EUPHRESCO Final Report

For more information and guidance on completion and submission of the report contact the EUPHRESCO Call Secretariat (euphresco@ages.at).

Project Title (Acronym)
Detection and Epidemiology of Pospiviroids (DEP)

Project Duration:

Start date:	31/10/08
End date:	30/10/09

1. Research Consortium Partners

Coordinator – Partner 1			
Organisation	Aarhus University, Faculty of Agricultural Sciences, Department of Integrated Pest Management		
Name of Contact (incl. Title)	Steen Lykke Nielsen	**Gender**:	Male
Job Title	Senior scientist – Head of research unit		
Postal Address	Forsøgsvej 1, DK-4200 Slagelse		
E-mail	Steenl.nielsen@agrsci.dk		
Phone	+45 89 99 19 00		

Partner 2			
Organisation	Central Science Laboratory		
Name of Contact (incl. Title)	Dr. Wendy Monger	**Gender**:	Male
Job Title	Scientist – Pest & Disease Identification		
Postal Address	Sand Hutton, York, YO41 1LZ, UK		
E-mail			
Phone	++44-(0)1904 462140		

Partner 3			
Organisation	Plant Protection Service		
Name of Contact (incl. Title)	Dr J. Th. J. Verhoeven	**Gender**:	Male
Job Title	Senior virologist		
Postal Address	P.O. Box 9102, 6700 HC Wageningen, the Netherlands		
E-mail	j.th.j.verhoeven@minlnv.nl		
Phone	+31317496853		

Partner 4			
Organisation	Agricultural Institute of Slovenia		
Name of Contact (incl. Title)	Mojca Viršček Marn, PhD.	**Gender**:	Female
Job Title	Senior researcher		
Postal Address	Hacquetova 17, 1001 Ljubljana, Slovenia		
E-mail	mojcavm@kis.si		
Phone	+386 1 2805 253		

Partner 5			
Organisation	INRA		
Name of Contact (incl. Title)	Dr Thierry CANDRESSE	**Gender**:	Male
Job Title	Directeur de Recherche		
Postal Address	Equipe de Virologie UMR GDPP IBVM, Campus INRA 78 Avenue Edouard Bourleaux 33883 Villenave d'Ornon cedex France		
E-mail	tc@bordeaux.inra.fr		
Phone	+33 (0) 557 12 23 89		

Partner 6			
Organisation	Austrian Agency for Health and Food Safety (AGES) Institute of Plant Health Dept. for Phytopathology, Fruit Production & Viticulture		
Name of Contact (incl. Title)	Dr. Richard Gottsberger	**Gender**:	Male
Job Title	Research Expert		
Postal Address	Spargelfeldstr. 191, 1226 Vienna		
E-mail	richard.gottsberger@ages.at		
Phone	++ 43-0-50 555-33343		

Partner 7			
Organisation	German Collection of Microorganisms and Cell Cultures, DSMZ, Plant Virus Department		
Name of Contact (incl. Title)	Dr. Stephan Winter	**Gender**:	Male
Job Title	Head of Department, Plant Virologist		
Postal Address	c/o JKI, Messeweg 11/12, 38104 Braunschweig		
E-mail	Stephan.Winter@jki.bund.de		
Phone	+49 531 299 3780		

Partner 8			
Organisation	Laboratoire National de la Protection des Végétaux		
Name of Contact (incl. Title)	Valérie Molinéro-Demilly (Ingeneer)	**Gender**:	Female
Job Title	Detection and Epidemiology of Pospiviroids (DEP)		
Postal Address	7 Rue Jean Dixmeras 49044 Angers Cedex 01 France		
E-mail	valerie.molinero-demilly@agriculture.gouv.fr		
Phone	00.33.(0)2.41.72.32.40		

Please make as many copies of this table as necessary.

