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Pflanzenkrankheiten und Pflanzenschutz

Studies on transmissibility, cytopathology and control of T*omato chlorotic dwarf viroid* and P*otato spindle tuber viroid*

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Abstract

PSTVd and TCDVd have wide host ranges and are able to cause huge economic losses in many high-valued crops in the *solanaceous* family. TCDVd has recently been detected to infect many species worldwide, posing the potential risk of spreading this viroid disease. Hence, more information about transmission modes and prevention methods need further investigation.

Mechanical transmission continues to play a very important role in spreading *Pospiviroids* via handling activities in the greenhouse. Nearly 100% of plants showed infection with both PSTVd and TCDVd when treated with contaminated rubber gloves, mechanically inoculated with plant sap and cut with contaminated razor blades. In addition, the transmissibility of TCDVd via *M. persicae* with assistance of PLRV is confirmed for the first time in this study. High temperature promotes the rate of PLRV assisted aphid transmission. Although the efficiency of TCDVd transmission via *M. persicae* is low, the risk of PLRV-assisted aphid transmission of TCDVd to other *solanaceous* crops seems to be likely.

The severity of induced symptoms is various depending on viroid species, host plants, inoculation time and the environmental conditions. Tomato is a susceptible symptomatic host for both PSTVd and TCDVd, whereas infected ornamental plants remain asymptomatic. High temperature (26-28°C) promotes foliage symptom development of TCDVd infection on tomato. In addition, the increase of plant age at the time of inoculation corresponds with a decrease of symptom expression. Distinguishable symptoms between mixed PLRV+TCDVd infection and single TCDVd infection could be observed only 6 to 8 weeks after inoculation and developed as newly emerging dwarfed and hard sprouts on top of the plant.

The macroscopic symptoms may correlate with a variation of cytopathogical changes in viroid infected tissues. Disintegration of cell walls of many parenchyma cells and appearance of vesicular plasmalemmasomes are probably associated with leaf distortions and curling leaves in plants with macroscopic symptoms. Moreover, malformation of grana and thylakoid membranes of chloroplasts were typical for TCDVd-infected tissues. In PLRV-infected tissues massive accumulation of carbohydrates as starch grains could be observed in chloroplasts being a consequence of blocked translocation of photoassimilates.

Besides, the viroid RNAs remain infectious for at least 4 months at -20°C storage condition and somewhat less after storage in a refrigerator at 4°C and most astonishingly after drying at room temperature. Thus, the importance of phytosanitary treatments during crop handling and plant cultivation should be highlighted. Sodium hypochlorite (NaOCl) is the most promising disinfectant against TCDVd in aqueous solution in plant sap and after treatment of contaminated handling tools in practical greenhouse work. Concentration of 0.5% NaOCl and an incubation time of 20 sec are sufficient to disinfect TCDVd on cutting tools and in solution. Comparable disinfection results could be achieved by flaming cutting tools with 96% alcohol.

Kurzfassung

PSTVd und TCDVd haben große Wirstkreise und sind in der Lage, große wirtschaftliche Schäden in hochwertigen Kulturen aus der Familie der Solananceen hervorzurufen. Beide Viroide können leicht auf vielseitige Art und Weise auf andere Arten oder innerhalb der gleichen Art übertragen werden. TCDVd wurde in letzter Zeit in vielen Pflanzenarten weltweit nachgewiesen. Dies erhöht die Gefahr der zunehmenden weltweiten Verbreitung dieser Viroidkrankheit. Daher ist es notwendig mehr Informationen zur Übertragbarkeit sowie zu präventiven Bekämpfungsmethoden zu erarbeiten.

Mechanische Übertragung spielt nach wie vor eine sehr wichtige Rolle bei der Verbreitung von Viroiden durch manuelle Bearbeitung im Gewächshaus. Nahezu 100% der Versuchspflanzen (Tomaten) wurden infiziert, wenn sie mit Viroid-kontaminierten Gummihandschuhen abgerieben oder mit kontaminierten Rasierklingen kontinuierlich in Folge angeschnitten oder mit Viroid-haltigem Pflanzensaft mechanisch inokuliert wurden. Darüber hinaus wurde die Übertragung von TCDVd durch die Blattlaus *Myzus persicae* mit Unterstützung durch das *Potato leaf roll virus* als Transportmittel nachgewiesen. Die Versuche wurden unter zwei unterschiedlichen Temperaturregimen (20-22°C und 26-28°C) durchgeführt. Hohe Temperaturen begünstigen die Rate der PLRV-unterstützten TCDVd-Blattlausübertragung. Obwohl die Effizienz der TCDVd-Übertragung durch *M. persicae* nicht hoch ist, erscheint die Gefahr der Übertragung auf weitere Kulturen von Nachtschattengewächsen wahrscheinlich.

Die Schwere induzierter Symptome ist unterschiedlich und hängt von der Viroid- und Wirtspflanzenart, Zeitpunkt der Inokulation und Umweltbedingungen ab. Tomate ist eine anfällige symptombildende Wirtspflanze für PSTVd als auch für TCDVd, wohingegen infizierte Zierpflanzen symptomlos bleiben. Hohe Temperaturen (26°-28°C) begünstigen die Bildung von Blattsymptomen an Tomaten. Außerdem korrespondiert das Pflanzenalter zum Zeitpunkt der Inokulation mit einer Abnahme der Symptomausprägung. Unterscheidbare Symptome zwischen einer TCDVd/PLRV Mischinfektion und einer TCDVd Einzelinfektion konnten erst 6-8 Wochen nach Inokulation beobachtet werden: Es entwickelten sich neue verzwergte und hartblättrige Triebe aus den oberen Achselknospen der Tomatenpflanzen.

Makroskopische Symptome können mit einer Anzahl von zytopathologischen Veränderungen in Viroid-infizierten Pflanzengeweben zusammenhängen. Gradueller Zerfall von Zellwänden vieler Parenchymzellen und Bildung vesikulärer Plasmalemmasomen wurden in Verbindung mit Blattdeformationen wie Blattkräuselung beobachtet. Darüber hinaus sind Deformierung von Grana- und Thylakoidmembranen in Chloroplasten typisch für TCDVd-infizierte Gewebe. In PLRV-infizierten Geweben konnte eine massive Ansammlung von Kohlehydraten in Form einer Vielzahl anomal großer Stärkekörner in den Chloroplasten, als Folge blockierter Translokation von Assimilaten festgestellt werden.

Versuche zur Aktivität von TCDVd und PSTVd in Blattmaterial unter verschiedenen Lagerungsbedingungen zeigten, dass die Pathogene für mindestens 4 Monate bei -20°C im

Tiefkühlschrank, etwas weniger bei Lagerung im Kühlschrank bei 4°C und erstaunlicherweise sogar zu 20 % in bei Raumtemperatur gelagerten, verrotteten Blättern infektiös bleiben.
Zur Desinfektion von TCDVd wurden verschiedene Chemikalien getestet, wobei Natriumhypochlorit (NaOCl) die beste Wirksamkeit zeigte. Alle Behandlungen, wie mechanische Inokulation mit Pflanzensaft und Desinfektion von Rasierklingen mit eingetrocknetem Inokulum konnten mit 0,5%iger NaOCl Konzentration bei einer Einwirkungsdauer von 20 Sekunden erfolgreich durchgeführt werden. Vergleichbar gute Desinfektionsergebnisse zeigte nur ein Abflammen der Schneidewerkzeuge mit 96%igem Alkohol.

List of abbreviations

Viroids and Viruses

ASBVd	*Avocado sunblotch viroid*
CCCVd	*Coconut cadang-cadang viroid*
CChMVd	*Chrysanthemum chlorotic mottle viroid*
CEVd	*Citrus exocortis viroid*
CSVd	*Chrysanthemum stunt viroid*
HSVd	*Hop stunt viroid*
PLMVd	*Peach latent mosaic viroid*
PLRV	*Potato leafroll virus*
PSTVd	*Potato spindle tuber viroid*
TASVd	*Tomato apical stunt viroid*
TCDVd	*Tomato chlorotic dwarf viroid*

General

Bp	Base pair
C_2H_5OH	Absolute alcohol 96%
CCR	Central conserved regions
CD	Central domain
Chl	Chloroplast
ClO_2	Chlorine dioxide
Cv	Cultivar
CW	Cell Wall
DSMZ	German Collection of Microorganism
Ext	External
Fig.	Figure
Int	Internal
M	Mitochondria
MiRNA	Micro Ribonucleic Acid
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
NEP	Nuclear encoded polymerase
Nm	Nanometer
nt	Nucleotides
PD	Pathogenic domain

Pd	Plasmodesmata
PEP	Plastid-encoded polymerase
Pol II	DNA - dependent RNA polymerase II
Ppm	Part per million
PSs	Plasmalemmasomes
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAse	Ribonuclease
RT	Room temperature
RT-PCR	Reverse transcription – polymerase chain reaction
Rz	Hammerhead ribozymes
S	Starch
SiRNA	Small interfering RNA
TCH	Terminal conserved hairpin
TCR	Terminal left regions
TEM	Transmission Electron Microscopy
TLD	Terminal left domain
TPIA	Tissue print immunoassay
TRD	Terminal right domain
V	Vacuole
VD	Variable domain
Vs	Vesicles

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Chapter 1: General introduction

1. Discovery of viroids

Viroids are the smallest known infectious agents and cause several dramatic diseases in a number of agricultural and horticultural crops. They are distinguished from viruses by absence of coat protein and by their extremely small size (Fig. 1, Diener 1987, Gross and Riesner 1980, Hull 2009). The term “viroid” (virus–like) was proposed by their discoverer T.O. Diener (1971); however, viroid diseases have been described earlier. Since 1921, a new disease on potato (*Solanum tuberosum*), named spindle tuber disease, which showed severe foliage symptoms including shoots growing upright, small branches and pointed leaves and spindle like misshaped tubers has been described in the United States of America. Because no causal agents related to micro-organisms were found, unknown viruses were supposed to be responsible for that (Martin 1922). In another case in avocado, a new disease with stem streaks and discolored lesions on fruits was at first reported in California (USA). It was supposed that the disease was as an expression of physiological characteristics or genetic disorder (Coit 1928). However, all causal agent proposals were not completely persuading. Until beginning of the 1970s, everything has become more explicit when groups of researchers in Canada, USA and Germany asserted that the infectious agent of spindle tuber disease on potato were unencapsidated ribonucleic acids (RNA) of an unexpectedly small size. Interestingly, this RNA was able to replicate in host cells without a helper virus, which was different from satellite RNAs (Singh and Clark 1971, Diener 1971, Sänger 1972).

Furthermore, this infectious RNA was not able to encode any proteins in susceptible host plants to accomplish its biological functions for infection such as replication and movement. It rather relied on host plant operative mechanisms. Due to these differences from other pathogens, Diener proposed the name viroid (virus-like) for this infectious RNA (Diener 1971). This name was based on the similarities on symptom expression between viroid and plant virus infection. Later, by visualization of *Potato spindle tuber viroid* (PSTVd) by electron microscopy, Sogo and colleagues provided further persuasive evidence for extremely small sized viroid particles (Sogo et al. 1973). Thereafter, with the success in sequencing of PSTVd,

this viroid was described as a covalently closed RNA molecule of 359 nucleotides and able to form secondary structures (Gross et al. 1982).

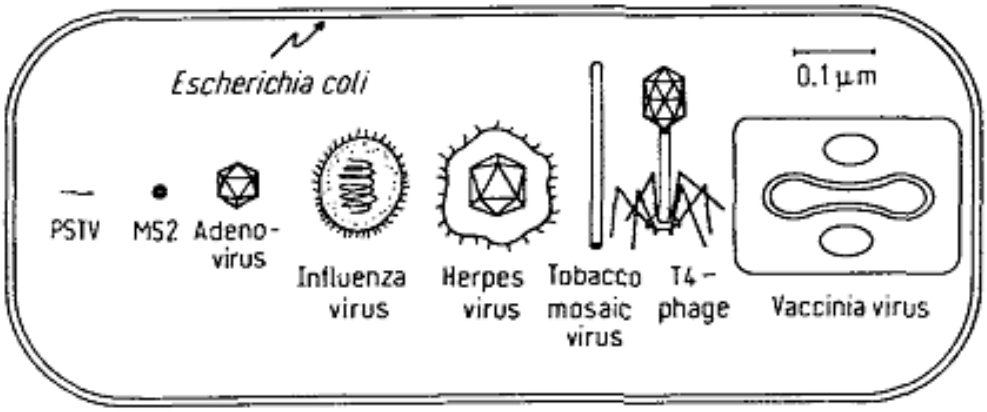

Figure 1 Comparison of the size of PSTVd with viruses and the bacterium *Escherichia coli* (Gross and Riesner 1980).

2. Economic impact of viroid diseases

Although viroids are just RNA molecules with only a few hundred nucleotides (nt) and not able to accomplish their biological functions by themselves, they cause huge economic losses in many high-valued crops. In general, the losses caused by viroid diseases are measured in two aspects. The first is considered as direct losses of plant yield, and quality of products induced by infection of viroids. These direct losses vary, depending on viroid species, crops, appearance in time and environmental conditions. The second aspect of the losses is calculated on the ability of epidemic spread to other crops over wide distances with infected plant material, and costs to prevent and control the diseases. This is considered as indirect losses caused by viroid diseases (Hadidi et al. 2003).

2.1 Direct losses due to viroid diseases

Viroid diseases have been responsible for dramatic economic losses in many important crops worldwide: from main agricultural crops over vegetables to ornamental plants. Plant yield losses, reduction of growth rate and quality of plant products have been reported. *Avocado sunblotch viroid* (ASVd) has been diagnosed in most of avocado-growing countries in the 1930s. Infected avocado showed streaks on stems, leaf distortion, discoloration and distortion of fruits, resulting in significant reduction in yield and unmarketable avocado products (Desjardins 1987). In the Philippines, Coconut cadang-cadang disease has caused the death of around 30 million coconut palms since the disease was first discovered in the 1950s.

The calculation of Randles (2003) showed that infected coconut palms reduce nut production around 5 years before their death, and the replanting takes another 5-8 years until plants are able to produce nuts again. Thus, in total, the interruption of production for each infected plant is about 10-13 years. An average annual loss due to coconut cadang-cadang disease has been estimated in the range of 20 to 50 million US dollars since 1950s (Randles 2003). *Chrysanthemum stunt viroid* (CSVd) was reported to be responsible for losses of around 3 million US dollars in Australia in 1987 (Hill et al. 1996). In Japan, hop stunt disease caused losses of 30-50% biomass of plants due to severe symptoms such as stunted growth and reduced size and number of leaves (Shikata 1987). In 1987, the yield loss because of spindle tuber disease of potato was estimated to be about 1% of the whole potato production in North America. The infection reduced not only yield, but also quality and was a threat to seed- and germplasm production (Diener 1987). In Canada, the infection of *Tomato chlorotic dwarf viroid* (TCDVd) caused stunted growth rates, as well as cracked and reduced fruit size of tomato, resulting in unmarketable fruits of tomato crops (Singh and Dilworth 2009). In Japan, TCDVd was also considered to have a significant impact on tomato production under glass in the Hiroshima region (Matsushita et al. 2008).

2.2 Indirect losses due to viroid diseases

This kind of losses includes the extra costs for prevention and control measures of the diseases, especially when viroids spread as an epidemic. Prevention and control include the costs for reliable diagnostics, eradication of infected plants, production of viroid-free seedlings, disinfection methods and replanting. These measures are generally closely linked to the mode of spread of the viroid. For instance, when mechanical transmission is the means of spread, eradication, replanting and disinfection to avoid mechanical transmission are highly recommended. When seed or pollen transmission is the mode of viroid spread, removal of infected plants and replanting with viroid free seedlings is recommended (Hadidi et al. 2003). In the case of ASBVd infection, the infected plant can remain asymptomatic and thus act as reservoir for mechanical transmission of the pathogen through hand-on activities of growers. Hence, the losses here included the costs for viroid free seedlings and diagnostic tests (Desjardins 1987). Furthermore, the costs for research in order to understand the mechanisms of viroid diseases should be considered as costs for prevention measures. For example, in the case of Coconut cadang-cadang in the Philippines, the costs for research of

the disease consisted of 0.27% of the overall costs of the disease including employment of experts, travel, infrastructure and other supports. These research costs are trivial compared to the economic loss, but its benefits are outweighing. It provided relevant knowledge on etiology and epidemiology of the cadang–cadang disease (Randles 2003).

The primary strategies to prevent the spread of viroid diseases among countries are quarantine and certification programs. In the European Union, three viroids, namely *Coconut cadang-cadang viroid, Potato spindle tuber viroid and Chrysanthemum stunt viroid*, are in the quarantine- or certification regulation list (EU council Directive 2000/29/EC). These regulations usually cause extra costs for international trading between countries. For example, in the case of *Pospiviroid* infection in the Netherlands (in the year 2006), asymptomatic *Pospiviroid* infections were detected in *solanaceous* ornamentals (*Solanum jasminoides* and others) and served as potential inoculum sources for other *solanaceous* crops, especially potato and tomato. Calculation on the Netherlands exports revealed that totals costs were estimated between 3 and 5 million Euros, including destruction of infected plants, disinfection, cleaning and reproduction. Furthermore, costs for inspection, diagnosis, prevention and certified allowance for export have to be calculated every year (Verhoeven 2010).

3. Viroid structure and classification

3.1 Viroid structure

Viroids are known as the smallest plant pathogens; consisting of a covalently closed single-stranded circular RNA which ranges in size from 246–467 nucleotides depending on species. In contrast to viruses, they do not code for any proteins or peptides and rely entirely on host proteins for their biological functions such as replication, processing and transport (Hadidi et al. 2003, Flores et al. 2004). By electron microscopical observation in purified preparations PSTVd appears as rod-like structured molecules of an average length of approximately 37 + 6 nm. The complete sequencing of PSTVd by using a radioactive labeling method, led Gross and colleagues (1982) to conclude that PSTVd molecules were able to form a unique rod-like secondary structure based on intra-molecular base pairing, serial arrangement of double-helical sections and internal loops. This rod-like secondary structure is assumed to play important roles in biological functions and providing binding signals to specific host factors

(Tabler and Tsagris 2004). When treated with heat, the internal base pairing will be denatured and a ring like structure appears (Fig. 2, Riesner et al. 1979).

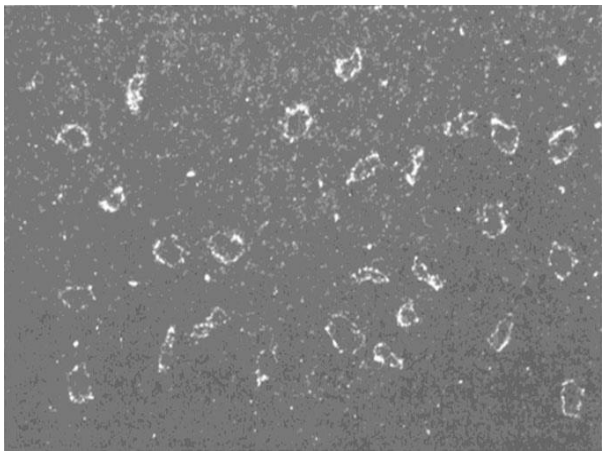

Figure 2 PSTVd particles (average length of 37+6 nm) after heat treatment showing the circular nature of viroid RNA. Transmission electron micrograph, at a magnification of 185.000x (Riesner et al. 1979)

On the basis of sequence homology among many viroid species, Keese and Symons (1985) proposed a model for five structural and functional domains in all viroids (Fig. 3A), except ASBV. These domains include (1) a central domain (C), able to form alternative structures that may regulate viroid replication, (2) a domain associated with pathogenicity (P), (3) a domain with high sequence variability (V), (4 and 5) two terminal domains: terminal left (TL) and terminal right (TR). The changes or variability by a few residues in the pathogenic domain may lead to changes of the isolate to produce mild, intermediate, severe, or lethal symptoms. The variable domain (VD) is the most variable region and has the least sequence homology amongst species and strains as compared to the other regions. However, unlike the P domain, the sequence differences in the V domain is not correlated to symptom expression or to emergence of other isolates (Keese and Symons 1985).

