asiaticus', a phloem-limited gram-negative bacteria, from infected citrus trees (Hoffman et al. 2013).

In this chapter, the efficacy of heat therapy with subsequent tissue culture to eliminate phytoplasmas from infected raspberry (*Rubus ideaeus* L.) and blackberry (*Rubus* subgenus *Rubus* (Stace 2010)) plants is evaluated in order to determine the feasibility of heat therapy as a routine tool to ensure the presence of phytoplasma

free mother plants in nurseries and thus the propagation of phytoplasma free plant material.

6.2.3 Materials and Methods

Plant Material

Phytoplasma infected raspberry (*Rubus ideaeus*) plants of the cultivar 'Tulameen' and blackberry (*Rubus* subgenus *Rubus*) plants of the cultivar 'Loch Ness' were kindly provided by Michael Petruschke (Agricultural Technology Centre (LTZ) Augustenberg, Karlsruhe, Germany) (Figure 27). These infected plants were originally multiplied from a sampled raspberry and a sampled blackberry plant which were dug out on a commercial fruit farm in Rielingshausen in Southern Germany (48°57'31"N 9°20'08"E). The plants were multiplied by splitting their root balls in order to have a sufficient number of infected plants for the heat therapy experiment.

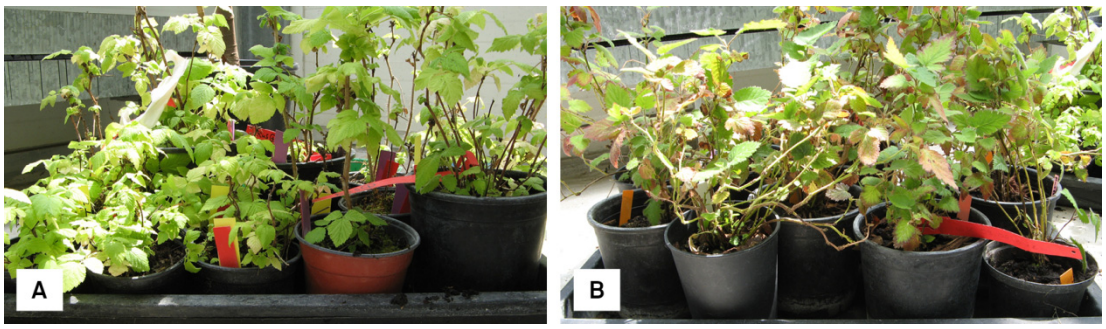

Figure 27: Phytoplasma infected raspberry (A) and blackberry (B) plants as received by Michael Petruschke (LTZ Augustenberg).

The plants were carefully pre-cultivated in order to help them cope with the heat treatment while still producing vigorous shoots with vital meristems. For the pre-cultivation, a substrate mixture of brill type 5 (Gebr. Brill Substrate GmbH & Co. KG, Georgsdorf, Germany) : sand : perlite = 1 : 1.2 : 0.3 was used and plants were watered manually as required. Liquid fertilizer (Kristalon Blaumarke, YARA GmbH & Co. KG, Dülmen, Deutschland) was applied individually according to growth rate. The woody canes were cut back in order to force numerous root shoots. With new root shoots of 5 to 10 cm in length the pots were transferred to the heating cabinet, where they were inserted in full depth in moist peat to give further heat protection. The size of the plants at the beginning of the heat therapy varied in a broad range which supposedly was caused by the infection status.

Heat Therapy

Heat therapy was performed in a heating cabinet built by the Institute of Agricultural Engineering of the University of Bonn in Germany (Figure 28). Size and dimensions of the cabinet (inner space for plants' growth: 1500 mm high, 1780 mm wide, 600 mm deep) allowed to treat up to 15 plants in plant pots with a volume of 3 l at the same time. Light was provided for 16 hours per day by four metal-halide lamps CHD Agro 400 (DH Licht GmbH, Wülfrath, Germany) which gave a photon flux density of 300 $\mu mol*m^{-2}*s^{-1}$ with full light spectrum.

Altogether, 25 raspberry plants and 33 blackberry plants were treated in the heating cabinet in three batches each. Heat therapy was carried out with a day-night rhythm of 39 °C for 16 hours and 36 °C for 8 hours. This rhythm proved to be tolerable by most *Fragaria*, *Malus*, *Prunus* and *Rubus* cultivars treated in more than 30 years. All batches of raspberry and blackberry plants were treated in the heating cabinet for 38 days before explants were taken for the tissue culture. During the heat treatment the vegetative root shoots grew up to 30 to 50 cm in length. Raspberry plants at the beginning of the heat therapy are shown in Figure 29 and raspberry plants at the end of heat therapy are shown in Figure 30. Again, the plants were watered manually on demand and fertilized (Kristalon Blaumarke, YARA GmbH & Co. KG, Dülmen, Deutschland) once a week. For the third batch of raspberry plants, however, explants for the tissue culture were additionally taken 16 and 8 days earlier (after 22 and 30 days) in order to investigate the suitability of shorter heat therapy periods for Rubus stunt phytoplasma elimination. Three infected raspberry plants were used as control plants and were cultivated without heat therapy and without tissue culture for the whole duration of the experiment.