2. Executive Summary

Project Summary

Please provide a summary suitable for web publication and which is understandable to the intelligent non-scientist.
Include: Title, main objectives, Methods, Results and Conclusions. (max. 2 pages)

Detection and Epidemiology of Pospiviroids (DEP)

The project ran one year from 31st October 2008 to 30th October 2009. The project was initiated as a pilot project by EUPHRESCO and granted under the so-called virtual pot competitive funding mechanism.
The main objectives were to provide new knowledge of the epidemiology and diagnosis of *Potato spindle tuber viroid* (PSTVd), *Citrus exocortis viroid* (CEVd), *Columnea latent viroid* (CLVd), *Chrysanthemum stunt viroid* (CSVd), *Tomato apical stunt viroid* (TASVd) and *Tomato chlorotic dwarf viroid* (TCDVd) with major efforts targeting the risk of transmission of pospiviroids from ornamental plants to crops of tomato and potato and the development and validation of diagnostic laboratory manuals to be presented to national and EC plant health authorities for surveillance and seed-testing purposes.
To address these challenges the following activities were carried out:

Surveying for new 'natural' host plants of the six targeted pospiviroids and investigation of host-pospiviroid interactions. Solanaceous and gesneriaceous plants were sampled at nurseries and botanical gardens. Eight new plant hosts of pospiviroids were found comprising CLVd in *Gloxinia*, Iresine-1 viroid in *Celosia*, PSTVd and TCDVd in *Calibrachoa* and TASVd in *Lycianthes* and *Streptosolen*. Isolates of four pospiviroid species from ornamental plants were successfully transmitted to potato and tomato by mechanical inoculation and further on to a series of potato and tomato plants for up to four passages. Only minor changes in genotypes were found and confirm the high stability of predominant pospiviroid genotypes.

Investigation of possible transmission pathways that might spread pospiviroids from ornamental plants to crops. Experiments were carried out to examine whether PSTVd can be transmitted by the western flower thrips and the onion thrips within ornamentals and from ornamentals to tomato by leaf sucking. No transmission was shown. Furthermore experiments were carried out to examine whether honeybees and bumblebees can transmit PSTVd during their feeding/pollinating activities within ornamentals and from ornamentals to tomato. No transmission was shown. It is concluded that the negative transmission result indicates that thrips are not vectors for pospiviroids and that PSTVd transmission by honeybees and bumblebees from ornamentals to tomato probably does not occur.
The effectiveness of mechanical transmission of PSTVd was studied within ornamentals and from ornamentals to potato and tomato. PSTVd can be transmitted by contact and crop handling, which may also lead to transmission from ornamentals to other crops.

Development of diagnostic RT-PCR-based assays for pospiviroids and validation of them by ringtesting. A real time RT-PCR assay for universal pospiviroid detection and specific assays for detection of CEVd, CLVd and TASVd have been developed, validated by ringtesting and they reliably detect pospiviroid isolates. Furthermore a laboratory manual for conventional RT-PCR for screening for pospiviroids based on the Pospi1 and Vid primer sets published by Verhoeven *et al.* (2004) has been validated by ringtesting and has shown to be a reliable and sensitive method to detect pospiviroids.

Development of reliable assays for the detection of PSTVd in tomato seeds. PSTVd is present in seeds of infected tomato and can lead to seedling infections. The viroid cannot be inactivated by any of the routinely used seed disinfection treatments and contaminated seeds are a potential source of PSTVd outbreaks. PSTVd can be detected in seeds by RT-PCR and/or real-time RT-PCR. Seed testing of larger sample batches requires real-time RT-PCR. The threshold of reliable PSTVd detection is at < 1:1000 infected/healthy seeds. However most crucial to detecting PSTVd in seeds is sample disruption and homogenization prior to RNA extraction. Variable PSTVd concentrations in individual seeds and lower percentage of seed infections in commercial seed production render it difficult to evaluate a detection threshold and sample size.