Within the family *Pospiviroidae*, the C domain contains a central conserved region (CCR) formed by two highly conserved nucleotide fragments, one in the upper strand and the other in the lower strand, those of upper strand being flanked by an inverted repeat (Flores et al. 2004, Claude and Fauquet 2004). So far, there are 5 different CCRs known amongst the *Pospiviroid* family (Flores et al. 2004). The nucleotides of the upper strand in CCR may form either a hairpin, oligomers or a palindromic structure which is possibly involved in viroid replication (Claude and Fauquet 2004). In addition, all members of the family *Pospiviroidae*

present either terminal conserved regions (TCR) or terminal conserved hairpins (TCH) in the terminal left domain (TLD). The presence of CCR, TCR and TCH are criteria to classify the main genus of the family *Pospiviroidae* (Flores et al. 2004).

In contrast to the members of the family *Pospiviroidae*, the members of the family *Avsunviroidae* lack the highly conserved region in the CD or TLD. Rather they are able to form hammerhead structures which are composed of 11 conserved residues and adjacent helices (Fig. 3B). This hammerhead structure is the most important motif of the family *Avsunviroidae*, which involves in many biological functions including replication and transport (Hadidi et al. 2003, Flores et al. 2009).

Table 1 Main features to classify the members of families *Pospiviroidae* and *Avsunviroidae* (Hadidi et al. 2003)

Features	***Pospiviroidae***	***Avsunviroidae***
Secondary structure	Rod-like	Quasi rod-like or branched
Conserved sequence regions	Present (CCR, and TCH or TCR)	Absent
Replication site	Nucleus	Chloroplast
Replication pathway	Asymmetric cyclus	Symmetric cyclus
Cleavage	No self-cleavage domain	Self-cleavage via hammerhead structures

CCR: central conserved region, TCH: Terminal conserved hairpin, TCR: Terminal conserved region.

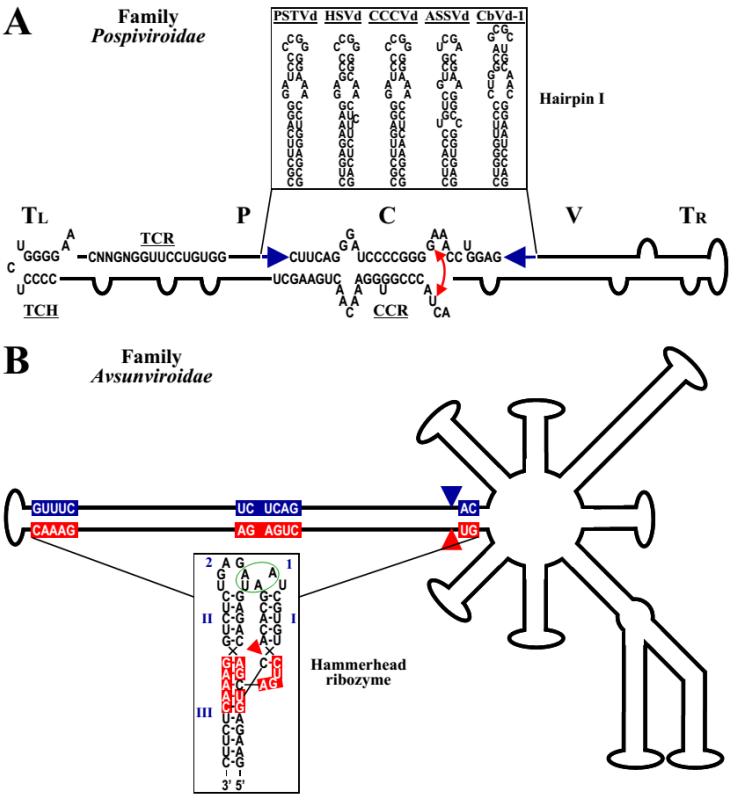

Figure 3 Genome structure of viroids (Verhoeven 2010). (A) rod-like secondary structure of the family *Pospiviroidae* with the central (C), pathogenic (P), variable (V), and terminal left and right (TL and TR, respectively) domains. CCR: central conserved region, TCR: terminal conserved region, TCH: terminal conserved hairpin. (B) Structure of branched RNA with hammerhead ribozyme of family *Avsunviroidae*: I, II and III: helices, loops 1 and 2, respectively.

3.2 Classification

According to three criteria: secondary structure, central conserved regions and replication features as illustrated in table 1 above, the 40 viroid species are classified into 2 families: *Pospiviroidae* and *Avsunviroidae* (table 3) (Verhoeven 2010).

The family *Pospiviroidae* is divided into 5 genera including *Pospiviroid, Hostuviroid, Cocadviroid, Apscaviroid and Coleviroid*, which are distinguished based on the type of CCR in the CD and the presence of either TCR or TCH in the TLD (Flores et al. 2004). In contrast, the family *Avsunviroidae* is divided into only three genera, *Pelamoviroid* with 2 species, *Avsunviroid* and *Elaviroid* with only one species each.

Table 2 Classification of viroids (Verhoeven 2010)

Avsunviroidae	
Avsunviroid	*Avocado sunblotch viroid*
Elaviroid	*Eggplant latent viroid*
Pelamoviroid	*Chrysanthemum chlorotic mottle viroid*
	Peach latent mosaic viroid
Pospiviroidae	
Apscaviroid	*Apple dimple fruit viroid*
	Apple fruit crinkle viroid (tentative species in the genus)
	Apple scar skin viroid (= dapple apple viroid; Japanese pear fruit dimple viroid; pear rusty skin viroid)
	Australian grapevine viroid
	Citrus bent leaf viroid
	Citrus viroid III
	Citrus viroid V (tentative species in the genus)
	Citrus viroid original source (tentative species in the genus)
	Grapevine yellow speckle viroid 1
	Grapevine yellow speckle viroid 2
	Grapevine yellow speckle viroid 3 (tentative species)
	Pear blister canker viroid
	Persimmon viroid (tentative species in the genus)
Cocadviroid	*Coconut cadang-cadang viroid*
	Coconut tinangaja viroid
	Citrus bark cracking viroid (=Citrus viroid IV)
	Hop latent viroid
Coleviroid	*Coleus blumei viroid I*
	Coleus blumei viroid II
	Coleus blumei viroid III
	Coleus blumei viroid IV (tentative species in the genus)
	Coleus blumei viroid V (tentative species in the genus)
	Coleus blumei viroid VI (tentative species in the genus)
Hostuviroid	*Hop stunt viroid(= cucumber pale fruit viroid; Citrus cachexia viroid)*
Pospiviroid	*Chrysanthemum stunt viroid*
	Citrus exocortis viroid (= Indian tomato bunchy top virus)
	Columnea latent viroid
	Iresine viroid 1
	Mexican papita viroid
	Pepper chat fruit viroid (tentative species in the genus)
	Potato spindle tuber viroid
	Tomato apical stunt viroid
	Tomato chlorotic dwarf viroid
	Tomato planta macho viroid

4. Viroid replication

The differences in genome structure of two viroid families led to the differences in their replication process, including replication sites, pathway of replication, replication enzymes and the RNA motifs involved in replication.

4.1 Replication sites

Experiments using conventional cell fractionation methods based on differential centrifugation, Diener (1971) demonstrated that PSTVd, type species of family *Pospiviroidae*, accumulates in the nucleus. On the contrary, ASVd, type species of family *Avsunviroidae*, was detected to predominantly accumulate in the chloroplast. In other experiments using in situ hybridization with confocal laser scanning and transmission electron microscopy to localize the viroid complementary strands after replication, it was detected that viroids replicate in the same organelles where they predominantly accumulate (Diener 1987, Navarro et al. 2000, Hadidi et al. 2003).

4.2. Replication models

Generally, the viroid replication through an RNA-based rolling circular mechanism has three steps with some variations in each family: 1) synthesis of the oligomeric strand by reiterative transcription of the initial circular template RNAs, 2) cleavage of the oligomeric into unit-length monomers, and 3) circularization of unit length monomers (Flores *et al*. 2009).

In more detail, the members of *Pospiviroidae* replicate via an asymmetric pathway of the rolling-circle mechanism whereas the *Avsunviroidae* replicate via a symmetric pathway (Fig. 4). For the *Pospiviroidae*, the (+) circular RNA is reiteratively transcribed by an RNA-dependent RNA polymerase into oligomeric, linear (-) strand RNAs and subsequently (+) strand RNAs. Then these oligomeric strands are properly cleaved and ligated into circular RNA via an RNase and an RNA ligase, respectively. Conversely, within the family *Avsunviroidae*, the initial monomeric (-) circular RNA is transcribed into oligomeric linear (-) strand RNAs, which are cleaved into unit-length monomers and then ligated into circular (-) RNA. Thereafter, the circular (-) RNA serves as a template for oligomeric, linear (+) strand RNAs synthesis, which subsequently are cleaved and circularized into unit-length monomers (Branch and Robertson 1984).

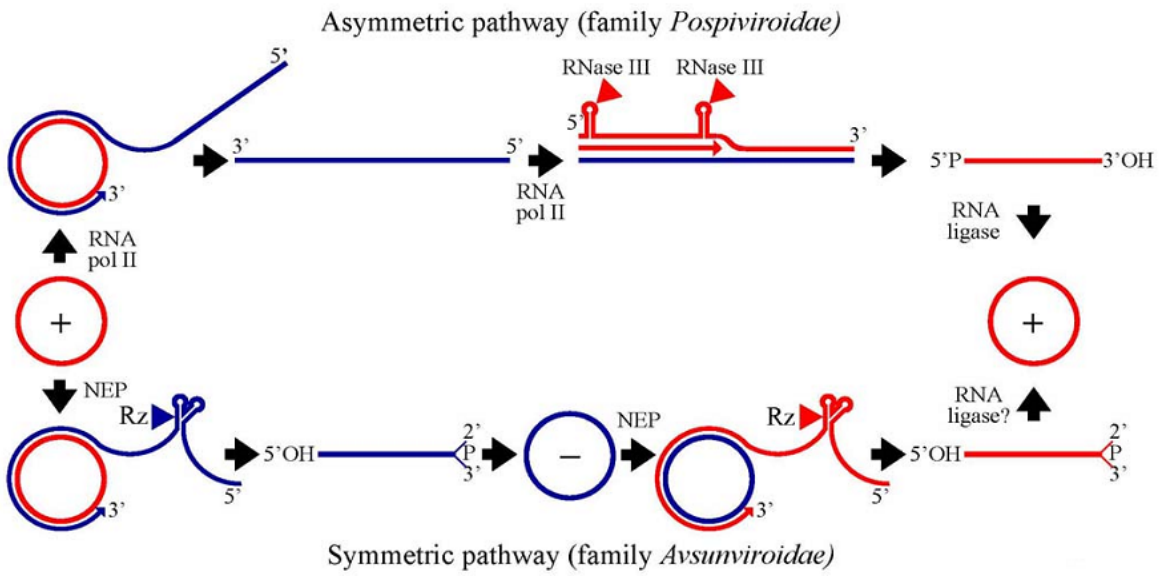

Figure 4 Asymmetric and symmetric rolling-circle replication pathways of members of the family *Pospiviroidae* and *Avsunviroidae*, respectively. Red and blue lines stand for (+) and (-) strands, respectively. RNA pol II is nuclear polymerase RNA II, NEP is nuclear-encoded chloroplastic RNA polymerase, Rz: Hammerhead ribozymes (Branch and Robertson 1984).

4.3. Riboenzyme and related enzymes

For the members of the *Pospiviroidae* family replicating in the nucleus-, nuclear DNA-dependent RNA polymerase II is supposed to be involved in the transcription process of viroid replication by catalyzing the elongation of PSTVd and closely-related viroids (Schindler and Mühlbach 1992). Within the members of family *Avsunviroidae* replicating in chloroplasts, two different DNA dependent RNA polymerases in plastids, the plastid-encoded polymerase (PEP) and the single–unit nuclear encoded polymerase (NEP), have been reported to involve in the transcription of the viroid replication process (Navarro et al 2000, Flores et al. 2009).

In terms of the cleavage and ligation process, different enzymes are involved. Within the family *Pospiviroidae*, the cleavage of oligomeric (+) strands is most likely mediated by a member of the RNase III family. A novel RNA ligase is meanwhile proposed to be involved in the ligation of monomeric linear (+) strands (Flores et al. 2009). The members of the family *Avsunviroidae* do not need any host enzymes to catalyze the cleavage, because they form hammerhead structures, functioning as ribozymes, i.e. self-cleaving RNase activities. However, it is unclear if the circularization process of monomers is effected by autocatalysis or mediated by a chloroplastic RNA ligase (Flores et al. 2009).

5. Movement of viroids

In order to establish systemic infection, viroid RNAs must penetrate into host cells by wounding, followed by intracellular movement to the replication site (chloroplast or nucleus). After replication, these viroids RNAs must traffic from initially infected host cell to neighboring cells and from organ to organ to infect the whole plant. Due to the fact that viroids are non-coding autonomously replicating RNAs, these infectious RNAs have to interact with its host components to accomplish functions ranging from replication and systemic movement (Hadidi et al. 2003, Flores et al. 2004, Verhoeven 2010).

5.1 Intracellular movement

Intracellular movement of viroids from the initially penetrated sites to the replication sites still remain poorly understood. After penetration into the host plants, viroids RNAs must enter either nucleus or chloroplast in order to replicate and exit these organelles after replication. The viroid RNAs may have the signals that they can directly enter into or exit from replication sites by changes of permeability of the membranes (Verhoeven 2010). Furthermore, special motifs of viroid RNAs may also contribute to the entry or exit of selective organelles (Ding and Itaya 2007).

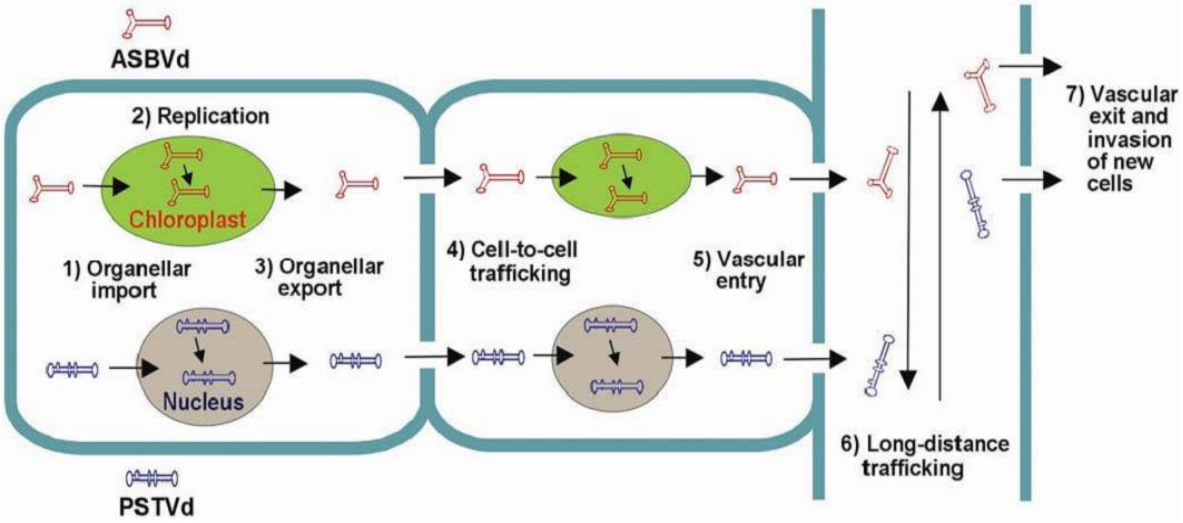

Figure 5 Systemic infection process of ASBVd and PSTVd (Ding and Itaya 2007)

5.2 Cell to cell movement

Similarly to plant viruses, viroids move from initially infected cells to neighboring cells via plasmodesmata (Pd) (Zhu et al. 2001, Hadidi et al. 2003, Takeda and Ding 2009). In an

experiment using micro-injection to inject infectious PSTVd RNAs labeled with a specific fluorescent dye (TOTO-1 iodide) into guard- and mesophyll cells, it is observed that PSTVd RNAs moved rapidly from cell to cell when injected in mesophyll cells. They concluded that PSTVd moves from cell to cell via Pds, but not through plasma membranes or cell walls (Ding et al. 1997). Another experiment also using micro injection methods showed that PSTVd is able to be transported via Pds when PSTVd was fused into non-mobile RNA transcripts. It is assumed that PSTVd may possess certain RNA motifs which are able to mediate the transport via Pds. However, the mechanism of this movement is still not well-understood. PSTVd is assumed to "piggyback" on cellular proteins or interact with Pd components for transport from cells to cells (Itaya et al. 1997).

5.3 Long-distance movement

Although both, viruses and viroids use long distance transport via sieve tubes and sieve elements, the trafficking principles of viroid RNAs are different from that of viruses. In the light of simplicity of viroid genomes, they have to interact with host cellular components in long-distance trafficking to infect the whole plant. Zhu and coworkers (2001) reported that long distance traffic of PSTVd through the phloem was into sink but not source leaves like viruses. Moreover, during the movement, PSTVd replicates actively in phloem parenchyma cells. PSTVd may exit sieve elements, enter to the phloem parenchyma and companion cells for replication, and then re-enter to sieve elements for further transport (Zhu et al. 2001). Some cellular proteins have been proposed to involve in viroid movement. Cucumber phloem protein 2 may interact with RNAs of *Hop stunt viroid* (HSVd) and facilitate the systemic movement of HSVd (Owens et al. 2001). In addition, two proteins extracted from melon phloem sap, CmmLec17 (17kDa) and an uncharacterized protein of approximately 14 kDa, have been shown to have RNA binding capacity and were proposed to facilitate long-distance movement of viroids (Gomez et al. 2005). However, evidence on the role of these proteins in long-distance viroid trafficking need to be confirmed. Based on the above available information, it has to be concluded that knowledge on movement of viroids in plants is still incomplete.

6. Transmission and pathogenesis

6.1 Transmission

Viroids can be transmitted in many different ways: by vegetative propagation, by mechanical transmission, by seeds and pollen, and by insects. Among these transmission ways, vegetative propagation is considered to be responsible for most of the spread of viroid infections. Especially, when “mother plants” remain asymptomatic which is often in case in ornamental plants, the risk of viroid spread will dramatically increase (Verhoeven 2010).

Mechanical transmission including foliar contact, contaminated fingers, pruning shears, grafting, fruit harvesting and many other hand-on activities have been proved as the second important means of viroid spread (Bonde and Merriam 1951; Matsushita et al. 2009; Verhoeven 2010; Li et al. 2015). This kind of transmission frequently happens under greenhouse conditions. Once a viroid is established under glass, the spread of the disease will occur quickly, based on many hand-on activities, through contaminated tools and cultivation equipment or by natural contact between plants (Seigner et al. 2008, Verhoeven 2010, Li et al. 2015). In the case of asymptomatic infection of ornamental plants, the risk of either accidental transmission to many crops during the practical activities or use for propagation will be catastrophic (Matsushita et al. 2008, Owens et al. 2009, Verhoeven 2010).

The spread of viroids through seed and pollen has been reported in many viroid species (Singh 1970, Singh et al. 1992, Singh and Dilworth 2009). The rate of transmission is quite variable depending on host plants and viroid species. This means of transmission may raise the risk of spreading viroid diseases worldwide via international trade of horticultural plants (Matsushita and Tsuda 2016).

Finally, the indirect transmissibility of viroids through insect vectors has been reported and may play an important role in the long-distance spread of viroid diseases. PSTVd was reported to be transmitted non-persistently via the aphid *Macrosiphum euphorbiae* with low frequency (Schumann et al. 1980, De Bokx and Prion 1981), and persistently by *Myzus persicae* (Sulzer) with the assistance of *Potato leafroll virus* (PLRV) as a shuttle virus (Salazar et al. 1995, Syller et al. 1997, Singh and Kurz 1997, Syller and Marczewski 2001). Besides aphid vectors, bumble bees have been reported to transmit TASVd and TCDVd (Matsuura et al. 2010). However, the

transmission is thought to occur by mechanical transmission through wounding of flowers by insect claws or result from pollination (Verhoeven 2010).

6.2 Pathogenesis

Viroid pathogenicity is considered as a complex process, resulting from a combination of interactions between specific structures of viroid RNAs and host components. In addition, environmental conditions may play important roles in viroid pathogenicity, especially the influence of temperature. Besides those factors, RNA silencing has been proven to be involved in the responses of host plants to the infection of viroids.