Figure 28: **Heating cabinet used in the heat therapy experiment with blackberry plants currently inside. (Figure courtesy of Dr. Christa Lankes)**

Figure 29: **Phytoplasma infected raspberry plants in the heating cabinet at the beginning of heat therapy. (Figure courtesy of Dr. Christa Lankes)**

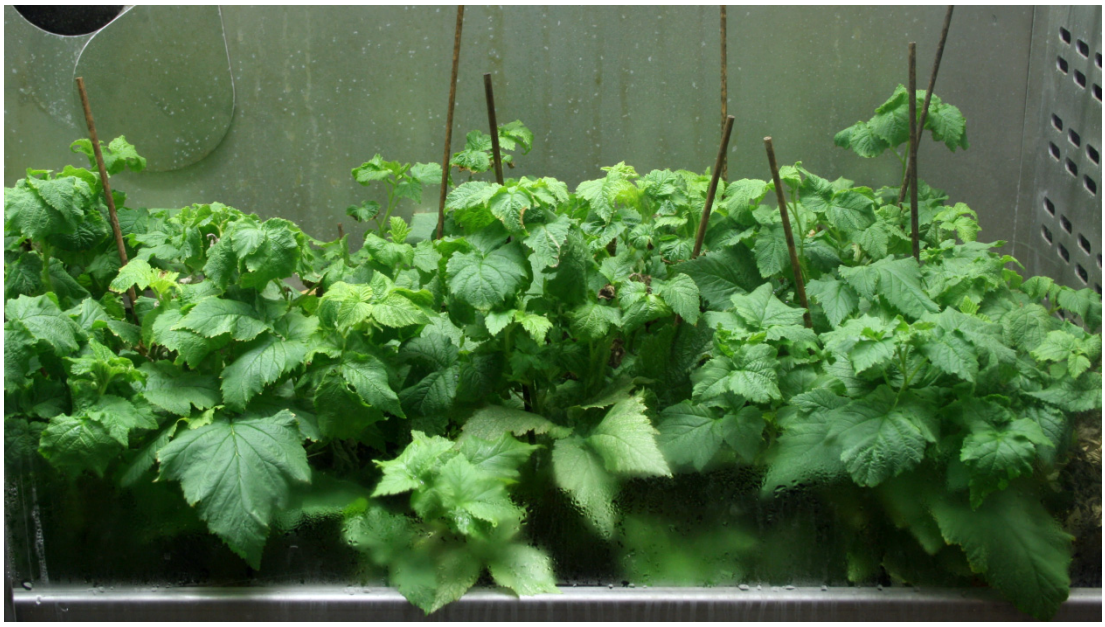

Figure 30: Phytoplasma infected raspberry plants in the heating cabinet at the end of heat therapy. (Figure courtesy of Dr. Christa Lankes)

Tissue Culture, Plant Regeneration, and Cultivation

For the tissue culture, shoot tips with a size of approximately 20 mm were dissected and surface-sterilized by shaking them for 20 min in a calcium hypochlorite solution according to Broome and Zimmerman (1984) and rinsing them with autoclaved tap water afterwards. *In vitro* establishment of the shoot apical meristem explants with sizes of 0.4 to 0.6 mm for raspberry plants and 0.5 to 0.8 mm for blackberry plants was carried out in microtiter plates with 24 wells on a Murashige and Skoog medium (Murashige and Skoog 1962) containing vitamins and macro- and micronutrients as depicted in Table 13. In the establishment phase, the synthetic cytokinin 6-benzylaminopurine was added to the medium at a concentration of 2 µmol/l. During the proliferation phase the auxin indole-3-acetic acid was additionally added to the medium at a concentration of 3 µmol/l. Due to working in subsequent batches, the duration of the *in vitro* culture period varied from 120 to 240 days. The hardening phase took about 30 days for all the batches and was carried out in a growing room at 23 °C for a 16 hours light period and 20 °C for 8 hours of darkness. Light was provided by two metal-halide lamps SONT Agro 400 (DH Licht GmbH, Wülfrath, Germany) which gave a photon flux density of 200 µmol*m^{-2}*s^{-1}. During hardening the plants did not receive any fertilizer. After the hardening phase, regenerated plants were cultivated outdoors in an insect proof horticultural tunnel covered with anti-insect mesh made out of saran plastic during the growing season and in an insect