Conclusions. Many ornamental plant species are hosts for pospiviroids and they can be successfully transmitted within ornamentals and from ornamentals to potato and tomato by contact and crop handling. Risk of transmission of pospiviroids from ornamentals to tomato and potato crops by sucking and pollinating insects seems to be negligible. Seed transmission in tomato is a fact, but variable viroid concentration in individual seeds renders it difficult to evaluate a detection threshold. There are available validated and reliable diagnostic protocols for pospiviroids based both on conventional and real-time PCR.

Literature cited

Verhoeven, J.Th.J., Jansen, C.C.C., Willemen, T.M., Kox, L.F.F., Owens, R.A. & Roenhorst, J.W. 2004. Natural infections of tomato by citrus exocortis viroid, columnea latent viroid, potato spindle tuber viroid and tomato chlorotic dwarf viroid. European Journal of Plant Pathology 110, 823-831.

Acknowledgement

The project grant was provided through EUPHRESCO and financed by the following national funders: Denmark: Ministry of Food, Agriculture & Fisheries, Danish Food Industry Agency. United Kingdom: Food and Environmental Research Agency, Policy Programme. The Netherlands: Ministry of Agriculture, Nature and Food Quality, Plant Protection Service. Slovenia: Ministry of Agriculture, Forestry and Food of the Republic of Slovenia. Austria: Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management. Germany: Julius Kühn-Institut, Bundesforschungsinstitut für Kulturpflanzen, Institut für nationale und internationale Angelegenheiten der Pflanzengesundheit, Braunschweig. France (INRA): French National Institute for Agricultural Research. France (LNPV): Ministry of Agriculture, Food, Fisheries & Rurality, General Food Directorate.

3. Report

Full scientific report of the DEP project

Table of contents

Introduction

The project titled Detection and Epidemiology of Pospiviroids (DEP) ran one year from 31st October 2008 to 30th October 2009. The project was initiated as a pilot project by EUPHRESCO and granted under the so-called virtual pot competitive funding mechanism. The total funding amounted 324,642 EURO.

For a research project a period of only one year is very unusual. The rather comprehensive content of the project was only made possible by the fact that several partners already were active in other financed projects on viroids, whereby synergism could be obtained. The project benefits from that for example through that results on other pospiviroids than the six included in the project description are included in this final report.

The present report presents a full scientific report of the project activities to EUPHRESCO including results that will be submitted for publishing in scientific journals. The DEP consortium therefore strongly request that the report is not published, but only forwarded to the reviewers and the EUPHRESCO partners with the claim that it is not yet a public report. To compensate for that the DEP consortium has, besides the max two pages summary, prepared a long detailed version suitable for presentation on the EUPHRESCO web home page. As soon as the results have been published, the scientific report can be regarded as a public report.

Acknowledgement

The project grant was provided through EUPHRESCO and financed by the following national funders: Denmark: Ministry of Food, Agriculture & Fisheries, Danish Food Industry Agency. United Kingdom: Food and Environmental Research Agency, Policy Programme.

The Netherlands: Ministry of Agriculture, Nature and Food Quality, Plant Protection Service. Slovenia: Ministry of Agriculture, Forestry and Food of the Republic of Slovenia. Austria: Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management. Germany: Julius Kühn-Institut, Bundesforschungsinstitut für Kulturpflanzen, Institut für nationale und internationale Angelegenheiten der Pflanzengesundheit, Braunschweig. France (INRA): French National Institute for Agricultural Research. France (LNPV): Ministry of Agriculture, Food, Fisheries & Rurality, General Food Directorate.