6.2.1 Viroid RNAs structure

A model of five structural and functional domains for PSTVd and related viroids (family *Pospiviroidae*) is proposed (Keese and Symons 1985). Many of the studies subsequently carried out to identify structural motifs modulating viroid pathogenicity. Schnolzer et al. (1985) showed that the substitution of some nucleotides in the pathogenic domain of PSTVd could alter the host responses from mild to severe expression. However, following investigations show that viroid pathogenicity becomes complicated and unpredictable when pathogenicity determinants are restricted not only to the PD, but also to other domains. Visvader and Symons (1985) suggested that expression of CEVd-induced symptoms may be influenced by sites of nucleotides within either PD or VD. Furthermore, sequence changes in the TL had a greater effect on pathogenicity of CEVd and TASVd than those in the PD (Sano et al. 1992). In the case of *Coconut cadang cadang viroid* (CCCVd), sequence mutations in the pathogenic domain and CD were proposed to influence symptom expression (Rodriguez and Randles 1993).

The knowledge about pathogenicity for members of the family *Avsunviroidae* which replicate in the chloroplasts and do not contain 5 structural domains in its rod-like secondary structure, is still very fragmentary. Investigation on CChMVd, De la Peña et al. (1999) showed that the variation of a single hairpin loop was able to convert a symptomatic strain into an asymptomatic one. PLMVd and its variants containing a 12-13 nt insertion in the hairpin loop exhibit severe symptom, so called “peach calico”, while the variants lacking this insertion remain symptomless (Malfitano et al. 2003).

6.2.2 Host components

The interaction between viroid RNA and cellular proteins has been assumed to trigger viroid pathogenesis. Wolff et al (1985) demonstrated that PSTVd RNA interacted with many different proteins in tomato including four histones and two other proteins approximately 31 kDa and 41 kDa in size. Riesner et al (1984) reported that DNA dependent RNA polymerase II (Pol II) purified from wheat interacted with both PSTVd terminal loops. Gomez and Pallas (2001) also gave evidence of the binding of HSVd to phloem protein 2 of cucumber. The binding of a chloroplast protein in avocado to ASBVd RNA in vivo has been proven to facilitate hammerhead self-cleavage (Daro and Flores 2002).

Besides interaction with host proteins, the sequence homology between viroid RNA and host genome has been proposed to play a role in symptom development of viroid diseases. The partial homology between lower strand of CD of PSTVd and mammalian U1 RNA (Diener 1987) and between sequence of lower strands of the PD and CD of PSTVd and the 7S RNA from tomato (Haas et al. 1988) was demonstrated. The interaction among these homologous structures was supposed to interfere with pre-rRNA processing or with the formation of signal recognition particles (Diener 2001).

6.2.3 Environmental conditions

The symptomatology of viroid diseases has been proven to be influenced by environmental conditions, especially higher temperatures. Marton et al (1982) found that CEVd infected tomato cells grew and survived to an optimum at temperatures above 30°C. In addition, Škorić et al (2001) showed that a mild CEVd strain expressed no symptoms under glasshouse conditions (at 24°C); however, it expressed severe symptoms when the infected plants were grown at 40°C. Severe symptoms were reversed to symptomlessness when the plants were grown again under glasshouse conditions with lower temperatures.

6.2.4 RNA silencing

Many studies have shown that RNA silencing is considered to be involved in viroid symptom expression (Barba and Hadidi 2009, Flores et al. 2005, Parisi et al. 2010, Navarro et al. 2012). RNA silencing or RNAi (RNA interference) is an important mechanism of gene regulation, which naturally takes place in different organisms, including protozoa, fungi, plants and

animals. Viroid replication in infected host plants is accompanied by the synthesis of small interfering RNAs (siRNAs), derived from both, the plus and minus strands, which are supposedly combined into the RNA-induced silencing complex (RISC) of host RNA silencing machineries (Papaefthimiou et al. 2001, Itaya et al. 2007). However, viroid siRNAs could escape from this immunity mechanism through many ways such as cellular localization, compact secondary structure of viroid RNA and high branching (Parisi et al. 2010). As a result, RISC may not target viroids in its place of replication and storage. The study of Hill and Lukiw (2014) indicated that siRNAs of viroid and micro RNAs (miRNAs), have similarity in structure, processing, and biological function. miRNAs possess 18–25 nucleotides, as well as also have siRNAs, and are non-coding single stranded RNAs. They are the smallest known carriers of highly selective genetic regulatory information in plants and animals. Therefore, the accumulation of viroid siRNAs in infected host plants could give rise to inconceivable adverse effects on certain normal host gene regulation by miRNAs in a sequence-specific manner, which may result in viroid pathogenicity (Sano et al. 2010).

7. The objectives of the thesis

Although viroids are only RNA molecules with a few hundred nucleotides, they cause huge economic losses in many important crops. There have been many studies on viroid pathogenicity; however, most of them have been focused on PSTVd. TCDVd is closely related but different from PSTVd, with 95-99% sequence homology (Singh et al. 1999). Because of the sequence homology, TCDVd is expected to exhibit similarities in pathogenicity to PSTVd. However, certain position changes or substitutions of single nucleotides in the sequence of viroids are able to cause changes in their pathogenicity (Wassenegger et al. 1996). Recently, TCDVd has been found worldwide in many *solanaceous* plants, and is asymptomatic in ornamental host plants (Ling et al. 2009, Viršcek Marn and Mavric Pleško 2010; Candresse et al. 2010, Fox et al. 2013) posing potential risk of further spreading this epidemic in wider areas. Hence, more investigations on practical aspects and modes of spread of the relatively new pathogen TCDVd is a must in order to contribute to the understanding of this species.

The specific objectives of the study were, to:

1) Investigate other possibilities of mechanical transmission of PSTVd and TCDVd by diverse practical greenhouse activities.

2) Determine the infectivity of PSTVd and TCDVd-infected plant material, stored under different temperature conditions.

3) Investigate the transmissibility of TCDVd via the vector *M. persicae* with assistance of *Potato leaf roll virus* as a shuttle under different growth temperature conditions.

4). Determine the correlation between macroscopic symptoms and cytopathic effects in TCDVd, PLRV and mixed infection (TCDVd+PLRV) in tomato tissues.

5) Evaluate the effectiveness of some chemical disinfectants against TCDVd-infection in tomato under glass.

Chapter 2: Mechanical transmission of *Pospiviroids* onto ornamental and *Solanaceous* plants

1 Introduction

Pospiviroids can be transmitted in different ways: by vegetative propagation, by mechanical transmission, by seed and pollen, and by insects (chapter 1, page 13). Among these ways of transmission, vegetative propagation is considered to be responsible for most of the spread of PSTVd in potato and ornamental plants (Verhoeven 2010). Besides, it has been proven that mechanical transmission plays as well an important role in the spread of *Pospiviroids*. This modality of transmission occurs easily by contact between infected and healthy plants and many hand-on activities of humans through contaminated tools and cultivation equipment (Seigner et al. 2008, Verhoeven 2010, Li et al. 2015). Especially, in some ornamental host plants, infection remains asymptomatic increasing the risk of accidental transmission to other crops or playing a role as inoculum source for vegetative propagation (Matsushita et al. 2008; Owens et al. 2009; Verhoeven 2010). The transmissibility of *Pospiviroids* via seed and pollen has also been reported for some viroid species for instance for PSTVd (Singh 1970; Singh et al. 1992), TASVd (Antignus et al. 2007), and TCDVd (Singh and Dilworth 2009). However, the rate of seed transmission is quite variable, depending on host plant- and viroid species. Finally, insects may also contribute to the spread of viroid diseases. PSTVd is reported to be transmitted non persistently via the aphid vector *Macrosiphum euphorbiae* with low frequency (Schumann et al. 1980; De Bokx and Piron 1981) and persistently by the aphid *M. persicae* (Sulzer) with PLRV as shuttle virus (Salazar et al. 1995; Syller et al. 1997; Singh and Kurz 1997; Syller and Marczewski 2001).

Although there have been studies about viroid transmission from plant to plant in tomatoes, the information about transmission of PSTVd and TCDVd in ornamental plants has been rare so far, especially for TCDVd. Asymptomatic TCDVd infection and cases of infection are reported in many locations, raising the potential of transmission of viroids in a wider area. In this chapter, the transmissibility of PSTVd and TCDVd from infected ornamental source (petunia) and tomato to brugmansia and tomato (S. *esculentum*) by different hand on

practices in the greenhouse was reported. Furthermore, the susceptibility of tomato seedlings for TCDVd at different developmental stages and leaf ages was also investigated in this study.

2. Materials and methods

2.1 Viroid sources and test plants

In this study, a mild PSTVd isolate from petunia and a severe TCDVd isolate from tomato (DSMZ-German collection of microorganisms and cell cultures, Braunschweig,) were used. PSTVd was maintained by propagating infected petunia plants. TCDVd was maintained in tomatoes by mechanical inoculation. Inoculum sources were kept under greenhouse conditions (23°C/18°C day/night temperatures and 16-hour photoperiod).

Figure 6 Viroid inoculum sources. a) PSTVd infected petunia without symptoms, (b) severely stunted TCDVd infected tomato, (c) PSTVd infected tomato

Tomato cv. Moneymaker and brugmansia (*Brugmansia suaveolens*) were chosen for transmission experiments. Brugmansia cuttings were propagated from healthy mother plants. When the seedlings had developed 3-4 true leaves, plants were transferred to plastic

pots with fertilized soil. All experimental plants were grown under greenhouse conditions as described above.

2.2 Transmission experiments

2.2.1 Mechanical transmission with cotton swabs

Inocula of PSTVd and TCDVd were produced by separately homogenizing infected tomato and petunia leaves in 0,01 M sodium potassium phosphate buffer pH 7.0 (1g leaf tissue/10ml buffer) with pestle and mortar. Then tomato-and brugmansia plants, which had been dusted with carborundum powder, were inoculated by slightly rubbing cotton swabs on the leaf surface. Subsequently, the inoculated plants were rinsed with tap water to remove carborundum and plant debris.

2.2.2 Mechanical transmission by contaminated razor blades

For demonstration of the infectivity of contaminated cutting tools, razor blades were disinfected with 96% ethanol and then contaminated with viroids by cutting into stems or petioles of infected tomato or petunia plants 8 to 10 times. For inoculation, groups of 5 plants of either tomato or brugmansia were cut 4 times consecutively into stems and leaves with contaminated razor blades. One razor blade was contaminated once and used to inoculate each group of 5 plants per inoculum. The inoculated plants were then grown under greenhouse conditions as described above.

2.2.3 Mechanical transmission by contaminated gloves

For the inoculation with contaminated gloves, only inoculum from TCDVd infected tomato was used. To acquire the viroid, young leaves of infected plants were rubbed between thumb, index and middle fingers of hands wearing rubber gloves. Contaminated gloves were then stripped and left under greenhouse conditions one minute, 10 minutes and two hours. For inoculation, groups of 5 tomato cv Moneymaker plants (3-4 true leaves) were rubbed slightly on non-carborundum dusted tomato leaves successively. One glove was used for only one group of 5 tomato plants.

All mechanical inoculation experiments were performed twice with 5 plants per repetition. The inoculated experimental plants were also kept in the greenhouse condition. Healthy tomato plants were used as negative controls.

2.2.4 Mechanical transmission of TCDVd by infected sap with different developmental stages and leaf ages

The mechanical transmission was carried out by rubbing TCDVd containing sap from infected tomatoes onto leaves of healthy tomato seedlings at different developmental stages and leaf ages, young or old leaves. Inoculum sap was prepared as described above.

When the tomato seedlings had 2-3 true leaves, each of them was transferred to 25x10cm plastic pots with fertilized soil. For a first inoculation, a group of 10 tomato seedlings at 7 days after emergence of the first true leaf were mechanically inoculated. Then other groups of 10 tomato seedlings at 21 days were inoculated by rubbing inoculum either onto the youngest (on top) or onto the oldest (lowest) leaves. The inoculation was carried out in the same way for tomato seedlings 35 days and 49 days. All inoculated plants were grown under greenhouse conditions. Four weeks after inoculation, the upper leaves of inoculated plants were collected and tested on viroid infection. With plants inoculated after 49 days, the testing was done additionally 6 weeks after inoculation.

2.2.5 Plant height and shoot fresh weight of tomato plants at different seedling-and leaf ages

In the experiment of mechanical transmission at different seedling ages and leaf ages, plant height and shoot fresh weight were measured for all tomato seedlings with all treatments at 2 months after development of the first true leaf. Mock inoculated plants underwent the same procedure as negative control. The data were analyzed by SPSS.

2.3. Sampling

For the experimental transmission by infected plant sap and contaminated gloves, the samples were collected 4 weeks after inoculation. Those inoculated by contaminated razor blades were collected 6 weeks after inoculation. The samples were transferred into sterile bags and stored at -80^{0}C until RNA extraction could be conducted.

2.4 Viroid detection

The examination of viroid infections was conducted by observing typical foliar symptoms of experimental plants 4 weeks after inoculation. The status of asymptomatic plants or plants with unclear symptoms was determined with one-step RT-PCR. For this purpose, pieces of

leaves were collected and immediately transferred into sterile extraction bags. The RNA extraction was done following the Qiagen (RNeasy Plant Mini Kit) instruction. The primers for RT-PCR were Pospi- and Vid-oligonucleotides designed by Verhoeven (2010):

Pospi Forward: GGGATCCCCGGGGAAA (16),
Pospi Reverse: AGCTTCAGTTGTYTCCACCGGGT (23),
Vid Forward: TTCCTCGGAACTAAACTCGTG (21)
Vid Reverse: CCAACTGCGGTTCCAAGGG (19),

A positive control of viroid infected tomato, negative control of non-viroid infected plant and non-template control (sterilized water) was included in the RT-PCR reaction.

3. Results

3.1 Mechanical transmission with three different inoculation methods

In these experiments, inocula of TCDVd from tomato and PSTVd from petunia to inoculate the tomato cv Moneymaker and/or brugmansia were used. As shown in table 3, all inoculated tomatoes became infected with PSTVd or TCDVd. TCDVd infected plants developed typical symptoms including stunted growth, short internodes, small and curly leaves 3-4 weeks after inoculation. While PSTVd infected tomato did not show clear symptoms 4 weeks after inoculation. The transmission of PSTVd to brugmansia (*B. suaveolens*) was less successful; only 5 out of 10 brugmansia plants showed infection after RT-PCR with very faint bands on electrophoresis gels. Moreover, the infected brugmansia plants remained asymptomatic.

For the transmission experiment using contaminated razor blades, the transmission rate was less successful compared to sap inoculation. All inoculated tomatoes were susceptible for TCDVd and expressed clearly visible symptoms 4 weeks after inoculation. However, only two of ten inoculated brugmansia were asymptomatically infected with TCDVd as tested by one step RT-PCR. The transmission of PSTVd by contaminated razor blades was less successful to tomato and brugmansia. While only 2 of 10 inoculated tomatoes showed infection with PSTVd, none of the brugmansias became infected.

For the transmission experiment using contaminated gloves after 1min, 10 min and 2 hours incubation time, the results showed that TCDVd-RNA was very stable under greenhouse

conditions. All the inoculated tomatoes showed infection and severe symptoms, even after a waiting time of two hours between contamination and inoculation.

Table 3 Mechanical transmission from tomato (TCDVd) and petunia (PSTVd)

Inoculum from tomato (TCDVd)					
Contaminated sap	Contaminated razor blades		Contaminated gloves		
Tomato	Tomato	Brugmansia	Tomato		
			1 min	10 min	2 hours
10/10*	10/10	2/10	10/10	10/10	10/10

Inoculum from petunia (PSTVd)			
Contaminated sap		Contaminated razor blades	
Tomato	Brugmansia	Tomato	Brugmansia
10/10	5/10	2/10	0/10

*: number of infected plants from 10, PSTVd and TCDVd infection was confirmed by typical foliage symptoms and one-step RT-PCR 4 weeks after inoculation.

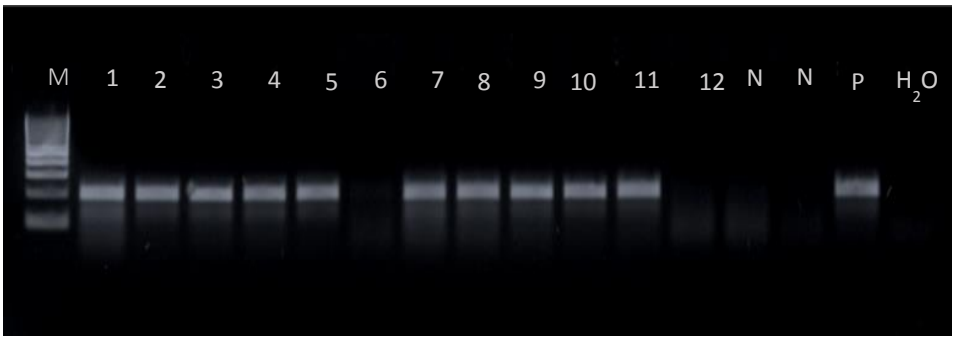

Figure 7 Detection of TCDVd transmission to tomato after RT-PCR on agarose gel electrophoresis. M: Marker, Lanes 1-5: tomato infected with TCDVd, Lanes 7-11: tomato infected with PSTVd, Lane 6, 12, 13, 14: healthy control, Lane 15: positive control, Lane 16: water control.

3.2 Mechanical transmission at different seedling ages and leaf maturity stages

The results of this experiment are shown in table 4. Tomato is very susceptible for TCDVd and can be considered as “indicator plant”. Although the treatment of 49 days old plants inoculated onto the young leaves resulted in only 2 infected-out of 5 plants 4 weeks after inoculation, all of them were TCDVd-positive after 6 weeks. The results of this experiment showed that tomato plants which were less than 35 days old (after emergence of the first true leaf at the time of inoculation), did neither influence TCDVd infection rate nor symptom development: All tomato plants that were less old than 35 days could be infected and expressed typical symptoms 4 weeks after inoculation. Tomatoes inoculated at 35 days, only

expressed symptoms when inoculated onto young leaves. From 49 days onward, regardless of the age of the inoculated leaf, the TCDVd infected tomatoes remained symptomless, even 6 weeks after inoculation, but infection could be detected by RT-PCR.

Table 4 Inoculation experiments with TCDVd on tomato at different seedling ages and leaf maturity stages

Plant-and leaf age at inoculation	4 weeks after inoculation		6 weeks after inoculation	
	Symptomatic[1]	Infected[2]	Symptomatic[1]	Infected[2]
7-day-old plant first true leaves	10/10	10/10	-	-
21-day-old plant young leaves	10/10	10/10	-	-
21-day-old plant old leaves	10/10	10/10	-	-
35-day-old plant young leaves	10/10	10/10	-	-
35-day-old plant old leaves	0/10	10/10	-	-
49-day-old plant young leaves	0/5	5/5	0/5	5/5
49-day-old plant old leaves	0/5	2/5	0/5	5/5

[1]: Number of symptomatic plants/total number of inoculated plants.

[2]: Number of infected plants/total number of inoculated plants. "–": not tested

3.3 Plant height and shoot weight of tomato plants after inoculation at different seedling and leaf ages

In general, there is a high correlation between plant height and shoot fresh weight of TCDVd infected tomato plants at different seedling ages post inoculation (Fig. 8). The tomato plants that were inoculated at 7, 21 and 35 days onto young leaves produced visible symptoms and significantly shorter shoots as well as lower biomass than healthy controls. However, there were no noticeable differences between healthy plants and inoculated asymptomatic tomato plants, when inoculation was done at day 35 onto old leaves and at day 49 on both young and old leaves. In particular, tomato plants inoculated at day 7 were only about 21cm in height and less than 35 g in fresh weight after 2 month post inoculation, whereas healthy control plants were 110cm in height and weighed about 350g in biomass. Within the same seedling ages, the treatments at 21 days and 49 days showed no statistical differences in plant height and plant fresh weight when the plants were inoculated either onto young or old leaves (Fig. 8). Only the treatment at 35 days showed significant differences between inoculation onto young and old leaves. These results demonstrate that TCDVd infection with foliage symptoms significantly affects growth and biomass production of tomato.