proof greenhouse chamber during winter. For the hardening phase, the plants were potted in 0.2 l pots and transferred to 0.330 l and later-on to 1.5 l pots for the cultivation in the insect-proof tunnel. Substrate mixture, watering, and fertilization were as mentioned above for the pre-cultivation. However, the substrate mixture was pasteurized for four hours at 80 °C by an electrical heater (Sterilo, Harter Elektrotechnik, Schenkenzell, Germany) in order to avoid soil-borne pathogens. Pictures of different phases in the performed tissue culture, plant regeneration, and cultivation of heat therapy treated plants are shown in Figure 31. Because the treatments of the plants had to be performed in several batches due to the limited volume of the heating cabinet, the raspberry and blackberry plants were regenerated and cultivated for different periods of time. The exact cultivation periods for all treatments and batches are shown in Table 14.

Out of the 25 infected raspberry plants that received heat therapy 100 plants were regenerated via tissue culture. For the blackberry plants, 65 plants were regenerated from 33 phytoplasma infected heat treated plants.

Table 13: Composition of macronutrients, micronutrients, and vitamins in the Murashige and Skoog medium used for the *in vitro* tissue culture.

		Concentration [mg/l]
Macronutrients	Potassium nitrate (KNO_3)	1900
	Ammonium nitrate (NH_4NO_3)	1650
	Calcium chloride ($CaCl_2 \cdot 2H_2O$)	440
	Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	370
	Monopotassium phosphate (KH_2PO_4)	170
Micronutrients	Ferric sodium ethylenediaminetetraacetate (NaFeEDTA)	20
	Manganese(II) sulphate ($MnSO_4 \cdot H_2O$)	18.9
	Zinc sulphate ($ZnSO_4 \cdot 4\ H_2O$)	8.6
	Boric acid (H_3BO_3)	6.2
	Potassium iodide (KI)	0.83
	Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	0.25
	Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	0.025
	Copper(II) sulfate ($CuSO_4 \cdot 5\ H_2O$)	0.025
Vitamins	Thiamine hydrochloride (Vitamin B1 hydrochloride)	0.4
	Myo-Inositol	100