Work packages and participants

Work Packages (WP)	
No. of WP	**Title** Detection and Epidemiology of Pospiviroids (DEP) Project leader: Steen Lykke Nielsen
1	Project Management and Co-ordination WP-leader: Steen Lykke Nielsen Other participants: All partners
2	Host range studies and host-viroid interactions WP-leader: Steen Lykke Nielsen Other participants: Partner 2, 3, 5 and 7
3	Transmission pathways WP-leader: J. Th. J. Verhoeven Other participants: Partner 1, 3 and 6.
4	Identification methods and diagnostic protocols WP-leader: Wendy Monger Other participants: Partner 1, 4 and 8
5	Seed test WP-leader. Stephan Winter Other participants: Partner 1, 4, 5 and 6

WP1. Project Management and Coordination

Objectives of WP1.

- To coordinate project activities, including project meetings and reporting.
- To manage all administrative, financial, legal and contractual aspects of the project.
- To publish and disseminate results obtained within the project to stakeholders.

Results

A Research Consortium Agreement (Appendix 1) was worked out by the Coordinator and signed by all partners.
A kick off meeting was held 4th-5th November 2008 in Denmark. Minutes of the meeting Appendix 2.
A midway project meeting was held 15th May 2009 as a telephone meeting with the WP-leaders. The minutes of the meeting (Appendix 3) was mailed to the EUPHRESCO Call Secretariat.
A final meeting was held 27th to 28th October 2009 in Slovenia. The minutes of the meeting (Appendix 4) was mailed to the EUPHRESCO Call Secretariat.
A final scientific report including validated diagnostic protocols and a max 2 pages summary was delivered the 21st December 2009 to the EUPHRESCO Call Secretariat.

A short version of the final scientific report suitable to be laid on the web home page of EUPHRESCO was delivered together with the final report the 21st December 2009 to the EUPHRESCO Call Secretariat.
Delivery of cost statements to each national funder will be the responsibility of each partner.

Discussion of results and their reliability
All tasks in WP1 have been fulfilled

Disseminations
The minutes of the midway and the final meetings were mailed to the EUPHRESCO Call Secretariat.
The final scientific report including validated diagnostic protocols was delivered to the EUPHRESCO Call Secretariat.
A short version of the final scientific report suitable to be laid on the web home page of EUPHRESCO was delivered to the EUPHRESCO Call Secretariat.

Work package 2 – Host range studies and host-viroid interactions

In the text the following acronyms are used for the viroids:
CEVd: *Citrus exocortis viroid*
CLVd: *Columnea latent viroid*
CSVd *Chrysanthemum stunt viroid*
IrVd-1: *Iresine viroid*
PSTVd: *Potato spindle tuber viroid*
TASVd: *Tomato apical stunt viroid*
TCDVd: Tomato chlorotic dwarf viroid

Objectives
To study the host range of PSTVd, CEVd, CSVd, CLVd, TASVd and TCDVd with special focus on solanaceous species (commercial grown ornamental crops and important wild species (weeds)) and ornamentals of Gesneriaceae. To study whether viroid genotypes from ornamental hosts will be able to infect tomato and potato, whether weeds/wild plants can function as (symptomless) viroid reservoirs and to study whether the viroid genome will adapt to the “new” host after transmission to tomato and potato.

Task 2.1. Establishment of a collection of isolates of the six pospiviroids

Summary
Partners’ who already possessed isolates of pospiviroids placed their collections at the consortium’s disposal, and the collections covered the needs for the different project activities.

Objectives of Task 2.1
To establish a collection of isolates of the six targeted pospiviroids at the partners to be used in the project activities.

Methods used and Results obtained
The partners' who already possessed isolates of pospiviroids placed their collections at the consortium's disposal both in form of stem cuttings from originally infected plants and viroids propagated in other host plants.
During the kick off meeting it was decided to use the PSTVd S1 isolate as standard in most trials.
Obtaining PSTVd infected tomato seeds for the WP5 activities made up a special problem. Time consuming production of infected tomato seeds had to be initiated before experiments could be started. This posed another challenge to find the right growth stage of the tomato plant for the inoculation, because if too early the plants were stunted and only limited fruit set was obtained and if too late too few infected seeds were obtained.