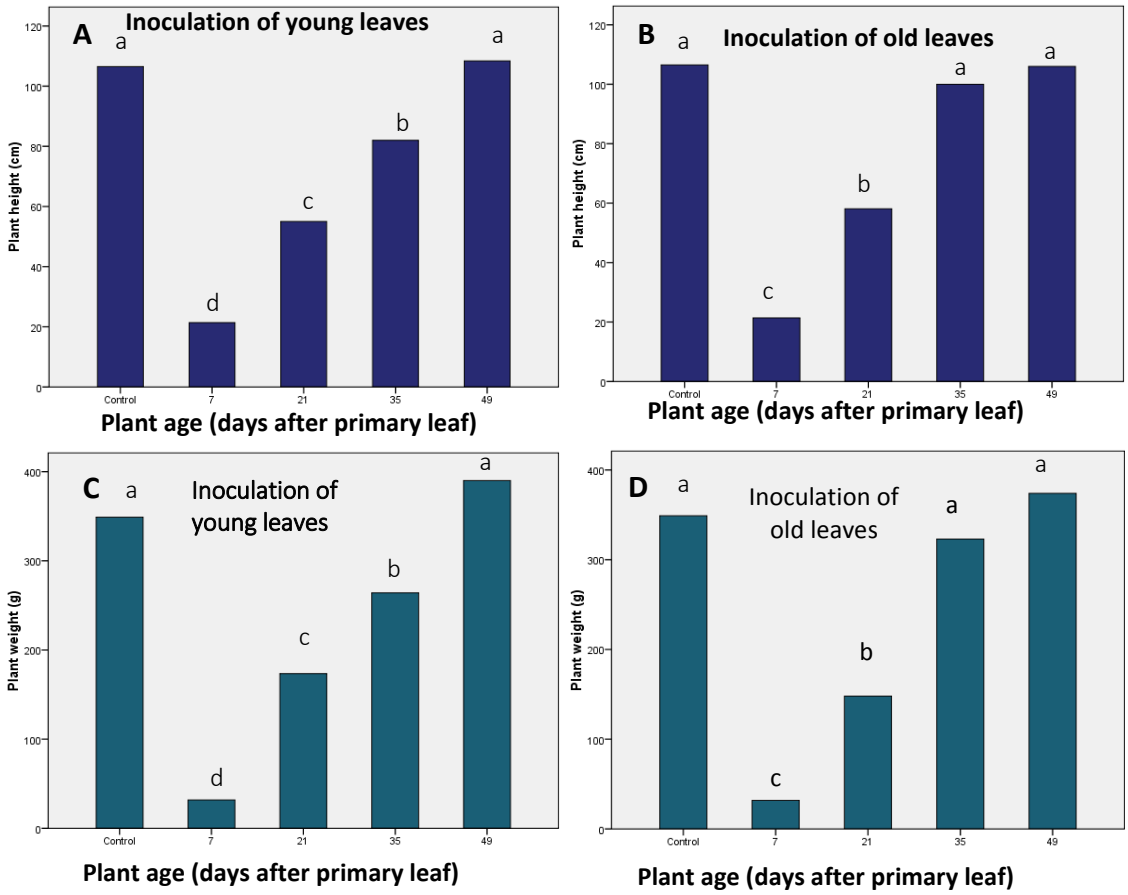

Figure 8 Comparison of plant height and shoot fresh weight after inoculation of tomato plants with TCDVd at different seedling ages (7, 21, 35 and 49 days after emerging of cotyledon leaves) and leaf maturity in comparison with healthy control. A, C: plant height and shoot fresh weight of tomato after inoculation of young leaves; B, D: plant height and shoot fresh weight of tomato after inoculation of old leaves.

Figure 9 Symptoms of TCDVd on tomato: a) stunted, bunchy top, reduced chlorotic and necrotic leaves, without fruits 2 months after inoculation; b) comparison between tomatoes inoculated at 7 days (on the left) and mock inoculated tomato; c) visible symptoms and asymptomatic tomatoes at different seedling-and leaf ages at time of inoculation (1: 7 days; 2a: 21 days-young leaf, 2b: 21 days-old leaf; 3a: 35 days-young leaf, 3b: 35 days-old leaf; 4: healthy tomato).

4. Discussion

Previous studies have reported the mechanical transmissibility of viroids. Up to now, most trials have been focused on PSTVd using *solanaceous* ornamental plants as inoculum source for transmission (Seigner et al. 2008, Verhoeven et al. 2010). In this investigation, inoculum prepared with a severe TCDVd strain and a mild PSTVd strain to inoculate tomato and brugmansia via hand on activities in the greenhouse were applied. The results showed that these two *Pospiviroids* can be transmitted easily to other *solanaceous* species. However, the rate of transmission varied depending on viroid-strain and -species in the inoculum. The transmissibility of the severe TCDVd isolate from tomato was higher than that of the mild PSTVd isolate. In the transmission experiment using razor blades, only 2 of 10 tomatoes became infected with PSTVd and none of the brugmansias showed infection. Tomato was the more susceptible host for both viroids than brugmansia. Moreover, brugmansia could only be infected with contaminated razor blades to 20% with TCDVd and not at all with PSTVd. It is supposed that unknown leaf components of brugmansia (Verhoeven 2010) or gene silencing (Flores et al. 2005) might inhibit the viroid infection.

The infectivity of PSTVd RNAs has been reported. The rate of transmission of potato spindle tuber disease was reported over 80% to potato in the field when tractor wheels distributed the infectious sap of this potato disease (Merriam and Bonde 1954). In another investigation, the infectivity of PSTVd remained over latency periods of two hours on fingertips (Verhoeven 2010). By applying PSTVd infected tomato leaves to eight common surfaces (cotton, wood, rubber, tires, leather, metal, plastic, human skin and string), Mackie et al. (2015) showed that PSTVd remained infective for 24 hours on all surfaces, but only for 30 minutes on human skin. The differences of the reports on PSTVd infectivity experiments could be due to different viroid titers, source plants for infective sap or temperature and air moisture. In our study, we found that the infectivity of TCDVd remained for at least 2 hours on contaminated gloves in the greenhouse. This highlights the risk of spreading TCDVd from tomato to other *solanaceous* plants in the greenhouse via crop handling.

Experiments on TCDVd transmission to tomato plants at different seedling ages and leaf ages revealed that the increase of plant-or leaf ages leads to decrease in symptom development of TCDVd in tomato. Actually, viroid pathogenicity has been proposed to result from the combination of many factors, including the specific structures of viroid RNAs, host

components, environmental conditions and the role of RNA silencing mechanism in host plants. Recently, various studies have shown that RNA silencing is considered to be involved in viroid symptom expression (Flores et al. 2005, Barba and Hadidi 2009, Parisi et al. 2010, Navarro et al. 2012) (as described at page 17).

Noticeably, in this study, the tomatoes which were less than 35 days old showed infection and clear foliage symptoms of TCDVd when they had been inoculated on either young or old leaves. When tomato plants are young and in the vegetative developmental stages, cell divisions happen exponentially to develop new leaves, roots and expand the length of the shoot (Tardieu and Granier 2000). Cell divisions make viroid replication escalate as well. Then the increase of siRNA will activate the gene silencing machinery in host plants and may result in symptom development. For old tomato (after 35 days old) TCDVd inoculation, the visible symptoms developed only when plants were inoculated onto the youngest leaves. In one investigation, Sano and Matsuura (2004) proposed that symptom expression may decrease in PSTVd-infected tomato plants in late infection stages. Due to the fact that in old tomato plants, cell divisions become less after some time this may lead to decrease of viroid replication.

In general, TCDVd and PSTVd can be transmitted successfully from petunia (ornamentals) and tomato to tomato and less well to brugmansia by infected sap, contaminated gloves and razor blades. Tomato is a very susceptible host for PSTVd and TCDVd and as such suitable as an indicator plant, whereas a transmission hardly succeeded on brugmansia. Our experiments showed that the increase of plant age corresponded with a decrease of disease symptoms. Asymptomatic plants represent a source for viroid spread. Furthermore, the high viroid stability and infectivity of contaminated plant remnants may represent a source of infection. The importance of phytosanitary treatments during crop handling and during plant cultivation was highlighted with these studies to prevent the spreading of viroids in practice.

Chapter 3: Transmission of *Tomato chlorotic dwarf viroid* by *Myzus persicae* assisted by *Potato leafroll virus*

1. Introduction

Potato leafroll virus (PLRV), genus *Polerovirus*, family *Luteoviridae* is a phloem-limited virus, being persistently transmitted only by aphid vectors, causing high yield losses on potato worldwide (Bagnall 1977; Casper 1988). PLRV has a rather narrow host range. Besides the main host potato, *Physalis floridana* and *Datura stramonium* are known indicator plants. On potato, PLRV can cause an abnormal accumulation of callose in the phloem and starch in chloroplasts, chlorosis, rolled and leathery leaves as well as plant stunting. However, the expression of symptom severity depends on host plant age, variety and environmental conditions (Faccioli et al. 1971; Bagnall 1977; Van der Zaag 1987).

TCDVd, similar to other *Pospiviroids*, can be transmitted easily by vegetative propagation, foliar contact, contaminated fingers, pruning shears during many hand-on activities. Seed transmission of TCDVd is also reported in petunia hybrids and tomato (Singh and Dilworth 2009; Matsushita and Tsuda 2016). While PSTVd is reported to be persistently transmitted by the aphid *M. persicae* (Sulzer) with PLRV as shuttle virus (Salazar et al. 1995; Querci et al. 1997; Singh and Kurz 1997; Syller and Marczewski 2001), the possibility of TCDVd transmission by aphid vectors has not been described up to now. The cases of TCDVd infection reported worldwide pose potential risk of further spreading this pathogen. Hence, investigation on virus-assisted aphid transmission of TCDVd is a “must” as an additional step towards its control.

2. Materials and methods

2.1 Aphids and test plants

A non-viruliferous clone of *M. persicae* (Sulzer) was fed and maintained on cabbage (*Brassica oleracea*) in cages. Apterous (wingless) aphids were used for transmission experiments.

Tomato cv. Moneymaker was chosen for the experiments. The tomato seeds were first sown in trays and when the seedlings had developed 2-3 true leaves, plants were transferred to

25x10 cm plastic pots with fertilized soil and maintained in a greenhouse until conducting the transmission experiments.

2.2 Virus and viroid sources

Potato (*Solanum tuberosum* L.) was inoculated with PLRV by *M. persicae* transmission. The plants were tested by tissue print immunoassay (TPIA) to confirm the infection and maintained in a growth chamber at 20-22^{0}C.

Inoculum of a severe TCDVd isolate from tomato (DSMZ, Braunschweig, Germany) was maintained in tomato under greenhouse conditions (23°C/18°C day/night temperatures and 16-hours photoperiod).

In order to obtain the mixed PLRV+TCDVd inoculum, young tomato plants (3-4 true leaves) were inoculated with a severe isolate of TCDVd by rubbing with cotton swabs and carborundum as abrasive. Immediately after inoculation with TCDVd, those tomatoes were exposed to PLRV-carrying *M. persicae* for 2 days, followed by spraying with a contact insecticide. Four weeks after inoculation, the plants were examined for infection by TPIA for PLRV and by one-step reverse transcriptase polymerase chain reaction (RT-PCR) for TCDVd.

2.3 Aphid transmission experiments

The experiments were performed in a growth chamber at 25^{0}C (pre-inoculation temperature) with a 16 hour-photoperiod. These aphids were exposed to doubly infected (TCDVd+PLRV) or singly TCDVd infected plants for 3 days to acquire the pathogens. Groups of TCDVd+PLRV carrying aphids were transferred to tomato seedlings (15-20 aphids per seedling) in the 2-3 leaf stage. Three days after inoculation, the aphids were killed by spraying them with a contact insecticide. Thereafter, the inoculated tomatoes were grown under two different temperature conditions, one in the growth chamber at 20-22°C and the other in the greenhouse during summer time from April 2016 till July 2016 (average temperature of 26-28°C). This experiment was done with 20 tomato plants per treatment (each growth temperature condition).

In addition, serial passages of TCDVd+PLRV into host plants (tomato) and non-host plants cabbage (*Brassica oleracea*) were carried out in order to exclude non-persistent TCDVd aphid-transmission. After three days acquisition of TCDVd/PLRV the aphids were transferred to 5

young tomato seedlings for a 2 days inoculation access period and continuously moved to other 2 groups of tomato seedlings with the same procedure before being killed with a contact insecticide. For the serial passages of virus and viroid into non-host plants, the TCDVd+PLRV carrying aphids were placed on host plants (tomato) for 2 days, and then transferred to (non-host) cabbage for 2 days. Thereafter, these aphids were again transferred to host plants (tomato) for 2 days, and then dismissed with a contact insecticide.

2.4 Tissue print immunoassay (TPIA)

TPIA was carried out to confirm PLRV infection. Nitrocellulose membrane sheets (Whatman Optitran BA-S 85 with 45 nm pore size) were cut to an appropriate size and a grid of 1x1 cm squares was drawn with a soft pencil and a ruler. PLRV infected potato and tomato, PLRV/TCDVd infected tomato (5 weeks after inoculation) and the mock inoculated tomato plants were dissected with new razor blades at stems and internodes. Cross sections of stem and midrib of these samples were printed onto each square of the nitrocellulose membrane. The membrane was blocked with polyvinyl alcohol 0,005% in PBS (Sigma Aldrich, MW 30.000-70.000) for 5 minutes, then rinsed three times for 5 minutes in PBST (phosphate-buffered saline with Tween 20) and incubated for 1 hour in conjugate buffer (PBST+2% Polyvinylpyrrolidone K25, Carl Roth GmbH) containing a 1:1000 (v/v) dilution of PLRV monoclonal antibody (DSMZ) for 1 hour. The membranes were rinsed 3 times in PBST and then incubated in conjugate buffer containing a 1:1000 (v/v) dilution of anti-mouse immunoglobulin-alkaline phosphatase conjugate for 1 hour. After rinsing in PBST three times for 5 minutes, membranes were incubated in substrate solution for 15 minutes. The substrate solution was prepared by dissolving NBT/BCIP tablets (Sigma-Aldrich) in distilled water.

2.5 TCDVd detection by one-step reverse transcriptase polymerase chain reaction (RT-PCR)

The examination of viroid infections was conducted by observing typical foliar symptoms of experimental plants 4-5 weeks after inoculation. The status of asymptomatic plants or plants with unclear symptoms was determined with one-step RT-PCR. For this purpose, pieces of leaves were collected and immediately transferred into sterile extraction bags. The RNA extraction was done following the Qiagen (RNeasy Plant Mini Kit) instruction. The primers for RT-PCR were Pospi- and Vid-oligonucleotides designed by Verhoeven (2010). A positive

control of viroid infected tomato, negative control of non-viroid infected plant and non-template control sterilized water was included in the RT-PCR reaction.

3. Results

3.1 Frequency of TCDVd transmission through *M. persicae* assisted by PLRV in mixed infection

In this study, the transmissibility of TCDVd by *M. persicae* using doubly infected (TCDV+PLRV) tomato served as inoculum sources to tomato host plants under two different growth temperature conditions, at 26-28°C in the greenhouse and at 20-22°C in a growth chamber. The results are illustrated in table 5. The efficiency of PLRV assisted TCDVd transmission by *M. persicae* was generally low. Five weeks after inoculation, the efficiency of TCDVd transmission by aphids at 26-28°C was higher than that at 20-22°C, which was characterized by 25% infection rate compared to that of 5%, respectively. The TCDVd transmission from singly TCDVd infected tomato by aphids did not occur, however, it was 100% for PLRV transmission.

Table 5 Transmission by *M. persicae* from doubly (TCDVd+PLRV) and singly TCDVd infected tomato plants to tomato under two growth temperature conditions (20 plants per treatment)

Temperature condition	Sources of inocula		
	PLRV	TCDVd	TCDVd+PLRV
Transmission rate at 26-28°C	100%*	0%	25%
Transmission rate at 20-22°C	100%	0%	5%

*: rate of infection, PLRV infection is confirmed by TPIA (Fig. 11), TCDVd infection is confirmed by one step RT-PCR (Fig. 10)

3.2 TCDVd transmission rate after serial passages into host and non-host plants

TCDVd+PLRV carrying aphids were passed over host plants (tomato) and non-host plants (cabbage, *Brassica oleracea*) to exclude the possibility of non-circulative transmission of TCDVd by aphids. The efficiency of TCDVd transmission by aphids from mixed infected plants after three serial passages into host plant tomato seedlings was 33% (table 6). When a group of non-host plants replaced the host plants in the second passage, still 20% of inoculated

tomatoes in the third passage showed infection with TCDVd. 100% of inoculated tomato showed infection with PLRV by aphids, irrespective if host plants or non-host plants were passed. However, no test plants were infected when aphids had previously sucked on plants with TCDVd alone. Thus, it can be concluded that TCDVd is transmitted by *M. persicae* only when PLRV is present.

Table 6 PLRV and TCDVd infection rate of tomato plants by *M. persicae* from TCDVd/PLRV mixed infection to tomato after passages virus/viroid into tomato plants and cabbage (5 plants per treatment)

Infection of inoculated tomato plants	Host plant passages			Non-host plant passage		
	1st tomato	2nd tomato	3rd tomato	1st tomato	2nd cabbage	3rd tomato
PLRV infection	100%*	100%	100%	100%	/	100%
TCDVd infection	0%	0%	0%	0%	/	0%
PLRV/TCDVd infection	20%	40%	40%	40%	/	20%

1st, 2nd, 3rd: first, second and third passages of tomato plants, *: infection rate, PLRV infection is confirmed by TPIA, TCDVd infection is confirmed by one step RT-PCR.

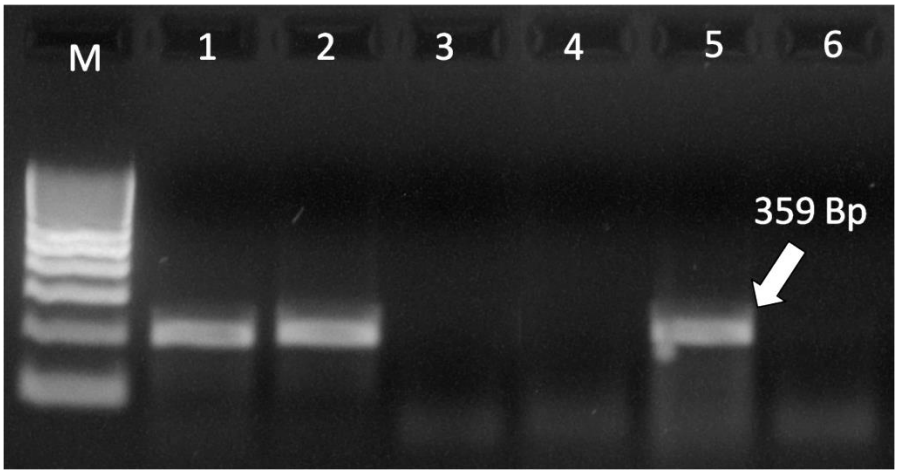

Figure 10 Detection of TCDVd infection on tomato after RT-PCR on agarose gel, M: Marker, lane 1: TCDVd, lane 2: TCDVd/PLRV, lane 3: PLRV, lanes 4, 5, 6: Healthy, positive and water control, respectively.