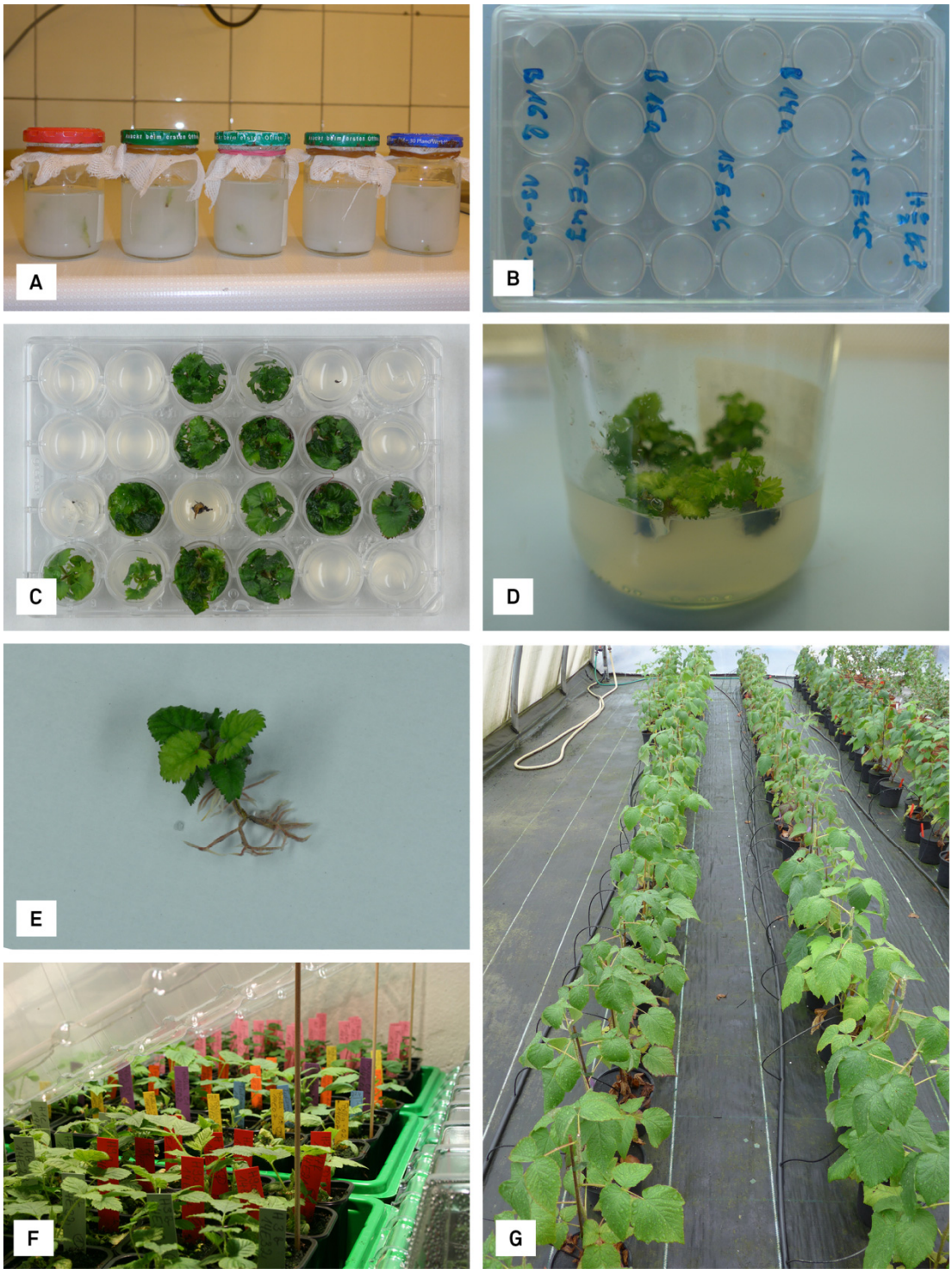

Figure 31: **Different phases in the performed tissue culture, plant regeneration, and cultivation of heat therapy treated raspberry and blackberry plants. (A) surface sterilization of shoot tips, (B) shoot apical meristems after 1 week on Murashige and Skoog medium, (C) shoot apical meristems after 4 weeks, (D) *in vitro* proliferation phase, (E) shoot after in vitro rooting, (F) plants during hardening phase, (G) cultivation of regenerated plants in an insect proof horticultural tunnel. (All pictures courtesy of Dr. Christa Lankes)**

Table 14: Time periods for all carried out heat therapy treatments, sampling dates for the phytoplasma detection after heat therapy, and consequential periods of regeneration and cultivation after heat therapy.

Treatment	Heat Therapy [dd/mm/yy]	Sampling Date [dd/mm/yy]	Regeneration and Cultivation Period [d]
Raspberry batch 1	12/09/14 – 23/10/14	10/05/16	565
Raspberry batch 2	27/10/14 – 04/12/14	10/05/16	523
Raspberry batch 3	10/12/14 – 15/01/15	10/05/16	481
Blackberry batch 1	23/03/15 – 29/04/15	31/03/16	337
Blackberry batch 2	12/05/15 – 19/06/15	31/03/16	286
Blackberry batch 3	29/06/15 – 13/08/15	31/03/16	231

Phytoplasma Detection

All plants were tested for the presence of phytoplasma DNA before the heat therapy and again at the end of their regeneration and cultivation periods of at least 481 days for the raspberry plants and at least 231 days for the blackberry plants (Table 14). Leaf samples were stored at -20 °C. For DNA extraction a mix of older and younger leaf tissue with a total weight of 1 g per sample was homogenized in a Bioreba extraction bag <Universal> (Bioreba AG, Switzerland) at room temperature in a mixture of 4 ml of CTAB buffer (3% CTAB, 0.1 M Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl) and 8 µl of 2-mercaptoethanol. The filtrate was incubated in a water bath at 65 °C for 20 min and was extracted with chloroform:isoamyl alcohol (24:1). Nucleic acids were obtained by isopropanol-precipitation. Extracted DNA was dissolved in deionized sterile water and stored at -20°C until use. The presence of phytoplasma DNA was tested by using the multiplex TaqMan qPCR assay for detection of phytoplasmas infecting Rubus species developed in chapter 4. Primers and probes were synthesized by Biolegio (Nijmegen, the Netherlands). The assay was run in 25 µl reactions using the KAPA PROBE FAST Master Mix (2X) Universal (Kapa Biosystems, Cape Town, South Africa) on an iQ5 real-time thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA), with an initial denaturation step of 20 sec at 95 °C followed by 40 cycles with 3 sec denaturation at 95 °C and 30 sec annealing and elongation at 60 °C. All samples were run in triplicate including positive controls, negative controls, and no-template controls.