Main conclusions
Sufficient numbers of isolates were obtained to carry out the project activities including obtaining viroid isolates for the ringtestings of the diagnostic assay protocols.
Deliverable D 2.1 Collection of relevant isolates and strains of pospiviroids fulfilled.

Task 2.2 Surveying for new host plants of pospiviroids

Summary
Surveys for new hosts of pospiviroids were carried out at nurseries, commercial growers and botanical gardens focusing on plant genera from the two families Gesneriaceae and Solanaceae. Eight new plant hosts of pospiviroids were found comprising CLVd in *Gloxinia*, IrVd-1 in *Celosia*, PSTVd and TCDVd in *Calibrachoa* and TASVd in *Lycianthes* and *Streptosolen*. Furthermore, weeds/wild plants close to glasshouse crops shown positive for pospiviroids were collected and tested for pospiviroids without finding any infection, so no indications were obtained for a role of weeds as infection reservoirs.

Objectives of Task 2.2
Finding new plant hosts of pospiviroids. Until now most plant hosts of pospiviroids have been found in the two families Solanaceae and Gesneriaceae. Therefore, the surveys were focused on these two families and especially on ornamental species, even though also other plant families were included. Furthermore, weeds/wild plants close to glasshouse crops shown positive for pospiviroids were collected to elucidate whether wild plants can function as (symptomless) viroid reservoirs

Methods used and Results obtained
Methods
Possible host plants of especially Solanaceae and Gesneriaceae were sampled at nurseries and botanical gardens.
In addition, solanaceous weeds were sampled close to nurseries and glasshouses where pospiviroids have been detected, previously.

Experimental design:
Pooled samples up to 5 plants were tested for pospiviroids with Pospi1 and Vid primers in common RT-PCR (Verhoeven *et al*., 2004). In case of positive results the individual plants were re-tested and the viroid was identified by sequencing of parts or the total viroid genome. In case of first reports pathogenicity was proven by inoculation to tomato, or independent confirmation of the presence of a pospiviroid obtained by molecular hybridization. The Danish survey comprised 42 samples of gesneriaceous species from 16 genera and 83 samples of solanaceous species from 19 genera. The Dutch survey comprised 17 samples of ornamental gesneriaceous species from 5 genera, 333 samples of ornamental solanaceous species from 17 genera, and 98 ornamental species distributed in 27 genera from various families. The French survey comprised 525 samples of Solanaceae from 17 genera and 21 samples of *Verbena* sp. and *Impatiens* sp.

Results
The results of the surveys are shown in Table 1.

Table1. Gesneriaceous and solanaceous genera/species found positive for pospiviroids. The plants and viroids marked in **bold** are new records.

Family	**Genus/species**	**Viroid**
Gesneriaceae	**Gloxinia gymnostoma**	**CLVd**
	Gloxinia nematanthodes	**CLVd**
	Gloxinia purpurascens	**CLVd**
Solanaceae	*Brugmansia*	PSTVd
	Calibrachoa	**PSTVd, TCDVd**
	Cestrum	TASVd
	Datura	PSTVd
	Lycianthes rantonnetti	PSTVd, **TASVd**, CEVd
	Physalis peruviana	PSTVd
	Solanum jasminoides	CEVd, PSTVd, TASVd, CSVd
	Streptosolen jamesonii	**TASVd**

Among plant species from other families CSVd was found in *Chrysanthemum* sp. and IrVd-1 in *Celosia plumosa*.
No pospiviroids were detected in the 55 weed samples of the species *Datura stramonium*, *Solanum dulcamara* and *Solanum nigrum* that were tested.

Discussion
The results show that pospiviroids are present in gesneriaceous and solanaceous ornamentals but not very frequent. It is interesting that only one viroid was found in the botanical gardens collections, where the plants are original wild species. It might indicate that the commercial plant production is the important source for propagation and transmission of viroids. The three *Gloxinia* species were situated close to each other in the botanical garden's glasshouse indicating a possible transmission from one originally infected species to the other. The failure to detect viroid infections in weeds collected close to nurseries with history of viroid outbreaks indicate that weeds probably can be considered of minor importance as infection source.