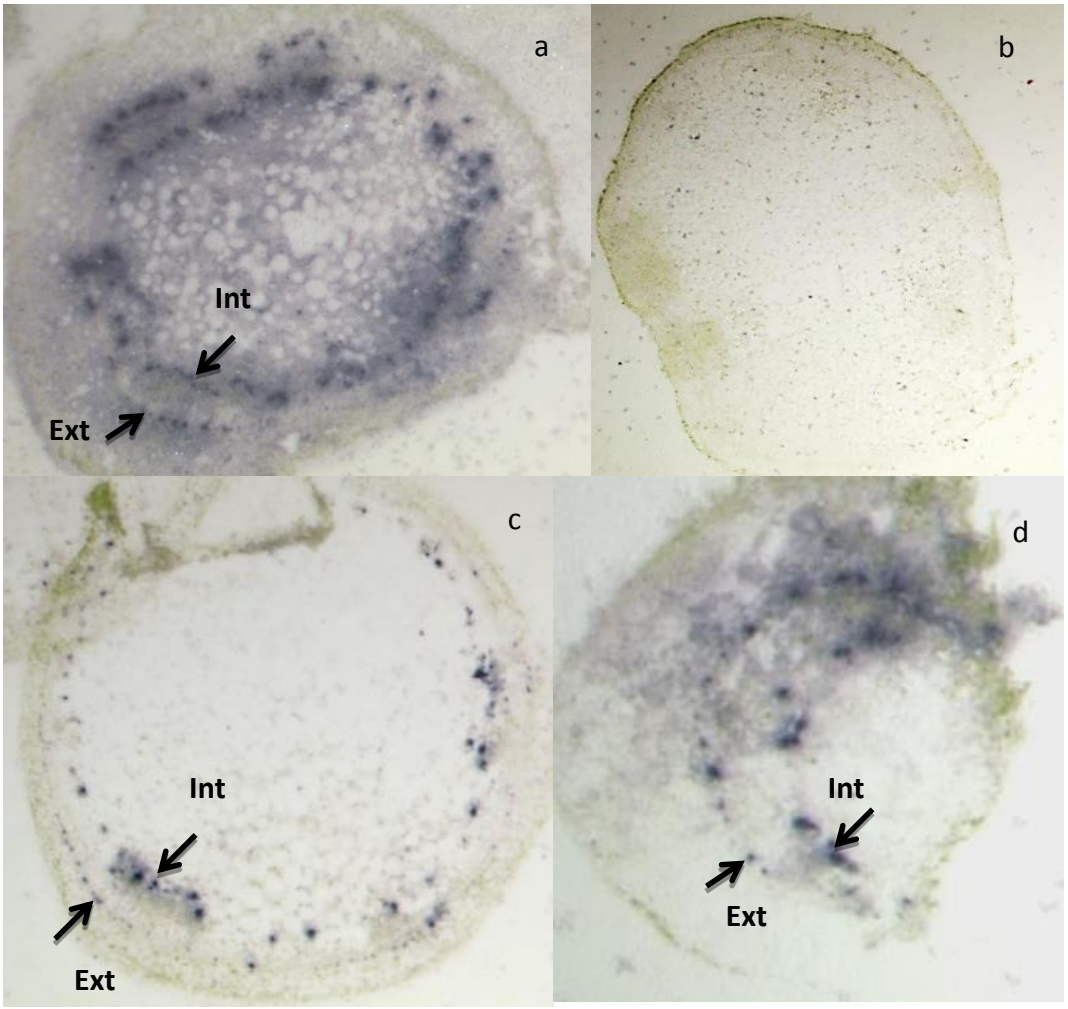

Figure 11 Immunoprints of stem tissue from plants systemically infected with PLRV a) infected potato, b) non-infected tomato, c) infected tomato, main stem, d) infected tomato, petiole. PLRV particles were visualized in phloem cells, staining dark blue with BCIP-NBT. Arrows indicate internal (Int) and external (Ext) phloem

3.3 Different symptoms of tomato after infection with PLRV, TCDVd and mixed infection with PLRV and TCDVd

The typical symptoms of PLRV infected potatoes are stunted growth, yellow or discolored lower leaves as well as upward rolled leaflets (Bagnall 1977; Van der Zaag 1987; Casper 1988). In this investigation, the responses of potato to PLRV infection were typical as described above (Fig.12a). In contrast, the responses of tomato to PLRV infection under greenhouse conditions were entirely different from that of potato, expressing a rather long latency period and weak or no symptoms 4 weeks after inoculation, although the infection was confirmed by TPIA. PLRV induced symptoms on tomato, developed not earlier than 6-8 weeks after inoculation. Symptoms could be described as downward turning of petioles, dwarfing, and

necrosis beginning from the central veins of leaf blades (Fig. 12b). The leaves became stiff and leathery. More severely, the upper leaves showed veinal net necrosis, developing into veinal browning, finally leading to death of the whole leaves of infected plants (Fig. 12c).

Figure 12 Symptoms of PLRV on potato and tomato a) stunted growth and upward rolling leaflets, petioles turn dark violet on potato, b) necrosis of leaflet veins and c) entire leaves on tomato, leading to death of the whole plants

The responses of tomato to TCDVd include growth reduction, stunting, curling or bunchy tops. The results of this study showed that the foliar symptoms of TCDVd infection on tomato developed faster at 26-28°C than those under lower growth temperatures at 20-22°C. At higher temperature, severe symptoms such as reduced stem growth, reduced leaves, short internodes and bunchy tops were observed beginning 3 weeks after inoculation. Moreover, no distinguishable symptoms were found between mixed PLRV+TCDVd infection on tomato plants and single TCDVd infections 3-4 weeks after inoculation (Fig. 13a and 13b). However, obvious differences could be observed not earlier than 6 to 8 weeks after inoculation. While TCDVd infected tomatoes were stunted, without fruit development, and finally dying, mixed infected TCDVd/PLRV tomatoes developed new sprouts consisting of hard, stunted shoots and leaves (Fig. 13c).

Figure 13 Comparison of symptom development between singly TCDVd infected-and doubly infected tomato plants. a) TCDVd infection 4 weeks after inoculation b) doubly infected TCDVd+PLRV tomato 4 weeks after aphid transmission c) Tips with new outgrowth of doubly infected tomato 8 weeks after aphid transmission

4 Discussion

4.1. PLRV assisted transmission of TCDVd by aphids

Previous studies have reported the successful transmissibility of PSTVd by M. *persicae* when the inoculum sources were doubly infected with PLRV and PSTVd. Up to now different transmission experiments have revealed varying transmission rates mostly from doubly infected PSTVd+PLRV potato inoculum sources to potato or to *Physalis floridana*. The transmission rates of PSTVd in PLRV infested fields in China were correlated with the severity of a PLRV infection (Querci et al. 1997). Viroid transmission by aphids did not occur from singly viroid infected inoculum sources. The transmission efficiency was variable according to post-inoculation temperatures and susceptibility of cultivars to PLRV (Salazar et al. 1995; Querci et al. 1997, Syller et al. 1997; Singh and Kurz 1997; Syller and Marczewski 2001). However, PLRV assisted transmissibility of TCDVd by aphids has never been described up to now. In this study, we found that the transmission rate was higher when the inoculated tomatoes were grown at 26-28°C than at 20-22°C. These findings confirm that post-inoculation temperature influences virus and viroid transmissibility. In fact, viroid transmission with assistance of vectors is a mutual interaction of several components such as species of vectors, availability of virus and viroid in the source inoculum, susceptibility of host plants, and environmental conditions. The effect of pre-inoculation (acquisition period) and post-inoculation (replication period) temperature on rate of infection and host susceptibility for PLRV by aphid transmission was proven (Singh et al. 1988; Chung et al, 2016). The optimum temperature for the acquisition period of PLRV was 25°C (Chung et al. 2016) and that for post-inoculation was between 25-30°C (Singh et al. 1988). Likewise, the rate of viroid infection was enhanced at higher temperatures (20-25°C) as compared to that at 15°C (Verhoeven 2010). In addition, the rate of viroid transmission by help of PLRV via aphids is much dependent on the availability of virus and viroid in the source inoculum. PSTVd was acquired by aphids after 3 days feeding on doubly infected plants ranging from 30-39% at 20-25°C during the acquisition period (Singh and Kurz 1997). The rate of encapsidation of PSTVd molecules into PLRV virions was estimated very low with one PSTVd molecule per 3000 to 5000 PLRV particles (Querci et al. 1997). Hence, with such a low percentage of viroids in the shuttle virus, a low level of viroid

transmission is not astonishing, particularly when the temperature has not been optimal for viroid replication.

Among the suggested possibilities concerning transmission mechanisms, trans-encapsidation of viroids into virions is the dominant hypothesis. Support for this was given by Singh and Kurz (1997) by using 3µg/ml RNAse to treat purified PLRV from singly-and doubly-infected *P. floridana*. By amplification products with RT-PCR, both PLRV and PSTVd were only detected from doubly-infected samples, whereas purified PSTVd alone was eliminated by treatment with the same amount of RNAse. Similarly, Querci et al. (1997) confirmed that PSTVd was encapsidated into PLRV virions and being protected from micrococcal nuclease treatment. If PSTVd would be externally associated with the virions, i.e. not encapsidated into virions, it should be destroyed by the action of ribonucleases inside the aphids. In order to be transmissible by aphids, PSTVd has to be uptaken into the aphid, pass through the gut walls, circulate in the hemolymph and exit through the salivary glands and salivary duct, as PLRV does (Gray and Gildow 2003). Moreover, the results of the transmission experiments by *M. persicae* on tomato, *Physalis* sp. and *Nicotiana benthamiana* (i.e. 100 aphids per plant) showed that no transmission occurred 3 weeks after inoculation (Van Bogaert et al. 2015). This implicates that viroid RNAs are probably degraded by RNAse activity inside aphids. Other experiments have also confirmed that no viroid transmission by aphid vectors from singly viroid infected sources takes place (Salazar et al. 1995; Querci et al. 1997; Singh and Kurz 1997; Syller and Marczewski 2001). Experiments with six common insect pests of tomato could neither transmit viroids (Schumann et al. 1980). In addition to the results of serial passages of virus and viroid into host- and non-host plants, we exclude the possibility of non-persistent TCDVd transmission by PLRV.

4.2 Plant reactions to infection with PLRV, TCDVd and mixed infection

The responses of tomato to PLRV infection are described in this study. The late severe foliar symptom development in tomato can be explained by a relatively slow virus replication in tomato phloem cells. The necrotic symptoms may correlate with an increased susceptibility of infected tissues to other stresses such as high temperature and water deficiency. PLRV can cause an abnormal accumulation of callose in sieve tubes, apparently due to the impaired translocation of carbohydrates (Faccioli et al. 1971). The metabolic changes can be expressed

as interveinal chlorosis, rolled and leathery leaves as well as stunting, being more severely expressed under high temperature conditions (Faccioli et al. 1971; Bagnall 1977; Van der Zaag 1987).

The responses of a mixed infection TCDVd+PLRV in tomato are for the first time described in this study. Generally, no differences were found between doubly infected tomato and singly TCDVd infected tomato 3-4 weeks after inoculation. However, obvious differences were seen in later stages of infection (6-8 weeks after inoculation) as new outgrowth with dwarf shoots. Viroid pathogenesis has been proposed to result from combination of factors like interaction between viroid RNA and host components, which is likely influenced by external conditions, especially temperature (Hadidi et al. 2003; Verhoeven 2010). In this investigation, we proved that TCDVd induced symptom expression is much dependent on growth temperature. Vector inoculated tomatoes with mixed inocula expressed the same foliar symptoms as mechanically TCDVd inoculated plants 3-4 weeks after inoculation when the plants were grown under high temperatures in the greenhouse.

5. Conclusion

The transmissibility of TCDVd via *M. persicae* with assistance of PLRV is herewith confirmed for the first time. High temperatures promote the rate of PLRV assisted aphid transmission. An important role of aphids as vectors in viroid transmission should neither be denied nor underestimated. Although the efficiency of aphid transmission is low, the risk of PLRV-assisted aphid transmission of TCDVd to other *solanaceous* crops seems to be likely.

Chapter 4: Stability of viroid RNAs and evaluation of disinfectants to reduce the spread of *tomato chlorotic dwarf viroid* in the greenhouse

1. Introduction

Pospiviroids, family *Pospiviroidae,* have wide host ranges and cause huge economic losses in many high-valued crops in *solanaceous* plant species. They can be easily transmitted to other species by various means of transmission (as described in chapter 1 and 2).

PSTVd was reported to retain the infectivity for several months when freeze-dried in plant sap (Singh and Bagnall 1968) and up to 6 years in freeze-dried leaves stored at room temperature (Singh and Finnie 1977). The infectivity of PSTVd on many common surfaces such as human skin, leather, plastic, string, and gloves varied from 30 min to 24 hours (Merriam and Bonde 1954; Verhoeven 2010, Mackie et al. 2015). However, no investigation have been made on the infectivity of viroids in freshly infected materials over the storage time up to now.

Due to the imminent risk of viroid infection under glasshouse conditions, sanitation and disinfection are considered to be important measures in order to eradicate or at least minimize their spread to- and within crops. In recent years, several investigations have been done on inactivation of viroid infections, focusing mainly on PSTVd (Singh et al. 1989, Timmermann et al. 2000, Li et al. 2015). Some common chemical disinfectants (e.g sodium hypochlorite, sodium hydroxide, trisodium phosphate and formaldehyde) have shown effectiveness in preventing the widespread of PSTVd infections. However, investigations about chemical disinfectants in TCDVd inactivation have not been well-documented.

In this chapter, the infectivity of *Pospiviroids* (TCDVd and PSTVd) in freshly infected leaves under different storage times and different temperature conditions is described. Moreover, the effectiveness of some chemical disinfectants against TCDVd infection on tomato under glasshouse condition was evaluated as well.

2 Materials and methods

2.1 Test plants and viroid source

For investigation of *Pospiviroid*- longevity, a mild PSTVd isolate from tomato and petunia and a severe TCDVd isolate from tomato were used in this study. Tomato plants were inoculated with PSTVd and TCDVd infected sap by means of mechanical transmission. PSTVd infected petunia was propagated by cuttings.

In disinfection experiments tomato cv. Moneymaker was used. Inoculum of a severe TCDVd isolate from tomato (DSMZ) was chosen for experiments. All infected plants were maintained in a greenhouse under controlled conditions as described.

2.2 Storage of infected plant materials under different temperature conditions

The infection was determined 4 weeks after inoculation by one-step RT-PCR. Then leaf disks of young leaves of each TCDVd or PSTVd infected plants were cut out with a 1 cm cork borer, being disinfected by alcohol flaming after each sample taking. The samples were divided into 4 portions (less than 100 µg per portion) for each temperature treatment (at room temperature 20°C, at 4°C and at -20°C). One of four samples was tested by one-step RT-PCR immediately. The others were stored and tested in intervals of 1-, 2- and 4 months after sampling. In addition, re-inoculation to susceptible plants (tomato) was done at the same time for testing viroid infectivity.

2.3 Disinfectants

Sodium hypochlorite (NaOCl)

NaOCl is a strong oxidant, has been reported to be very effective against virus and viroid dissemination (Singh et al. 1989, Matsuura et al. 2010, Oliver et al. 2015). In this study, NaOCl was tested in three different concentrations (3%, 1% and 0.5%). Moreover, with the lowest concentration of NaOCl (0.5%) a disinfection experiment was performed by two methods: rub- and cut-inoculation with different exposure times (10 sec, 1min, 5 min and 10 min) to sap inoculum.

Chlorine dioxide (ClO_2)

ClO_2 is also a strong oxidant, an alternative of NaOCl (Rav-Acha et al. 1985). Moreover, ClO_2 is less corrosive than ozone and more compatible with construction materials (Chauret et al. 2001). ClO_2 was tested at 4 concentrations in water (3ppm, 5ppm, 10ppm and 15ppm). The concentration of ClO_2 was calibrated by a DPD (N, N – diethyl-p-phenylenediamine) colorimetry method as described by the manufacturer´s instructions (Hach Company, Harp 1995). The mechanism of this method based on the reaction with N, N–diethyl-phenylenediamine to the extent of one fifth of its total available chlorine content, which corresponds to the conversion of ClO_2 to chlorite. A pink color is built, the intensity of which is proportional to the chlorine glycine concentration in the samples. The coloring then is measured with a photometer (Hach Company).

Sodium hydroxide (NaOH), sodium bicarbonate ($NaHCO_3$) and sodium carbonate (Na_2CO_3): These chemicals were tested at 0.5% concentration.

Alcohol 96% (C_2H_5OH) and flaming

Absolute alcohol and flaming is commonly used to disinfect contaminated metal tools in laboratories and greenhouses. In this study, 96% alcohol followed by flaming 2 to 3 sec was investigated for the inactivation of TCDVd.

Table 7 List of disinfectants and their application concentrations used to evaluate efficacy as disinfectants against TCDVd infection

Disinfectants	Concentrations	Stock solution
NaOCl	3%, 1%, 0.5%	12%
ClO_2	3ppm, 5ppm, 10ppm, 15ppm	3000ppm
NaOH	0.5% pH 13	1M
Na_2CO_3	0.5% pH11	1M
$NaHCO_3$	0.5% pH 8.15	1M
C_2H_5OH + flaming 2-3s	96%	96%

All chemicals solutions were filled in brown glass bottles and wrapped in aluminum foil after preparation in order to prevent decomposition from temperature and light. Then they were immediately used.

2.4 Preparation of viroid inocula and disinfectants

TCDVd inoculum was prepared by grinding severely symptomatic tomato leaves with buffer using pestle and mortar. Then the same volume of doubly concentrated disinfectants was mixed with the TCDVd inoculum to keep the final concentration of disinfectants in the mixtures as calculated.

2.5 TCDVd disinfection experiments

Rub-inoculation was used for most of the chemical disinfectants to inactivate TCDVd infection. Disinfectants with different concentrations were mixed with the prepared viroid inoculum sap and allowed to stand for 15 min. Inoculation process was conducted by using a cotton swab dipped into the mixtures of inoculum and disinfectants, and then gently rubbed onto the surfaces of carborundum dusted tomato leaves. Thereafter, the inoculated tomatoes were rinsed with tap water in order to remove plant debris and reduce the potentially toxic effect of disinfectants. Each treatment was repeated with 8 plantlets. Mock inoculated plants were used as negative control.

For demonstration of TCDVd inactivation by absolute alcohol following 2-3 seconds flaming, cut-inoculation with the use of new razor blades was conducted. Contaminated razor blades were produced by dipping them into inocula and subsequent air drying. These blades then were dipped into absolute alcohol and immediately flamed 2 to 3 seconds. After cooling, 5 to 10 slashes were made into stem or leaves of healthy plants with the flamed razor blades.

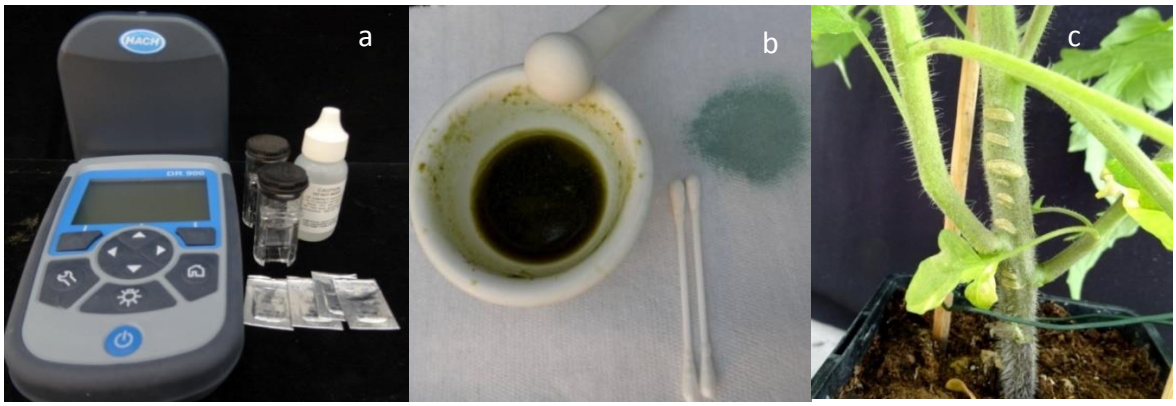

Figure 14 TCDVd disinfection by chemical disinfectants. a) DPD method with a photometer to calibrate the concentration of ClO_2, b) Rub-inoculation with cotton swabs and carborundum, c) Cut-inoculation into a stem of tomato plants by using razor blades.