6.2.4 Results

All raspberry and blackberry plants were tested positive for the presence of phytoplasma DNA prior to the heat therapy and tissue culture. After heat therapy, tissue culture, and cultivation periods between 231 and 565 days (Table 14) all 100 regenerated raspberry plants and all 65 regenerated blackberry plants were tested negative for the presence of phytoplasmas. This includes 16 regenerated raspberry plants which received heat therapy for only 30 days instead of 38 days and 7 regenerated raspberry plants which received heat therapy for only 22 days (Figure 32). All three raspberry control plants tested positive for phytoplasma DNA in the multiplex TaqMan qPCR assay after completion of the experiment with C_q-values ranging from 29.26 to 32.43.

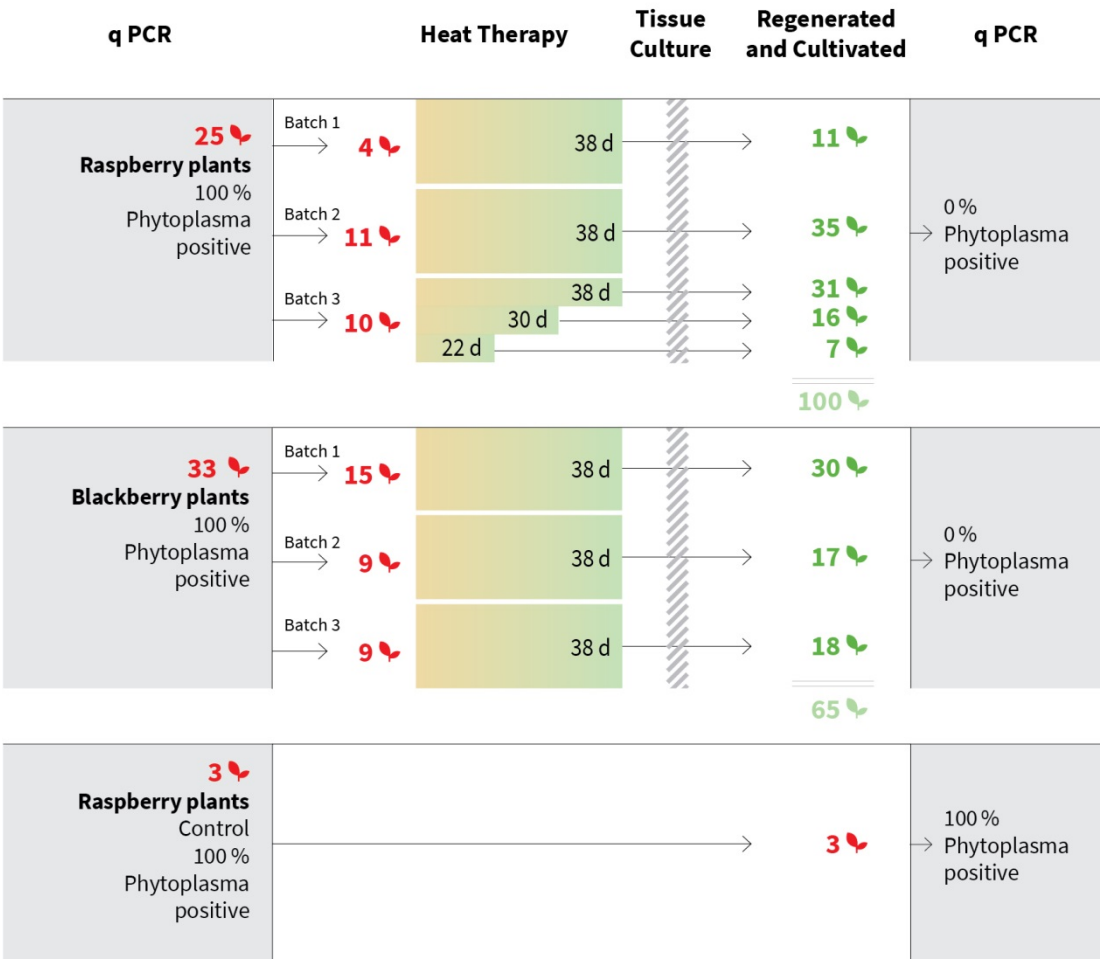

Figure 32: Schematic diagram of the experiment including number of source plants, number of source plants per batch of heat therapy, duration of heat therapy per batch, number of regenerated and cultivated plants and number of plants tested positive by qPCR in percent. (d) days; number of plants symbolized by leaf symbol.