Main conclusions
Eight new plant hosts of pospiviroids have been found i.e., CLVd in three *Gloxinia* species, IrVd-1 in *Celosia*, PSTVd and TCVd in *Calibrachoa* and TASVd in *Lycianthes* and *Streptosolen*. No indications were obtained for a role of weeds as infection reservoirs.
Deliverable D 2.3. *A report of new natural hosts of the six pospiviroids and of their experimental host range in selected weeds* fulfilled.

Disseminations.
The results were presented on an Ad-hoc EPPO meeting on tomato viroids in Ljubljana, 2009-10/28-29.
The results will be submitted as new disease reports or disease notes.

References
Verhoeven, JThJ, Jansen, CCC, Willemen, TM, Kox, LFF, Owens, RA and Roenhorst, JW (2004) Natural infections of tomato by *Citrus exocortis viroid*, *Columnea latent viroid*, *Potato spindle tuber viroid* and *Tomato chlorotic dwarf viroid*. European Journal of Plant Pathology 110: 823-831.

Task 2.3 Production of infectious cDNA clones of the six targeted pospiviroids

Summary
Full-length cDNAs were amplified and sequenced for 5 of the 6 targeted viroids (PSTVd, TASVd, CSVd, TCDVd and CLVd). The cDNAs were cloned in plasmid pUC9 in order to generate infectious constructs according to Candresse *et al*. (1990). Infectivity assays have been delayed by problems in the Partner 5 greenhouses during the summer and beginning of fall period. Infectivity has been validated for the PSTVd, TASVd , CSVd and TCDVd constructs while assays are still ongoing for the CLVd construct. The various constructs have been made available to the partners of DEP and have been deposited for future reference and distribution in the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) collection.

Objectives of Task 2.3
The objectives of task 2.3 were the development for use as control or reference of full-length infectious cDNA plasmid constructs for 6 pospiviroids (PSTVd, TASVd, CSVd, TCDVd, CLVd and CEVd).

Methods used and Results obtained
Methods
Full length monomeric cDNAs linearized a the *SmaI* site of the central conserved region (CCR) were PCR amplified, using specially designed primers, from total nucleic acids extracts obtained from plants infected with the various target pospiviroids obtained either from the collection of INRA or from DEP project partners. Problems were encountered with the CEVd sample, provided by one of the partners as a dormant infected potato tuber. No amplification was obtained during initial assays and the plant was later lost. CEVd cDNA cloning was not further pursued.
The amplified cDNAs were blunt-end cloned in *SamI*-linearized plasmid pUC9. This clon-

ing strategy generates an 8 to 11 bp duplication of the CCR (depending of the cDNA cloning orientation within the vector). This duplication is sufficient to allow recovery of infected plants when the recombinant plasmid is mechanically inoculated to host plants (according to Candresse *et al*. 1990).
Recombinant plasmids carrying the proper insert were then sequenced to validate the constructs and identify the viroid variant cloned.
Purified plasmids were mechanically inoculated to tomato (cv. Rutgers) seedlings and plants analyzed for viroid infection 1.5 month post-inoculation. Sequence of the progeny molecules was determined to confirm that they were indeed derived from the inoculated constructs.