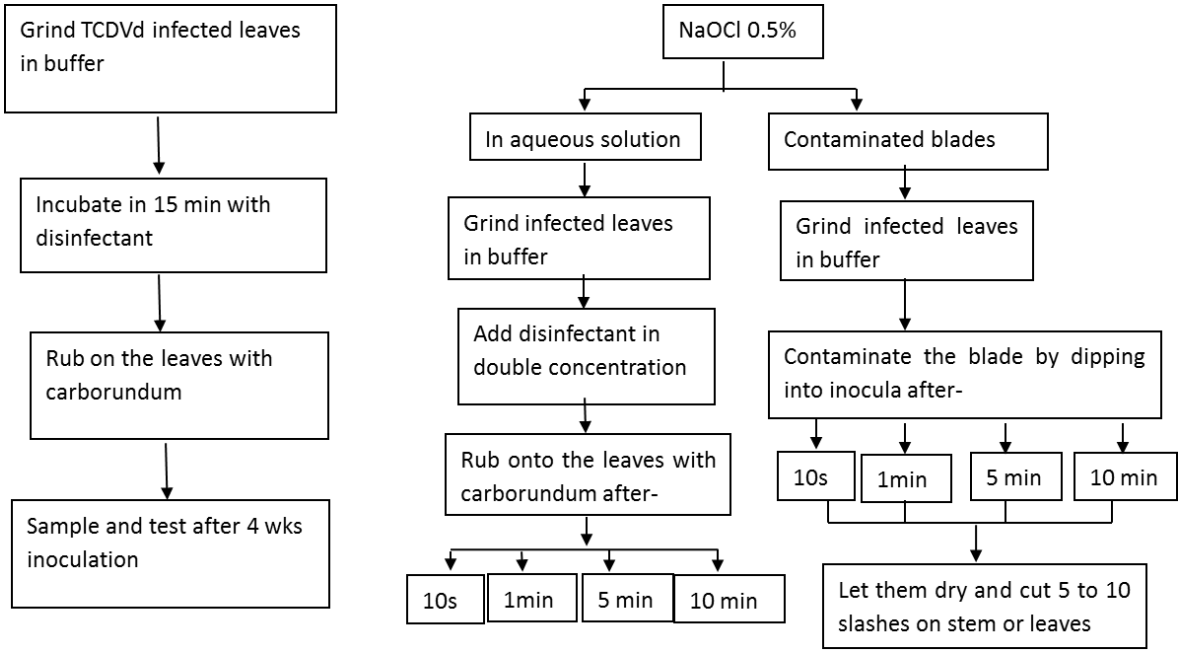

Figure 15 Diagram process of disinfection experiments applied for all experimental chemicals (left) and for NaOCl 0.5% by rub- and cut- inoculation (right).

In addition, in order to determine optimum contact time of a NaOCl solution needed to disinfect TCDVd on surfaces of contaminated tools and in aqueous solution, NaOCl at a concentration of 0.5% was used. Different contact times of 10 sec, 1 min, 5 min and 10 min were tested by cut-inoculation with razor blades and rub-inoculation with cotton swabs (as described above). Each treatment (contact time) was repeated with 5 tomato plants. Mock–inoculated plants served as negative controls, and inoculum without disinfectants supplied with the same amount of buffer was used as positive control.

2.6 Viroid detection

Viroid infection was confirmed by visible symptoms 4 weeks post inoculation. In case of asymptomatic or unclear symptoms, the infection was tested by using one step RT-PCR (as described in chapter 2).

3. Results

3.1 Stability of viroid RNAs under different storage conditions

The results of the infectivity test of PSTVd and TCDVd under different storage conditions (at room temperature 25°C, at 4°C and at -20°C) are shown in table 8. Basically both *Pospiviroids*

remained strongly infective at -20°C after 4 months storage. All of the infected leaves showed very clear viroid bands in agarose gel after RT-PCR (Figs. 17).

At 4°C, the viroid RNA infectivity remained strong up to 2 months of storage, decreasing gradually after that time. After 4 months storage at 4°C only 4 out of 5 samples of TCDVd infected tomato tissues showed viroid bands after RT-PCR, whereas 3 out of 5 and 1 out of 5 samples of PSTVd infected tomato and petunia, respectively, proved to be infectious. Noticeably, infected leaf tissues were partly degraded after 4 months storage. At room temperature the viroid infectivity was still strong after one month storage. However, after 4 months storage, 4 of 5 TCDVd infected tomato tissues remained infectious, as opposed to only 1 of 5 and 2 of 5 of PSTVd on tomato and petunia respectively. Leaf tissues became dried, developed a brown color and were infected with mold fungi.

Table 8 The infectivity of viroid RNA of a PSTVd isolate in petunia and a TCDVd isolate in tomato stored at room temperature and at low temperatures (+4°C and -20°C)

Viroid	TCDVd in tomato			PSTVd in tomato			PSTVd in petunia		
Storage temperature	RT	4°C	-20°C	RT	4°C	-20°C	RT	4°C	-20°C
After harvesting	5*	5	5	5	5	5	5	5	5
1 month	4	5	5	4	5	5	3	5	5
2 months	5	5	5	1	5	5	2	5	5
4 months	4	4	5	1	3	5	2	1	5

*: Amount of infected samples (out of 5 samples); RT: at room temperature.

The infectivity of the severe TCDVd isolate was obviously greater than that of the mild PSTVd isolate. All TCDVd infected leaves stored at room temperature (ca. 20°C) and at low temperatures (4°C and -20°C) were still very infectious, whereas the infectivity rate of PSTVd infected tomato leaves showed 100% only after -20°C storage. The infectivity of the mild PSTVd isolate was rather low at room temperature and at 4°C after 2 months of storage.

RNAs showed relatively high infectivity under different storage conditions. However, all strains were 100% infective after 4 months of storage at -20°C. The severe TCDVd isolate seemed to be more stable than the mild PSTVd isolate (after 4 months of storage).

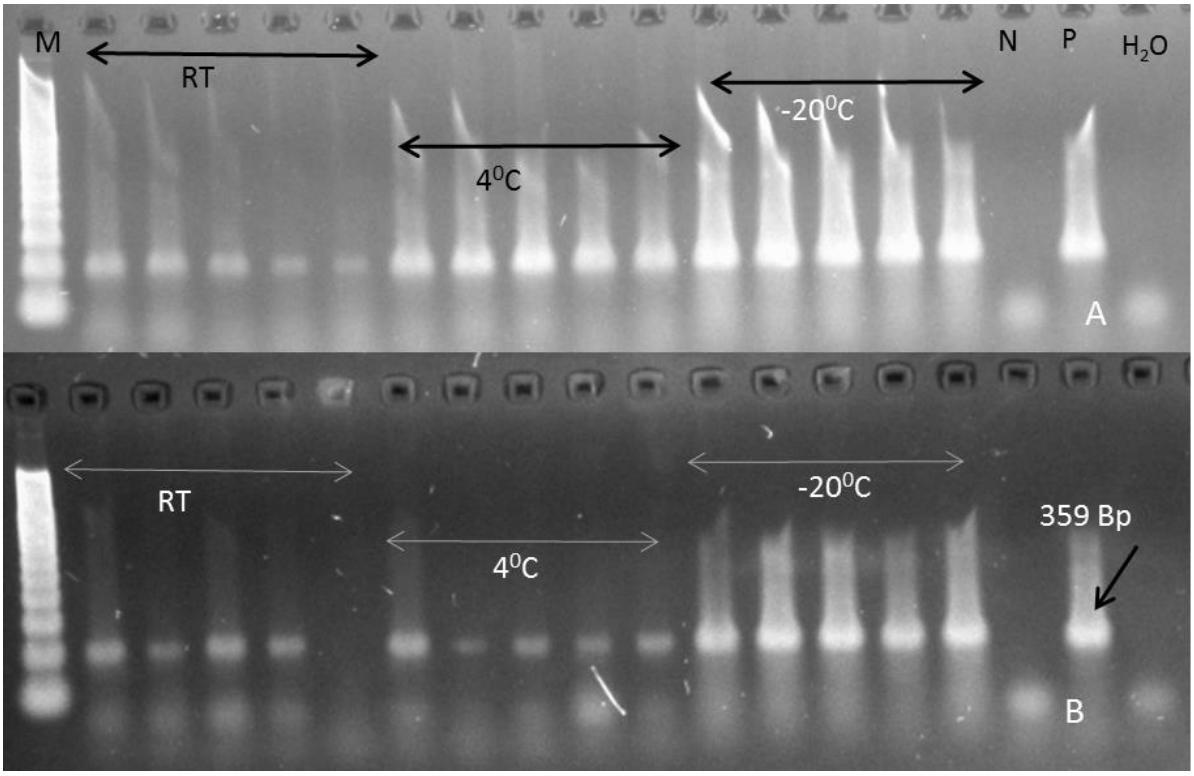

Figure 16 Detection of TCDVd infection in tomato on 2% agarose gel after RT-PCR. A) after 2 months, B) after 4 months storage.

3.2 Disinfection experiments with TCDVd with various chemicals

To evaluate the disinfective potential against TCDVd infection in tomato, six chemical disinfectants were investigated. Results of TCDVd disinfection experiments are shown in table 9. After evaluation, only three of those chemicals were able to completely deactivate TCDVd infectivity. TCDVd inocula in phosphate buffer were completely disinfected by addition of NaOCl (3%, 1% and 0.5%) and NaOH 0.5% (pH 13) for 15 min exposure time, although tomato plants suffered somewhat after inoculation. None of 8 inoculated tomato plants of each treatment expressed symptoms or showed viroid bands after RT-PCR amplification. Likewise, flaming with absolute alcohol showed an absolute effectiveness against TCDVd infection by cut-inoculation. All of the inoculated tomato plants that were treated with an adequate amount of water developed typical severe symptoms.

In this study, we found that the strong oxidant ClO_2 was not successful in disinfecting TCDVd by rub inoculation after 15 minutes exposure time. Although only 4 of 8 inoculated tomato plants developed typical symptoms after disinfection at concentrations of 10ppm and 15ppm after 15 min. incubation time, all of them showed infection with TCDVd 4 weeks post

inoculation. Similarly, Na_2CO_3 0.5% and $NaHCO_3$ 0.5% were not able to inactivate TCDVd infectivity in this investigation.

Table 9 Effectiveness of various solutions at different concentrations to deactivate TCDVd infectivity

Disinfectants	Concentration	Symptomatic[1]	Infected[2]
NaOCl	3%	0/8	0/8
	1%	0/8	0/8
	0.5%	0/8	0/8
ClO_2	3ppm	8/8	-
	5ppm	8/8	-
	10ppm	4/8	8/8
	15ppm	4/8	8/8
NaOH	0.5% pH 13	0/8	0/8
Na_2CO_3	0.5% pH11	5/8	8/8
$NaHCO_3$	0.5% pH 8.15	4/8	8/8
Alcohol + flaming 2-3s	96%	0/8	0/8

[1]Number of symptomatic plants from 8 inoculated plants, [2]: number of infected plants from 8 inoculated plants as detected by RT-PCR, ”-“not tested

3.3 The effect of 0.5% sodium hypochlorite after different exposure times

Results of the TCDVd disinfection experiment by using 0.5% NaOCl at four different exposure times (10 sec, 1 min, 5 min or 10 min) are shown in table 10. NaOCl 0.5% was able to completely disinfect TCDVd in a rub-inoculation experiment within the very short exposure time of 10 sec. None of the 20 experimental plants expressed symptoms or showed viroid bands after RT-PCR amplification in all of the 4 different exposure time treatments. Only one of five cut- inoculated tomatoes was infected with TCDVd after a treatment exposure time of 10 sec, whereas longer exposure times could completely deactivate TCDVd infection. This result indicates that the disinfective action of NaOCl 0.5% against TCDVd in aqueous solution was slightly more effective when applied in the inoculum than on the contaminated dried surfaces of razor blades.

Table 10 The effectiveness of a 0.5% NaOCl solution to deactivate TCDVd infectivity by rub- and cut-inoculation at different exposure times

Disinfectants	Exposure time	Symptomatic	Infected
NaOCl 0.5% in aqueous solution (rub-inoculation)	10 sec	0/5[1]	0/5[2]
	1 min	0/5	0/5
	5 min	0/5	0/5
	10 min	0/5	0/5
NaOCl 0.5% with contaminated razor blades (cut-inoculation)	10sec	1/5	1/5
	60 sec	0/5	0/5
	5 min	0/5	0/5
	10 min	0/5	0/5

[1]number of symptomatic plants from 8 inoculated plants, [2]: number of infected plants from 8 inoculated plants as determined by RT-PCR

4. Discussion

The infectivity of viroid RNAs in freeze-dried leaves or infected sap has been reported (Singh and Bagnall 1968, Singh and Finne 1977, Verhoeven 2010). In this study the stability of PSTVd and TCDVd in infected leaves in unsealed sterile plastic bags at room temperature, 4°C and -20°C were investigated. The results showed that both viroids (TCDVd and PSTVd) are very stable and remained infective after 4 months storage at -20°C, opposed to storage at room temperature and 4°C. The findings of this study provide relevant information for practical activities in greenhouses as well as storage of test samples for diagnostic purposes. The RNA degradation process was reported to minimize at low temperature (Levy and Miller 1998). Minus 20°C, however, is not sufficient to protect single stranded RNA from degradation over longer time. Thus the stability of viroids due to internal base pairing is a prerequisite for their infectivity. The activities of exogenous enzymes of bacteria and fungi at room temperature and 4°C conditions will lead to RNA degradation and reduce viroid RNAs infectivity over time, although drying out of the tissues had reduced their activity.

Sanitation and disinfection are two important measures to eradicate or at least minimize the spread of viroid diseases during handling. TCDVd has recently been detected worldwide, however, there is less information about it than for PSTVd. Hence, the evaluation of the effectiveness of some disinfectants against TCDVd infection on tomato was conducted in this study. The results here show that NaOCl at a concentration of 0.5% or more in 15 minutes incubation time completely deactivated TCDVd infectivity in tomato. This finding is in accordance with some investigations achieved from prior work. NaOCl at concentration of 3%

completely eliminated PSTVd infection from contaminated knives (Singh et al. 1989). Matsuura et al. (2010) also confirmed that NaOCl at a concentration of 0.5% or more was very effective in disinfecting TCDVd contaminated scalpels with 15 sec dipping. In 1971, Hayatsu et al. have reported that sodium hypochlorite reacts very fast with purines and pyrimidines and assumed that the process of inactivation of nucleic acid will also be rapid. The degradation of nucleic acid might derive from intensive and complicated oxidation processes, including chloramination. Hypochlorous acid (HOCl), an oxidizing agent present in NaOCl solution, reacts with purine and pyrimidine and promotes the replacing of hydrogen with chlorine, leading to decomposition of RNAs (Estrela et al. 2002).

Growers usually concern how fast a disinfectant could operate to inactivate pathogens. Some disinfectants work effectively against TCDVd; however, within a 15 minute reaction time they seem to be inappropriate for practical application. The results of experiments using NaOCl 0.5% at 4 different exposure times for disinfection of TCDVd infection showed that NaOCl 0.5% was very effective to disinfect viroid RNAs in aqueous solution, within 10 seconds exposure time. However, the disinfectivity of NaOCl 0.5% against TCDVd contaminated razor blades took a longer time than 10 sec. These contaminated blades might carry much plant tissue residues on surfaces. Thus a very short time could not be enough to completely destroy the infectivity of pathogen. Hence, the suggestion is that contaminated tools should be treated longer than 10 seconds to get the highest effectiveness.

In this study, chlorine dioxide at concentrations up to 15ppm was not effective against TCDVd infection with rub-inoculation at 15 min incubation time. ClO_2 is known as a strong oxidant, widely used as disinfectant against bacteria and viruses as an alternative of NaOCl disinfectants (Black and Veatch 2010). In one study, Hauchman et al. (1986) presented that chlorine dioxide could effectively inactivate bacteria and viruses and infectious RNA based on the reaction of chlorine dioxide with nucleotides. However, according to Noszticzius et al. (2013), chlorine dioxide is a rather selective oxidizer, and does not/or very slowly react with many compounds such as carbohydrates, lipids, nucleobases, and amines. This might lead to the reduction of the effectiveness of chlorine dioxide in solution.

Prior work confirmed that disinfectants with high pH value could accelerate the reaction with nucleic acid (Hauchman et al. 1986; Estrela et al. 2002). Similarly, Estrela et al. (2002) reported antimicrobial effectiveness of NaOCl, based on its high pH (hydroxyl ions). Sodium hydroxide

has also been shown to be effective in deactivating most viruses, bacteria, yeasts, and endotoxins (Healthcare 2009). Here a group of three chemicals with high pH, namely NaOH (pH 13), Na_2CO_3 (pH 11.5), and $NaHCO_3$ (pH 8.15) at a concentration of 0.5% were used to disinfect TCDVd infection. However, only NaOH showed effectiveness in TCDVd disinfection. This indicates that only a very high pH value might have a direct effect on the disinfective potential of chemicals for viroids.

Flaming with absolute alcohol 96% is an often applied disinfection method for cutting tools, used in practice to eliminate the spread of plant pathogens in greenhouses. Roistacher et al. (1969) proposed that flaming with absolute alcohol may not help against viroid infection when flaming was not successful to fully disinfect Citrus exocortis viroid (CEVd), at that time still considered to be a plant virus. Own results confirm that flaming of contaminated razor blades dipped in 96% alcohol completely inhibited TCDVd transmission by cut-inoculation. This method may not be practical in large scale application due to time consume; however, it may be useful for scientists in detection and handling the testing samples.

In conclusion, it is important to thoroughly clean up greenhouses between crop cycles and to disinfect tools in order to eradicate pathogens and to protect plants from e.g. viroid infection by crop handling activities (Olivier et al. 2015). The results in this study confirm that sodium hypochlorite was the most promising disinfectant against TCDVd in both aqueous solution and on contaminated handling tools in practical greenhouse work. Concentration of NaOCl 0.5% and an incubation time of 20 sec are sufficient to disinfect TCDVd on cutting tools and also in solution. Flaming of knive blades with alcohol is a comparably effective disinfection measure for heat resistant tools.

Chapter 5: Cytopathic effects in tomato leaves infected with PLRV, TCDVD and doubly infected with both pathogens

1. Introduction

Macroscopic symptoms induced by TCDVd, PLRV and doubly infected PLRV+TCDVd in tomato have been described in the previous chapter (chapter 3). As demonstrated, the foliar symptoms induced by PLRV in tomato are quite different from those in potato, expressing a rather long latency period or weak symptoms 4 weeks after inoculation. In contrast, the responses of TCDVd and double infection of PLRV and TCDVd induced in tomato cannot be clearly distinguished at 4 weeks after inoculation.

PLRV a phloem-limited virus, replicating in phloem companion cells of potato, can cause an abnormal accumulation of callose in the phloem (Bagnall, 1977; Casper, 1988). In contrast, TCDVd replicates in the nucleus with a rolling circle mechanism (Flores et al. 2009), moves from cell to cell through plasmodesmata and over long distance through phloem sieve tubes (Flores et al. 2004, Hull 2009, Biwas 2017), based on the interaction between viroid RNAs and host factors (Diener et al. 1972, Flores et al. 2005, Verhoeven 2010).

Ultrastructural alterations induced by viroid infection have been studied in some nucleus-replicating viroids such as *Citrus exocortis viroid* (CEVd) in *Gynura aurantiaca*, PSTVd in tomato, *Chrysanthemum stunt viroid* (CSVd) in chrysanthemum tissues (Semancik and Vanderwoude 1976, Wahn et al. 1980, Hari 1980, Rosenberg et al. 1985), as well as in chloroplast-replicating viroid species such as *Peach latent mosaic viroid* (PLMVd) in *Prunus persica* (Rodio et al. 2007). Cytopathic effects induced by TCDVd and PLRV as well as doubly infected tomato tissues have not been investigated up to now and will be described in this chapter.

2. Materials and methods

2.1 Inoculum sources and plant materials

Tomato leaves infected with a severe strain of TCDVd, as well as PLRV infected potato have all been obtained from DSMZ. A mixed infection of PLRV+TCDVd in tomato (Money Maker) was produced by mechanical inoculation with TCDVd followed by persistent aphid transmission with *Myzus persicae*.

2.2 Tissue preparation and fixation

Leaf tissue was collected from healthy, TCDVd-, PLRV- and doubly-infected tomato 4 weeks post inoculation. These leaf samples were cut into small pieces of 2x4 mm with a new razor blade. All subsequent infiltration steps were carried out in snap-on lid glasses incubated on a Pelco sample rotary mixer. The leaf pieces were immediately immersed in buffered fixative solution for at least 2 hours at room temperature. The fixative solution was prepared by mixing 8% formaldehyde (freshly prepared from paraformaldehyde), 8% glutaraldehyde and 0,005g calcium chloride with 0.2M cacodylic acid sodium salt trihydrate buffer, pH at 7.35 to end concentrations of 2% aldehyde (Karnovsky 1965). Subsequently, the leaf pieces were washed in cacodylic acid buffer 10 times for 10 min each, followed by post-fixation in 2% Osmium tetraoxide for 2 hours. Thereafter, they were rinsed in cacodylic acid buffer 10 times for 10-15 min each.

2.3 Dehydration and infiltration with resin and embedding in flat embedding moulds

The leaf pieces were continuously dehydrated in ethanol solution. Dehydration was performed in a graded series of ethanol concentrations to avoid the shrinkage of cell contents.