6.2.5 Discussion

Because *Rubus* species like raspberry (*Rubus ideaeus*) and blackberry (*Rubus* subgenus *Rubus*) plants are produced by vegetative propagation and the time between plant infection and the development of Rubus stunt symptoms can be up to 11 months (de Fluiter and van der Meer 1953), making sure phytoplasma free *Rubus* mother plants are used to produce planting material is of prime importance to stop the spread of Rubus stunt. In this study, we report for the first time on the successful elimination of phytoplasmas in raspberry and blackberry plants by heat therapy with subsequent tissue culture. So far, there is only limited literature about the elimination of phytoplasmas from infected plants via heat therapy and tissue culture. Nonetheless, single heat therapy, single tissue culture, and both methods coupled have been reported to be effective in certain host plants.

Kunkel (Kunkel 1941) used a hot room to investigate the efficacy of heat therapy to eliminate aster yellows in Madagascar periwinkle (*Catharanthus roseus* (L.) G.DON) without subsequent tissue culture. Kunkel (Kunkel 1941) found that periwinkle plants treated at 38 °C and 42 °C for 14 days, respectively, did not show any disease symptoms 10 and 12 months after the heat therapy, respectively. Periwinkle plants that were treated at lower temperatures or shorter periods of time all developed symptoms after heat therapy, however, the longer the treatment the longer newly developed shoots stayed without symptoms. Furthermore, Kunkel (Kunkel 1941) was able to cure wild tobacco (*Nicotiana rustica* L.) by heat therapy at 40 °C for three weeks, while two weeks of heat therapy were not sufficient to kill the phytoplasmas in the roots. Kunkel also reported heat therapy without tissue culture to be effective against peach yellows in peach trees (Kunkel 1936), false blossom in cranberry plants (Kunkel 1945), and potato witches' broom phytoplasmas from periwinkle plants (Kunkel 1943).

The first report of heat therapy coupled with subsequent meristem tissue culture to eliminate phytoplasmas from plants is from Hollings and Stone (1970). They treated 'Mistletoe' chrysanthemum plants at 35 °C for 14 to 37 weeks before doing meristem-tip culture. Even though all plants were symptomless for an initial period of five weeks and only three plants showed symptoms after nine weeks, only 2 plants out of 72 stayed without chrysanthemum stunt symptoms for the total duration of the study.

Chalak et al. (2013) performed a heat therapy at 38 °C for 40 days during the culture process of stem cuttings and cultures of shoot tips from grapevine (*Vitis vinifera* L.). After heat therapy, newly developed shoots underwent subculturing for 30 days before they were tested by nested PCR for a first time. Afterwards, explants were multiplied for two more 40 day subcultures and were subsequently tested by nested PCR again. Only 76% of shoots from the stem cutting culture with heat treatment and 76% of shoots from the shoot tip culture with heat treatment were negative in the first nested PCR after the first subculture, but all shoots were negative for phytoplasma DNA in the second nested PCR after the end of the third subculture (120 days of culture in total). In the same study, the authors also used shoot tip culture without heat therapy. While only 36% of these shoots were negative in the first nested PCR, again all shoots were negative after the third subculture. Unfortunately, Chalak et al. (2013) did not test regenerated plants for the presence of phytoplasmas after hardening and cultivation for longer periods of time. Chalak et al. (2005) did the same treatment with Lebanese almond (*Prunus dulcis* (MILL.) D. A. WEBB) varieties just at 35 °C for 30 days and all samples were negative for almond phytoplasma DNA in a regular PCR after three months of subculture, however, regular PCR is known to be often not sensitive enough to detect phytoplasma DNA in low titers (Jarausch et al. 2001; Delić 2012).

Similarly to Chalak et al. (2013), Wang and Hiruki (1996) applied heat therapy of 35 °C to paulownia tissue cultures with typical witches' broom symptoms for five weeks with subsequent meristem tissue culture. No symptoms of witches' broom appeared in a series of subcultures after the heat therapy and plants were tested for phytoplasmas by PCR. Out of 33 analyzed plants, 31 were tested negative, however, only regular PCR was used.

While Dai et al. (1997) reported that 10 to 30% of regenerated mulberry (*Morus alba* L.) plants remained infected up to three years after *in vitro* stem culture, Parmessur (2002) showed that phytoplasmas can be eliminated in sugarcane by tissue culture of apical meristems and embryogenic callus without performing heat therapy in advance. Phytoplasma elimination by tissue culture without heat therapy should be investigated in the future for *Rubus* species as heat therapy is a time consuming and therefore expensive process.