Results
Full-length cDNAs were amplified, cloned in plasmid pUC9 and sequenced for 5 of the 6 targetted viroids (PSTVd, TASVd, CSVd, TCDVd and CLVd).
The sequence of the various isolates is provided below:

TASVd
CGGGATCTTTCGTGAGGTTCCTGTGGTGCTCACCTGACCCTGCAGGCATCAAGAAAAAAGATAGGAGCGGGAA
GGAAGAACAGGTCGACGGATCCCCGGGGAAACCTGGAGGAAGTCGAGGTCGGGGGCTTCGGATCATTCCTGGT
TGAGACAGGAGTAATCCCAGCTGAAACAGGGTTTTCACCCTTCCTTTCTTCTGGTTTCCTTCCTCTCGCCGGA
AGGTCTTCGGCCCTCGCCCGGAGCTTCTCTCTGGAGACTACCCGGTGGAAACAACTGAAGCTTCCACTTCCAC
GCTCTTTTTCTCTATCTTTGTTGCTCTCCGGGCGAGGGTGAAAGCCCGTGGAACCCTGAATGGTCCCT

PSTVd
CGGAACTAAACTCGTGGTTCCTGTGGTTCACACCTGACCTCCTGAGCAGTAAAGAAAAAAGAAGGCGGCTCGG
AGGAGCGCTTCAGGGATCCCCGGGGAACCTGGAGCGAACTGGCAAAAAGGACAGCGGGGAGTGCCCTAGCGGC
CGACAGGAGTAATTCCCGCCGAAACAGGGTTTTCACCCTTCCTTTCTTCGGGTGTCCTTCCTCGCGCCCGCAG
GACCACCCCTCGCCCCTTTGCGCTGTCGCTTCGGCTACTACCCGGTGGAAACAACTGAAGCTCCCGAGAACCG
CTTTTTCTCTATCTTATGTGCTTCGGGGCGAGGGTGTTTAGCCCTTGGAACCGCAGTTGGTTCCT

CSVd
CGGGACTTACTTGTGGTTCCTGTGGTGCACTCCTGACCCTGCTGCTTGAAAGAAAAAGAAATAGGGCGAAGAA
CAGGTCGACGGATCCCCGGGGAAACCTGGAGGAAGTCCGACGAGATCGCGGCTGGGGCTTAGGACCCCACTCC
TGCGAGACAGGAGTAATCCTAAACAGGGTTTTCACCCTTCCTTTAGTTTCCTTCCTCTCCTGGAGAGGTCTTC
TGCCCTAGCCCGGTCTTCGAAGCTTCCTTTGGCTACTACCCGGTGGAAACAACTGAAGCTTCAACGCCTTTTT
TTCCTATCTTCTTTAGCACCGGGCTAGGGAGTAAGCCCGTGGAACCTTAGTTTTGTTCCCT

TCDVd
CGGAACTAAACTCGTGGTTCCTGTGGTTCACACCTGACCTCCTGTGCAGAAAAGAAAAAAGATAGGCGGCTCG
GAGGAGCGCTTCAGGGATCCCCGGGGAAACCTGGAGCGAACTGGCAAAAGGCGGCAGGGAGCTTGTGGAAGGC
GAAACAGGAGTAATCCCGTGTAGAAACAGGGTTTTCACCCTTCCTTTCTTCTGCGGTTTCCTTCCTTTGCGCG
CCACTCGACCCCTCGCCCCCTTGCGCTGTCGCTTCGGCTACTACCCGGTGGAAACAACTGAAGCTCCCGAGAA
CCGCTTTTTCTCTATCTTGCTGCTACCGGGGCGAGGGTGTTTAGCCCTTGGAACCGCAGTTGGTTCCT

CLVd
CGGAACTTTTCTCGTGGTTCCTGTGGTTACACACCTGACCCTGCAGCCATGCAAAGAAAAAAGAACGGCGGAG
AGAGCGCAAGAGCGGTCTCAGGAGCCCCGGGGCAACTCAGACCGAGCGGGGATCGCGGACCGAGGGCGGAAGC
CTGCTTCAGACAGGAGTAATCCCCGCTGAAACAGGGTTTTCACCCTTCCTTTCTTCGGGTTTCCTTCCTCTGC
TTGCGGCCTCGCCCGGAGTCTTCGAATCCAGCGCAGGTTCTGACGCGACCGGTGGCATCACCGAGTTGCTCAA
GCCTCAATCCGCTTTTTCTTCATCTTAGCTTGGTCTCCGGGCGAGGGTGAAAAGCCCGTGGAACCGCAGAAGG
TTCCT