(1) Ethanol 15%
(2) Ethanol 30%
(3) Ethanol 50%
(4) Ethanol 70%
(5) Ethanol 80%
(6) Ethanol 90%

} for 10- 15 min each

(7) Ethanol 100 % (two changes) for 30 min each

(8) Propylene oxide 100% (two changes) for 10 – 15 min each

When alcohol dehydration was complete, propylene oxide was used as inter-medium to improve incubation of the leaf tissues with resin (Hawes 2012).

After dehydration, the tissues were infiltrated with different concentrations of Agar low viscosity resin to propylene oxide 1:3, 1:1, 3:1, and 1:0, consecutively. For each concentration, the infiltration process lasted at least 12 hours. Thereafter, each tissue sample was polymerized in 100% Agar low viscosity resin in flat embedding moulds at 60^0C for 8 to max.24 hours.

2.4 Sectioning and collecting sections

After polymerization, the tissue blocks were trimmed with a razor blade. Semi-thin sections (about 500nm) for observation by light microscopy and ultrathin sections (ca. 80 nm) for transmission electron microscopy (TEM) were cut on a Reichert Ultracut E Ultramicrotome. Semi-thin sections were cut with glass knives, transferred subsequently onto a drop of water on a glass slide, and dried for a few minutes on an electric hot plate at 70^0C. These sections were then stained with 0.5% toluidine blue in 0.01 M sodium tetraborate buffer (pH 7.4), followed by washing in tap water and distilled water to remove excess stain, and dried again on a hot plate at 70^0C. The slides containing tissue sections were placed in xylene for 5 min, mounted and sealed in Entellan rapid mounting medium (Merck), and then dried at room temperature in a fume cupboard before observation under a light microscope. Once the area to be studied had been accurately checked by light microscopy, tissue specimens of the same tissue block were ultrathin sectioned (thickness of 70-80 nm) with a Diatome® diamond knive and mounted on Pioloform filmed slot grids, dried and contrasted.

2.5 Contrasting of ultrathin sections

To observe the morphology of the cells, a two steps staining procedure using uranium and lead salts was performed (Reynolds 1963, Hawes 2012). The ultrathin sections were placed onto a copper or nickel slot grid. These grids were transferred onto drops of 2% uranyl acetate in deionized water for 3 min, followed by rinsing twice in distilled water, and then placed on drops of a lead citrate solution for 20 seconds under CO_2-free conditions. The grids were then rinsed twice in distilled water, followed by air-drying at room temperature before storing in a grid box.

The ultrathin sections were observed with a transmission electron microscope (EM 109 T Carl Zeiss, Oberkochen, Germany) and specimens were photographed with a 1 K -Frame transfer CCD camera (A.Tröndle, Moorenweis Germany).

3. Results

3.1. Cytopathic effects of TCDVd infected tomato tissues

Ultrastructural alterations of TCDVd-infected tomato leaf tissue 4 weeks after inoculation are demonstrated in figure 17. Disintegration of cell walls could be observed in many parenchyma cells, leading to release of all cell components into the extracellular spaces (Fig. 17a). A further alteration was the appearance of vesicular plasmalemmasomes, which are formed by invagination of the plasmalemma (Fig. 17b, c). In addition, the appearance of vesicles with a single- or multilayer membranous structure were also observed in the cytoplasm (Fig. 17d). In healthy tomato tissue, vesicular structures were seen as well. However, vesicles in infected tissues were more numerous, and round or elongated with multilayer membranes sometimes being concentric. A common pathological alteration in infected cells was malformation of grana and thylakoid membranes, often with wave-like appearance, causing misshaped or distorted chloroplasts (Fig. 17f, g).

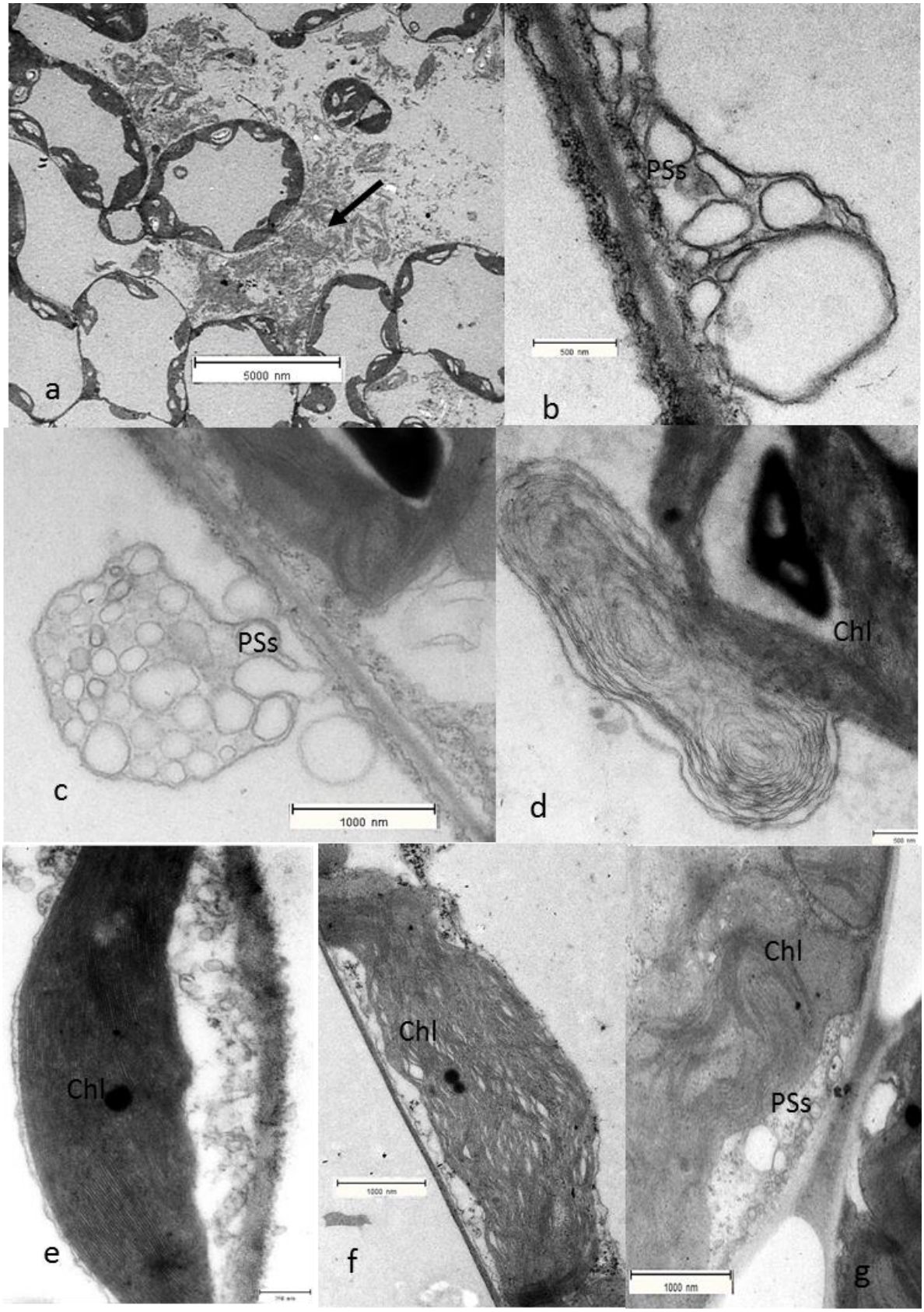

Figure 17: Cytopathic effects induced by TCDVd in infected tomato tissues. a) Cell distortion, b, c) plasmalemmasomes (PSs) with internal vesicular structures, d) vesicles with multilayer membranes in the cytoplasm e) Normal chloroplast, f, g) chloroplast with irregularly stacked thylakoids and malformation g). PSs: Plasmalemmasomes, Chl: Chloroplast, Vs: Vesicles.

3.2. Cytopathic effects of PLRV infected tomato tissues

Foliar symptoms of PLRV in tomato 4 weeks after inoculation at the time of sample collection were weak, showing only slight necrosis in mid veins of the leaves. The ultrastructural alterations of PLVR infected tomato leaf tissues are shown in figures 18 and 19. In phloem tissues, some parenchyma cells began to deteriorate as can be seen by the irregular shape and overall electron dense appearance of nuclei as well as strong vesiculation of those cells (Fig. 18a). The cell walls become thinner between cambial cells and abnormal in shape Fig. 18b. One of the alterations in PLRV infected tomato tissue as compared to non-infected tissue was the occurrence of multilamellar vesicles with diverse structures, sometimes protruding from the cytoplasm into the vacuole (Fig. 19a, b). These different sized vesicles appear singly in the cytoplasm or fused with the tonoplast. Some of them had two membranes and irregular forms. Furthermore, numerous huge starch grains were generally to be observed in chloroplasts, leading to distortion or disappearance of other components of these organelles such as thylakoid membranes or grana (Fig. 19d). Mitochondria were inflated and lost their matrix. Some of the mitochondria were probably degenerated, resulting in release of their matrix to the cytoplasm (Fig. 19e, f). In some phloem cells, the proliferation of rough endoplasmic reticulum was seen (Fig. 19e).

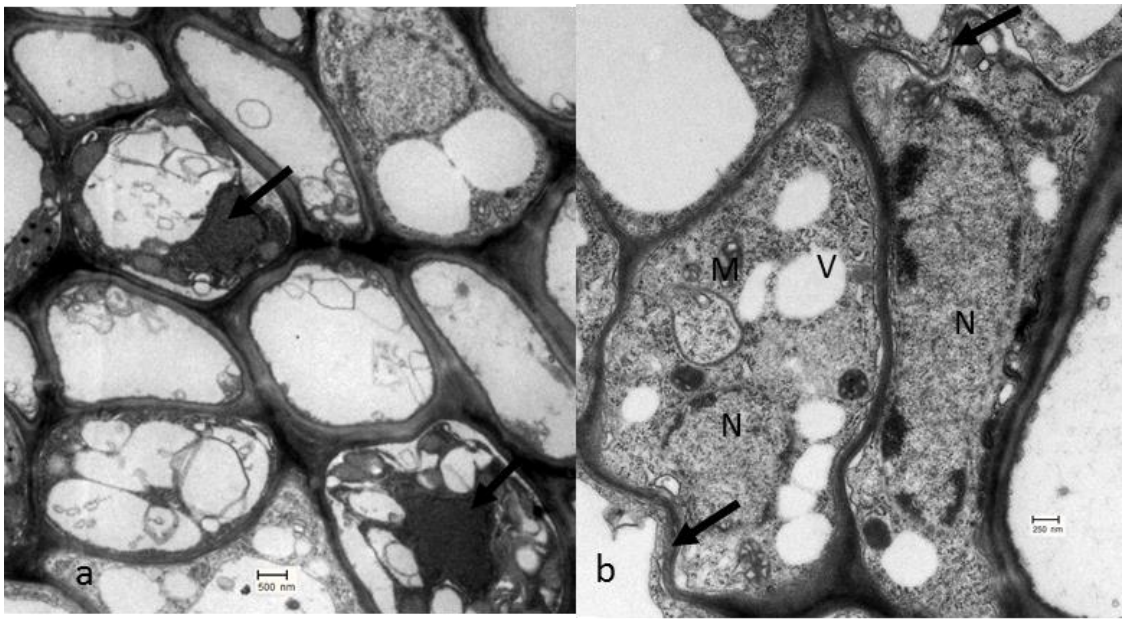

Figure 18 Cytopathic effects induced by PLRV in infected tomato tissue (general view) a) degeneration of nuclei in phloem parenchyma cells (arrow) and abnormal vesiculation, b) cell wall distortion (arrow). N: nucleus, V: Vacuole, M: Mitochondria,

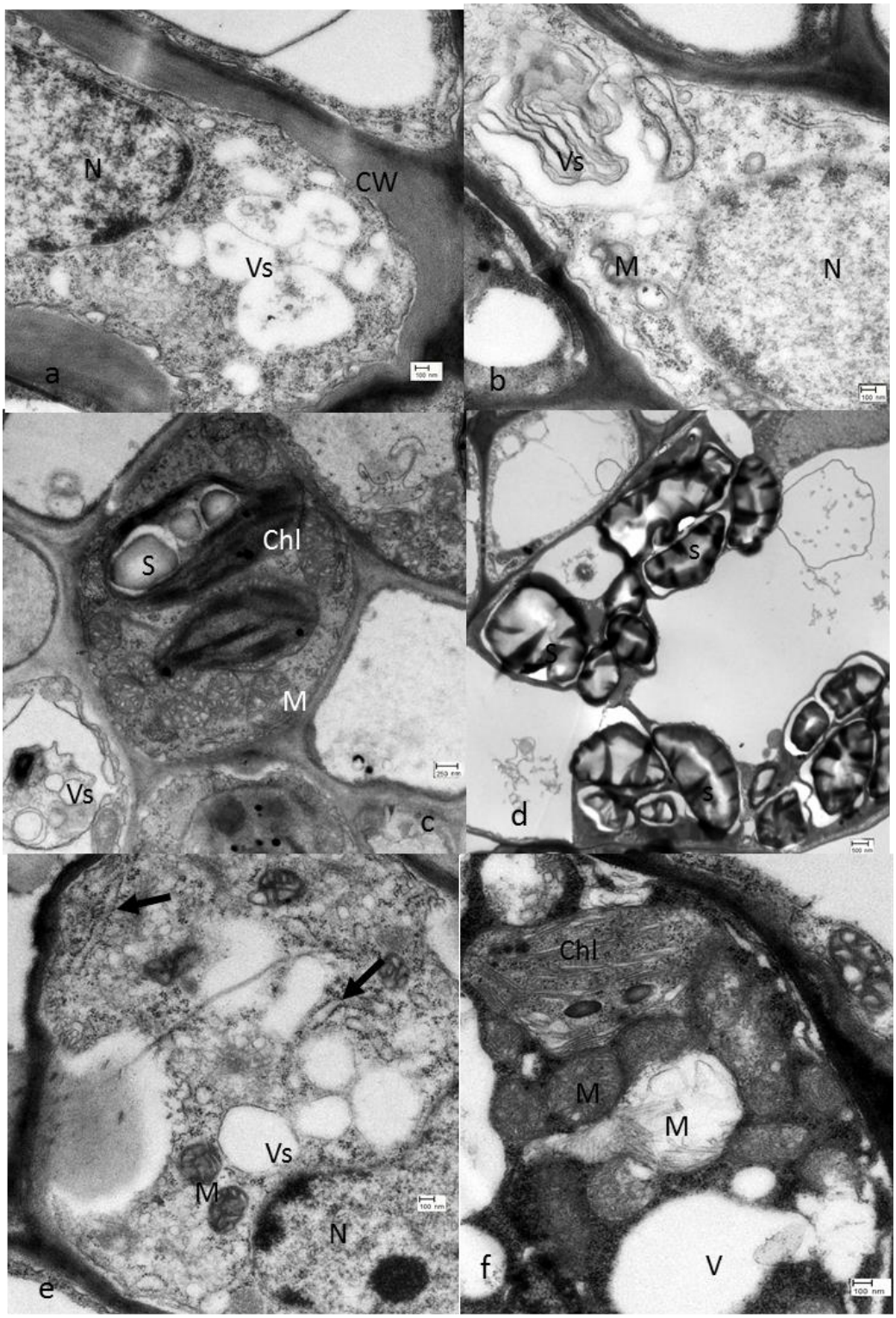

Figure 19 Cytopathic effects induced by PLRV in infected tomato phloem cells a) multiple vesicles with and filamentous and granular content in cytoplasm of phloem cell, b) multilayered membranous protrusions in phloem parenchyma cell c) healthy tomato tissue, d) multiple starch grains in chloroplasts in bundle sheat cells, e) proliferation and dilation of rough endoplasmic reticulum (arrow) dictyosom with vesicles (black arrow), small mitochondria darkly stained with dilated cristae f) degeneration of a mitochondrion. V: vacuole, Vs: Vesicles, N: nucleus, M: mitochondria, CW: cell wall.

3.3. Cytopathic effects in cells with mixed infection of TCDVd and PLRV

Basically, no distinguishable foliar symptoms between doubly PLRV/TCDVd and singly TCDVd infected tomato plants could be observed 4 weeks after inoculation. Ultrastructural alterations induced by a double infection of TCDVd+PLRV in tomato tissues are shown in Figs. 21. Similar to PLRV infected tissues of tomato, obvious alterations can be observed in mitochondria and chloroplasts. Mitochondria enlarge and cristae are dilated (Figs. 21d, f). In chloroplasts numerous very large starch grains occur generally, resulting in malformation of chloroplast shape (Fig. 21b). Moreover, in some cells, the disintegration of chloroplast membranes was also seen. Besides, the emergence of plasmalemasomes (Figs. 21e, f) and vesicles with diverse structures are clearly detectable (Figs. 21c, d), which do rarely appear in healthy tomato tissues.

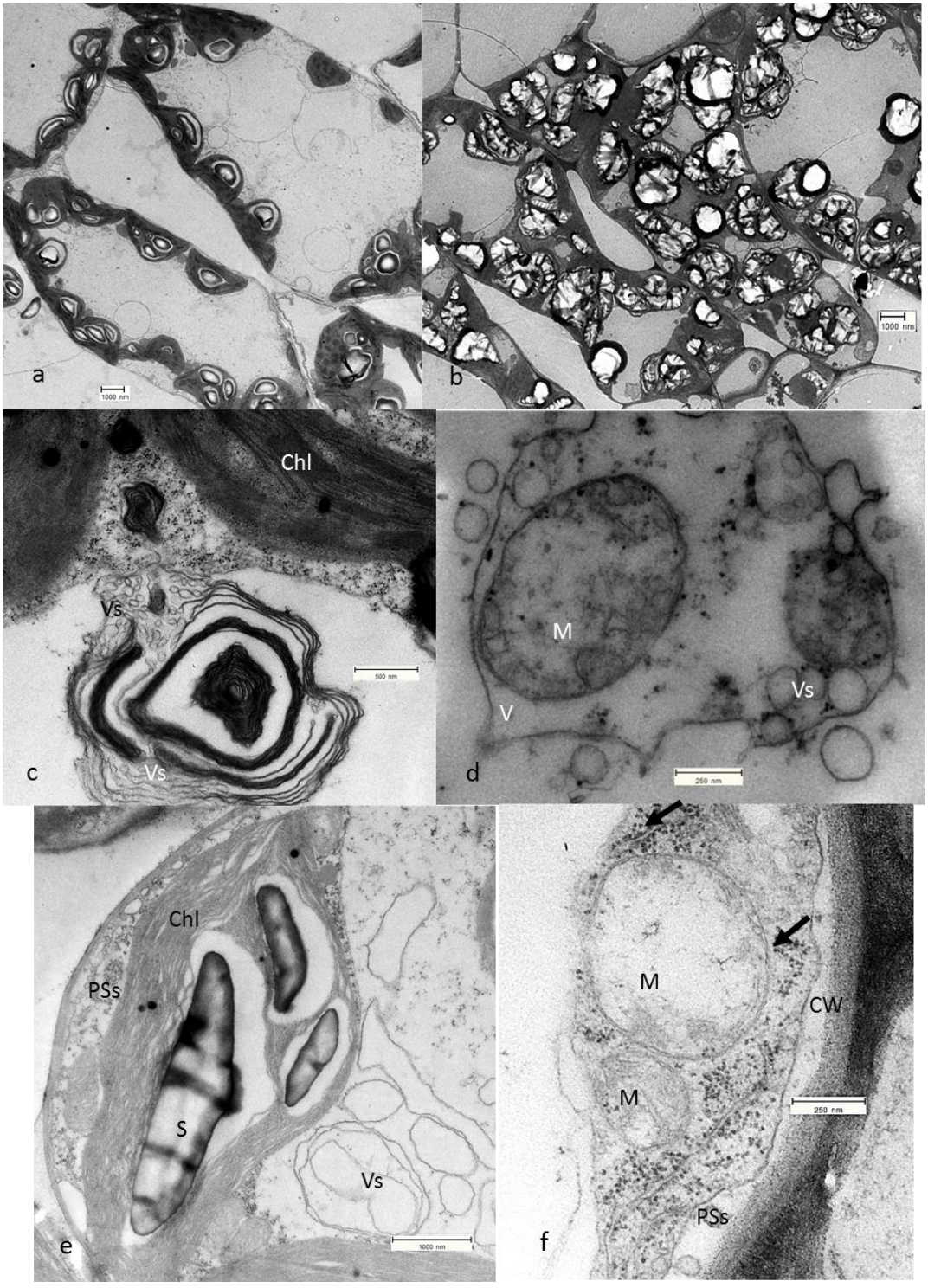

Figure 20 Cytopathic effects induced by doubly infected TCDVd and PLRV in tomato tissues in palisade cells. a) healthy tomato tissue, b) infected palisade cells with multiple huge starch grains; c) darkly staining probable cell wall appositions and vesicles with concentric membranous structures; d) vesicles with vesicular structures in cytoplasm and dilation of mitochondria; e) PSs with vesicular structures; f) dilation of mitochondria. V: Vacuole, Vs: Vesicles, M: Mitochondria, PSs: Plasmalemmasomes, Chl: Chloroplast, arrow: possible PLRV particles.