From the limited literature available on phytoplasma elimination by heat therapy and tissue culture it can be concluded that its success relies heavily on the duration and temperature of the heat therapy and type of tissue culture. Furthermore, the species of host plant seems to play an important role. Nonetheless, there are reports of successful elimination of phytoplasmas by heat therapy without tissue culture and vice versa, providing potential treatment options for host plants which are either sensitive to heat or are difficult to handle in tissue cultures.

As plant health of *Rubus* spp. is currently not regulated under a growing material regulation or certification scheme in Germany, we hope that the results of this study will help to establish such regulations in order to ensure *Rubus* plant health.

In conclusion, heat therapy with subsequent tissue culture is a suitable method for phytoplasma elimination in raspberry and blackberry plants and is therefore an important step during the propagation process of disease-free plant material. Further investigations on reduced treatment duration of heat therapy and the effectiveness of tissue culture without heat therapy should be made in order to optimize costs for routine treatments of mother plants and nuclear stock material.

7 General Conclusion

Rubus stunt is an economically important phytoplasma disease in *Rubus* species as it is widespread throughout Europe and North America and fruit malformations can be so severe that berry harvests can be completely lost (Vindimian et al. 2004). As Rubus stunt has a long period of latency of up to one year (Converse 1991) and disease symptoms can be unclear (Davies 2000) an early molecular detection is of major importance to prevent the spread of this disease. However, concentrations of phytoplasmas in *Rubus* plants are often too low to be detected by regular PCR (Jarausch et al. 2001) and nested PCR, the most commonly used tool for the detection of phytoplasmas since the early 1990s (Christensen et al. 2013; Monti et al. 2013), is laborious and has an increased risk for contaminations (Nikolić et al. 2010; Delić 2012). qPCR avoids these disadvantages because no post-PCR procedures are necessary, as the reporter fluorescence can be monitored in real time. Furthermore, qPCR employing TaqMan probes equipped with different fluorogenic dyes allows the detection of multiple targets in a single reaction, offering a simple and effective way to monitor DNA extraction performance as an internal control (Mumford et al. 2006). The multiplex TaqMan qPCR assay developed in this thesis (chapter 4) is able to detect specifically elm yellows phytoplasmas, phytoplasmas in general, and insect or plant DNA as an internal control. It now offers a practical tool for fast and sensitive detection of phytoplasma DNA in *Rubus* species and putative insect vectors for diagnostic laboratories and routine testing in plant nurseries. As control strategies of Rubus stunt entirely rely on preventative measures, a detection method like the one developed here represents an important basic component in managing this disease.

In the screening for putative insect vectors of Rubus stunt in Germany (chapter 5), *Macropsis fuscula* (ZETTERSTEDT), the only described insect vector of Rubus stunt, was present only in low numbers. This is consistent with the findings of Vindimian et al. (2004) who did not capture any *Macropsis* spp. in epidemiological studies in Italy, even though the Rubus stunt disease incidence was high in the same plantations. These results emphasize the hypothesis, that the spread of Rubus stunt by *M. fuscula* only plays a minor role. This should be taken into account for Rubus stunt disease control strategies, as the application of insecticides against phytoplasma insect vectors in general is already unsatisfying because it is impossible to eliminate

all vectors from a field (Bertaccini 2007) and insect resistance and environmental regulations have limited the viability of long-term applications of insecticides (Perilla-Henao and Casteel 2016). But in the case of Rubus stunt it is especially inexpedient as the limited knowledge there is about Rubus stunt insect vectors points to the fact that the number of insect vectors is apparently quite low and undesired side effects of insecticides should be avoided as much as possible. Nonetheless, insect vectors play an important role for wild reservoirs of Rubus stunt phytoplasmas in plants like wild *Rubus* species, as well as mallow (*Malva sylvestris* L.) and dog rose (*Rosa canina* L.) (Jarausch et al. 2001; Vindimian et al. 2004; Borroto Fernández et al. 2007; Cieslinska 2011).

Because most known phytoplasma insect vectors are rather thermophilic species and climate change will affect their spread and incidences (Maejima et al. 2014), further experiments should be carried out on putative insect vectors of Rubus stunt, including transmission assays of the insects found to contain phytoplasma DNA in this thesis. In addition, so called next generation insect control strategies like blocking insect vector transmission by using certain chemicals like lectins, carbohydrates, or antibodies in order to saturate the pathogen-binding site in the insect or on the bacteria surface (Perilla-Henao and Casteel 2016) should be investigated. This has been shown to be a promising approach for the xylem-limited vector-borne bacterial plant pathogen *Xylella fastidiosa* (Killiny et al. 2012) and also with some success in laboratory experiments with antibodies on '*Candidatus* Phytoplasma asteris' and the two leafhopper vectors *Macrosteles quadripunctulatus* KIRSCHBAUM and *Euscelidius variegatus* KIRSCHBAUM (Rashidi et al. 2015). Furthermore, the employment of RNA interference (RNAi) is a promising next generation approach to control insect vectors (Li et al. 2013; Yu et al. 2016) that needs to be further explored for phytoplasma diseases.

In contrast to the low occurrence of *M. fuscula*, the fact that 8 out of 60 plants (including 7 plants out of 15 from the cultivar 'Autumn Bliss') that were received from a commercial plant nursery for the susceptibility trial (chapter 6.1) were already infected with Rubus stunt phytoplasmas suggests, that vegetative production of infected *Rubus* planting material can play a major role in the spread of this disease. This observation is in consistence with reports of important contributions of vegetative propagation to the spread of other phytoplasma diseases such as

paulownia witches' broom disease (Wang and Hiruki 1996) or bois noir and flavescence dorée in grapevine (Mannini 2007).

Unfortunately, assessing the disease severity of Rubus stunt turned out to be difficult due to the equivocality of the symptoms making a comparison of susceptibilities of different cultivars complicated. In conclusion, a recommendation of a more tolerant variety cannot be given and would not be expedient at this point until a fully resistant variety is available. This is because visual identification of infected plants in commercial plantations is already difficult as symptoms are often unclear, but removal of infected plants is an important part of a successful Rubus stunt control strategy.

Assessing the susceptibility of phytoplasma diseases is challenging as the expression of symptoms is influenced by environmental conditions, agronomical features, and disease progression (Ermacora and Osler 2019), as well as by phytoplasma strains with different virulences (Kison and Seemüller 2001) or mixed infections. Therefore, experiments regarding the susceptibility of phytoplasma diseases need to be carried out using artificial inoculation and controlled cultivation conditions. Research on resistant or tolerant phytoplasma host plants remains challenging as the mechanisms are not yet completely understood (Bertaccini and Duduk 2009). Nonetheless, further research on phytoplasma disease resistance is of high importance as resistant cultivars would be a highly efficient control measure for these difficult to control diseases.

Testing with the developed multiplex TaqMan qPCR assay of different leaf tissues, namely old and young leaf tissues, at different points of time throughout the growing season revealed, that it is of high importance to take mixed samples of old and young leaves. This is based on the fact that phytoplasmas in *Rubus* species seem to be neither spread equally throughout the plant nor follow any systematic movement during the growing season. It is known that phytoplasmas can readily pass the sieve pores of the sieve tubes and may be passively translocated from source to sink organ with the assimilate flow (Kube et al. 2012). However, experiments using localized inoculation by insect vectors (Wei et al. 2004) and investigating seasonal colonization of pear trees (Garcia-Chapa et al. 2003) showed that movement of phytoplasmas cannot be explained solely by assimilate flow. As phytoplasmas lack

genes coding for cytoskeleton elements or flagella, active movement is, however, highly unlikely (Christensen et al. 2005).

The production of healthy *Rubus* planting material can be considered as the current main foundation in Rubus stunt disease management. Heat therapy with subsequent tissue culture proved to be an efficient method for the elimination of phytoplasmas in raspberry and blackberry plants (chapter 6.2) and should be implemented into the production of *Rubus* mother plants in order to ensure their healthiness. Furthermore, routine screening of planting material should be performed in plant nurseries so the absence of phytoplasmas from planting material can be guaranteed.

The results from this thesis can considerably improve Rubus stunt control strategies. However, further studies on the symptomology, the pathogenesis, and the mechanisms of tolerance or resistance against Rubus stunt should be carried out, as well as further monitoring of insect vectors, in order to optimize control strategies. This is highly important in order to keep the production of *Rubus* berry fruit attractive for growers.

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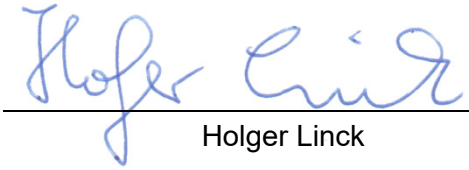
11 Statutory Declaration

I declare that I have prepared the submitted dissertation independently and without unauthorized third-party help and that no other than the in the dissertation listed facilities have been used. All text passages that are quoted literally or analogously from other published papers and all information that are based on verbal statements are identified as such. I have observed the principles of good scientific practice as defined in the statutes of the Hochschule Geisenheim University and the Justus-Liebig-Universität Gießen for safeguarding good scientific practice when carrying out the analyses of my research mentioned in the dissertation.

Weingarten, 7 August 2019



Holger Linck