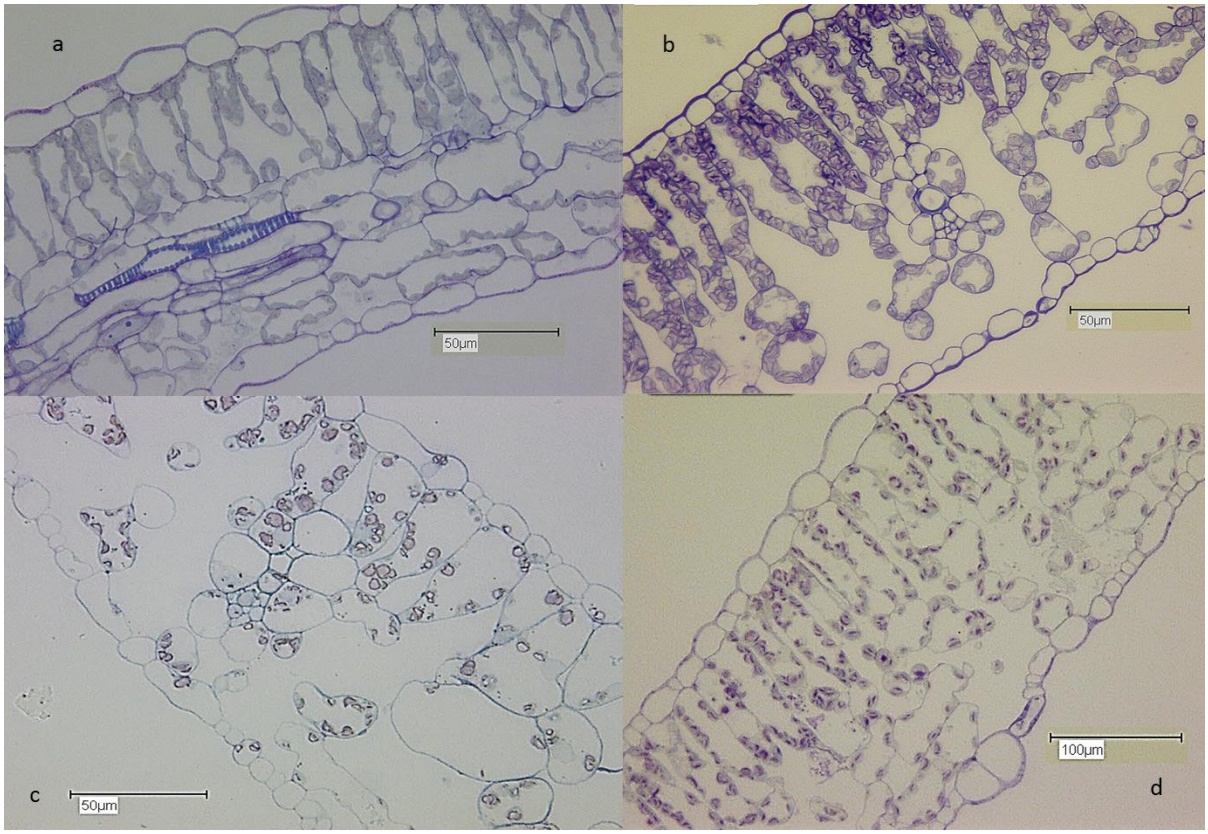

Figure 21 Sections through palisade cells of tomato tissue, light microscopy photograph, a) TCDVd infected tissue with differentiated cells in shape, b) PLRV infected tissue with multiple starch grains in chloroplasts, c) Mixed infection TCDVd+PLRV cells with huge starch grains, leading to disappearance of chloroplasts, and disappearance of many cells probably due to cell disintegration, d) Healthy tissue with normal distribution of cells and chloroplasts.

Table 11 Ultrastructural alterations in tomato tissues induced by viroid - and PLRV infection

Virus/Viroid	TCDVd	PLRV	PLRV+TCDVd	Healthy tissues
Foliage symptoms	Curling leaves, stunted growth	Necrosis in mid-vein of the leaves	Curling leaves, stunted growth	No symptoms
Cell wall	Disintegration of many parenchyma cell walls	Abnormal	Abnormal	Normal
PSs	Vesicles inside PSs	Not seen	Vesicles inside PSs	Not seen
Vesicles	Vesicular and membranous bodies	Vesicular and membranous bodies	Vesicular and membranous bodies	Vesicular
Chloroplast	Malformation of grana & thylakoid membranes: stacking of grana disturbed, wavy membrane appearance	Distortion of thylakoid membranes because of starch accumulation	Distortion of thylakoid and grana because of irregular membrane arrangement & huge starch grains	Well-organized
Starch grains	Not many	Numerous or huge starch grains	Numerous or huge starch grains	Not many
Mitochondria	Normal	Dilated	Dilated	Normal

4. Discussion

Some previous work has reported the ultrastructural effects in infected tissues induced by nucleus-replicating and chloroplast-replicating viroids. In general, the malformation of cell walls or the occurrence of cell wall related plasmalemmasomes (PSs) and the distortion of chloroplasts were reported as cytopathic effects in viroid infected tissue. The cytopathological studies of *Gynura aurantiaca* Citrus exocotis viroid (CEVd) infected leaf tissues, the appearance of paramural bodies between plasmalemma and cell wall was reported as a primary effect of alteration (Semancik and Vanderwoude 1976). However, later studies confirmed that the appearance of PSs in cytoplasm was not considered as primary change since these were also found in healthy tissues (Wahn et al. 1980, Gruner and Santore 1991). In the current study, PSs were observed in TCDVd- and doubly infected TCDVd+PLRV tomato tissue, but not in healthy tomato tissues. The origin and function of PSs remains unclear; however, they may be involved in the formation of the cell wall (Esau et al. 1966) or

considered as artifacts of the section preparation process (Fowke and Setterfiled 1969) or they may function as exosomes (An et al. 2007).

The misshaping and distortion of chloroplasts caused by malformation of grana and thylakoid membranes, and the appearance of vesicles, different in number and diverse in shape were reported as a cytopathic effect induced by viroids (Semancik and Vanderwoude 1976, Wahn et al. 1980, Kojima et al.1983, Di Serio et al. 2012). These alterations were also observed in single TCDVd-, and doubly PLRV+TCDVd infected tissues. PLRV infected tomato tissues reacted mainly with chloroplast deformations due to tremendous starch accumulation.

Information about the cytopathic effects of PLRV infection on plant cells has been very limited so far. Presumable PLRV particles were observed in companion cells and sieve elements. These particles were assumed to be virus particles because of their slightly bigger size and darker staining as compared to ribosomes. The cytopathological effects in PLRV infected potato tissues were described as the appearance of two types of vesicles formed by invagination of the nuclear envelope or freely occurring in the cytoplasm, the dilation of mitochondria and accumulation of starch in chloroplasts (Shepardson et al. 1980). Furthermore, slightly swollen mitochondria and degeneration of some phloem parenchyma cells, indicated by irregular shape and dark contrast of the nuclei as well as strong vesiculation, were observed as well. The overall virus specific changes in phloem cells were much less in tomato, than described in infected potato (Shepardson et al. 1980). This might be due to a rather late stage of infection at the time of sampling.

In this study, one of significant ultrastructural changes in PLRV- and mixed PLRV+TCDVd infected tomato tissue was the appearance of multiple huge starch grains in the chloroplasts. In fact, in many *Luteovirus* and *Polerovirus* infections of plants, the metabolism and translocation of carbohydrates are inhibited. It is discussed if this is due to the impairment of sugar translocation because of blockage of the sugar transporter Triose-phosphate translocator or by necrosis of phloem cells. PLRV is one of the phloem-limited viruses that induce necrosis of the phloem (Loebenstein and Gaba 2012). As a result, a massive accumulation of insoluble carbohydrates in form of starch grains occurs in chloroplasts of many species of virus-infected leaves (Biswas 2017, Hamacher, personal communication) that was also observed in PLRV infected tomato leaves. In mixed PLRV+TCDVd infected tissue. It still remains unclear how these two pathogens interact with each other. On the histological

level a severe degeneration of mesophyll parenchyma cells could be observed in mixed infections. The encapsidation of TCDVd by PLRV, however, will happen during PLRV replication in phloem parenchyma or companion cells, as PLRV does not escape the phloem.

Chapter 6: Summary

The transmissibility, cytopathology and disinfection of *Tomato chlorotic dwarf viroid* (TCDVd) and *Potato spindle tuber viroid* (PSTVd) were investigated. The results obtained from the present studies can be concluded in details as follows:

- Mechanical transmission with various methods plays a very important role in spreading pospiviroids, especially TCDVd, in greenhouses via handling activities. However, the rate of transmission was various depending on viroid strain and host species. The transmissibility rate of a severe TCDVd strain from tomato was higher than that of a mild PSTVd strain. Moreover, tomato was a more susceptible host for both TCDVd and PSTVd than brugmansia.

  TCDVd remained infectious on the surface of gloves for at least 2 hours under greenhouse conditions. The stability of TCDVd raises the risk of mechanical transmission especially under glass.

  The increase of plant age and leaf maturity at the time of inoculation led to a decrease in foliage symptom development of TCDVd in tomato. Tomato plants at 35 days after emergence of the first true leaves, expressed TCDVd symptoms only when they were inoculated onto young leaves. However, tomato plants from 49 days onward, did not develop symptoms, although being infected and regardless if inoculated onto the youngest or the oldest leaves.

  There is a high correlation between plant height and shoot fresh weight of TCDVd infected tomato plants with and without visible symptoms. The infected tomato plants with visible symptoms developed significantly shorter shoots as well as lower biomass than healthy and asymptomatically infected tomato.

- The transmissibility of TCDVd via *M. persicae* with assistance of PLRV was confirmed for the first time in this study. High temperatures (26-28°C) promoted the rate of PLRV assisted aphid transmission as compared to lower temperatures (20-22°C). Moreover, circulative aphid transmission was confirmed by serial passages by *M. persicae* over non

host and host plants. This was proven by the fact that after passage over non-host plants, TCDVd+PLRV carrying aphids could still transmit the viroid to host plants.

The severity of symptom development was various depending on species, host plants and environmental conditions. TCDVd infected tomato expressed severe symptoms under high temperatures. There were no differences between single TCDVd infection and mixed TCDVd+PLRV infection 3-4 weeks after inoculation. However, 6-8 weeks after inoculation, obvious differences could be observed. While TCDVd infected tomatoes were stunted, without fruit development, and finally leading to death, mixed infected (TCDVd+PLRV) tomatoes developed new sprouts consisting of hard, stunted shoots and leaves.

- The infectivity of viroid RNA under storage was significantly different depending on viroid species, viroid isolate and storage condition. We found that *Pospiviroids* stored -20^{0}C remained fully infective even after 4 months of storage. At room temperature and 4^{0}C, the infectivity did not decrease after one and two months, and then gradually declined. Moreover, the infectivity of the severe TCDVd isolate was higher than that of the mild PSTVd isolate under the same storage conditions.

  TCDVd was completely deactivated by sodium hypochlorite (NaOCl) with concentrations of 0.5% and more, by NaOH 0.5% (pH=13) as tested by rub-inoculation and by flaming cutting tools with 96% alcohol as tested by cut-inoculation. NaOCl 0.5% was able to completely disinfect TCDVd in aqueous solution and on the surface of razor blades within a very short exposure time. However, the strong oxidant ClO_2 was not able to disinfect TCDVd when employed at concentrations up to 15ppm with 15 minutes incubation time, as tested by rub-inoculation. The chemicals Na_2CO_3 0.5% and $NaHCO_3$ 0.5% were not successful in disinfecting TCDVd.

- The cytopathic variations of TCDVd infected tomato tissues were associated with macroscopic symptoms. In TCDVd infected tissues, cell wall disintegration led to release of all cell components into the extracellular spaces. This change was associated with curling and stunting of infected tomato leaves. Moreover, vesicular plasmalemmasomes which are formed by invagination of the plasma membrane and vesicles with multilayer membranes in cytoplasm were observed. In addition, malformation of grana and

thylakoid membranes of chloroplasts in infected tissue were also observed by electron microscopy, causing misshaped or distorted chloroplasts.

The cytopathic effects of PLRV infected tomato tissues included the appearance of numerous huge starch grains in chloroplasts of mesophyll cells, vesicles with diverse structures in cytoplasm and the increase of mitochondrial size in phloem parenchyma and companion cells. PLRV particles were assumed to be seen in phloem companion cells and sieve tubes, because of their slightly bigger size and heavier staining characteristics as compared to ribosomes.

Cytopathic effects in mixed infection of TCDVd and PLRV tomato tissues were not really specific, but similar to both PLRV tomato infected tissues and TCDVd infected tissues. The appearance of numerous large starch grains in chloroplasts, slightly swollen mitochondria, the appearance of vesicular plasmalemmasomes and vesicles with diverse structures and amounts could be observed.

To sum up, the studies demonstrated the danger of mechanical transmission of *Pospiviroids* via contaminated tools as well as the potential risk of aphid vector transmission with the assistance of PLRV to other *solanaceous* plants. In addition, asymptomatic infection may play an important role in spreading viroid diseases. Therefore, the importance of sanitary measures in first instance disinfection must be highlighted in order to eradicate or at least minimize spread of viroids to other crops.

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APPENDICES

Appendix 1: RNA extraction from plant tissue (Qiagen kit)

1. **Step 1**: Determine the amount of plant material. Do not use more than100mg. Weighing tissue is the most accurate way to determine the amount.
2. **Step 2**. Immediately place the weighed tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2ml micro centrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw. Proceed immediately to step 3.

   RNA in plant tissues is not protected until the tissues are flash-frozen in liquid nitrogen. Frozen tissues should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

3. **Step 3**. Add 450µl Buffer RLT or Buffer RLC to a maximum of 100mg tissue powder. Vortex vigorously. A short 1–3 min incubation at 56°C may help to disrupt the tissue. However, donot incubate samples with a high starch content at elevated temperatures, otherwise swelling of the sample will occur.

   Note: Ensure that β-ME is added to Buffer RLT or Buffer RLC before use.

4. **Step 4**. Transfer the lysate to a QIA shredder spin column (lilac) placed in a 2ml collection tube, and centrifuge for 2min at full speed. Carefully transfer the supernatant of the flow-through to a new micro centrifuge tube (not supplied) without disturbing the cell-debris pellet in the collection tube. Use only this supernatant in subsequent steps. It may be necessary to cut off the end of the pipet tip to facilitate pipetting of the lysate into the QIA shredder spin column. Centrifugation through the QIA shredder spin column removes cell debris and simultaneously homogenizes the lysate. While most of the cell debris is retained on the QIA shredder spin column, a very small amount of cell debris will pass through and form a pellet in the collection tube. Be careful not to disturb this pellet when transferring the lysate to the new microcentrifuge tube.

5. **Step 5**. Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Proceed immediately to step 6.

   Note: The volume of lysate may be less than 450 µl due to loss during homogenization.

   Note: Precipitates may be visible after addition of ethanol. This does not affect the procedure.

6. **Step 6**. Transfer the sample (usually 650µl), including any precipitate that may have formed to an RNeasy spin column (pink) placed in a 2ml collection tube (supplied). Close the lid gently, and centrifuge for 15s at ≥ 8000xg (≥10,000rpm). Discard theflow-through.* Reuse the collection tube in step 7.

   If the sample volume exceeds 700µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation.*

   Optional: If performing optional on-column DNase digestion (see “Eliminating genomic DNA contamination”, page 21), follow steps D1–D4 (page67) after performing this step.

7. **Step 7**. Add 700µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at ≥8000xg (≥10,000rpm) to wash the spin column membrane.

   Discardtheflow-through.* Reuse the collection tube in step 8.

   Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Be sure to empty the collection tube completely.

   Skip this step if performing optional on-column DNase digestion (page 67).

8. **Step 8**. Add 500µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15s at ≥8000xg (≥10,000rpm) to wash the spin column membrane. Discard the flow-through.

   Reuse the collection tube in step 9.

   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use

9. **Step 9**. Add 500µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at ≥ 8000xg (≥10,000rpm) to wash the spin column membrane.

   The long centrifugation dries the spin column membrane, ensuring that no ethanol is carried over during RNA elution. Residual ethanol may interfere with downstream reactions.

   Note: After centrifugation, carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

10. **Step 10**. (Optional): Place the RNeasy spin column in a new 2ml collection tube (supplied), and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.

    Perform this step to eliminate any possible carryover of Buffer RPE, or if residual flow-through remains on the outside of the RNeasy spin column after step 9.

11. **Step 11**. Place the RNeasy spin column in a new 1.5ml collection tube (supplied). Add 30–50µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at ≥8000xg (≥10,000rpm) to elute the RNA.
12. **Step 12**. If the expected RNA yield is > 30µg, repeat step 11 using another 30–50µl RNase free water, or using the eluate from step11 (if high RNA concentration is required).Reuse the collection tube from step 11. If using the eluate from step 11, the RNA yield will be 15–30% less than that obtained using a second volume of RNase-free water, but the final RNA concentration will be higher.

Appendix 2: One step RT-PCR for viroid determination

1. Master mix
   - 25µl 2x RT buffer
   - 21 µl distilled water
   - 1 µl primer forward
   - 1 µl primer reverse
   - 1 µl Taq mix plantinum
   - 1 µl RNA
2. RT-PCR program

-	50^0C	30 min	1 cycle
-	95^0C	3 min	1 cycle
-	95^0C	1 min	35 cycles
-	58^0C	1 min	
-	72^0C	1 min	
-	72^0C	10 min	1 cycle
-	10^0C	storage	

Appendix 3: Tissue print Immunoassay for virus confirmation (modified method from DSMZ)

1. Sample preparation: cut plant material (stalks, shoots) with a razor blades and print (4x per samples) the intersection shortly onto the membranes (Whatman).

2. Block the membrane for 5 minutes with polyvinyl alcohol in PBS 1x on shaker.

3. Wash the membrane in PBS-tween 3 times, 5 minutes each time on shaker.

4. Incubate membrane in monoclonal antibody (1:1000) in conjugate buffer for 1 hour.

5. Incubate the membrane in anti-mouse antibody in conjugate buffer for 1 hour.

6. Wash membrane 3 times in PBS1x buffer, 5 minutes each.

7. Enzyme substrate reaction 10-15 minutes.

8. Stop reaction by incubating the membrane in water.

9. Dry membrane between water and observe under binoculars.

Appendix 4: Powder pillow procedure for Chlorine dioxide calibration (Hach Company)

1. Start program 76 Chlor Diox DPD.

2. Prepare the blank: fill the sample cell with 10 mL of sample, put the stopper in the blank.

3. Prepare the sample: Fill a second sample cell with 10 mL of sample. Put the stopper in the prepared sample.

4. Clean the blank sample cell.

5. Insert the blank into the cell holder.

6. Push ZERO the display shows 0.00 mg/L ClO_2.

7. Add 4 drops of Glycine Reagent to the sample cell.

8. Swirl to mix.

9. Add the contents of one DPD Free Chlorine Powder Pillow to the sample cell.

10. Swirl the sample cell for 20 seconds to mix.

11. Wait 30 seconds for any undissolved powder to settle. Undissolved powder will not affect accuracy.

12. Clean the prepared sample cell.

13. Within one minute of the reagent addition, insert the prepared sample into the cell holder.

14. Push READ. Results show in mg/L ClO_2.

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