Infectivity of the PSTVd , TASVd, CSVd and TCDVd constructs has been verified by inoculation of tomato (cv. Rutgers) plants. Assays are ongoing for CLVd constructs due to technical problems in the greenhouse for the initial infectivity assays.
The various constructs have been made available to the partners and have been deposited in the DSMZ collection (DEP Partner 7).

Main conclusions
Five of the 6 planned constructs have been obtained, with infectivity validated for 4 of them. The infectivity of the last construct (CLVd) should pose no problems since it was prepared in a fashion similar to that used for the other infectious constructs.

Disseminations.
The various constructs have been made available to the partners and have been deposited for future reference and distribution in the DSMZ collection (DEP Partner 7).

References
Candresse, T, Owens, RA and Diener, TO (1990). The role of the viroid central conserved region in cDNA infectivity. Virology, 175: 232-237.

Task 2.4 and 2.5. Transmission of viroids from ornamentals to tomato and potato and sequence analysis

Summary
Isolates of *Citrus exocortis viroid* (CEVd; 1), *Potato spindle tuber viroid* (PSTVd; 7), *Tomato apical stunt viroid* (TASVd; 1) and *Tomato chlorotic dwarf viroid* TCDVd; 2), from ornamental plants, and *Pepper chat fruit viroid* (PCFVd; 1) from pepper were successfully transmitted to potato and tomato by mechanical inoculation. After determination of the sequence, each genotype was inoculated to a series of potato and tomato plants and propagated for up to four passages. Newly arisen genotypes were inoculated 0 to 3 times depending on the passage, in which they were encountered. In total, 385 nucleotide sequences were determined, in which 17 new predominant genotypes were identified. Generally, a specific mutation was only found in one of the inoculated plants and only consisted of a single substitution or deletion. After four series of inoculations, the number of different mutations varied between 0 and 4 per genotype. Most mutations were found in isolate B1 from TCDVd. The PSTVd genotypes most frequently found in ornamental plants did not mutate (B1and S1) or only once (S2), and genotypes of TASVd and PCFVd did not mutate either. Also the predominant pospiviroid genomes in the original ornamental hosts had not mutated during the two years overlapping the period of the experiments, except for a single mutation in one genotype of PSTVd. These results confirm the high stability of predominant pospiviroid genotypes.

Objectives of task 2.4 and 2.5

a) Mechanical transmission of pospiviroids from ornamentals to potato and tomato. Many new pospiviroid-host combinations have been found during the last few years. This raises the issue whether infected ornamentals may act as sources of inoculum for

potato and tomato. In this study we examine whether pospiviroids can be transmitted from ornamentals to potato and tomato by mechanical inoculation.

b) Determination of genetic variability of original predominant pospiviroid genotypes after transmission from ornamentals to potato and tomato.
In previous experiments sequencing of uncloned PCR products from *Potato spindle tuber viroid* infected plants of *Brugmansia* spp. revealed many different predominant genotypes, whereas in *Solanum jasminoides* only a few predominant sequences were found. Generally, a single predominant genotype was present in each plant or group of plants tested because the obtained sequences did not show ambiguity. Also for the newly characterized *Pepper chat fruit viroid* (genus *Pospiviroid*) sequencing of cloned PCR products revealed a single predominant genotype. In the current study we investigate the genetic variability, i.e. the combined action of mutation and selection, of the predominant genotypes from five pospiviroids upon transmission from ornamental plants and pepper (*Capsicum annuum*) to potato and tomato.

Methods used and results obtained

Isolates of *Citrus exocortis viroid* (CEVd; 1), *Potato spindle tuber viroid* (PSTVd; 7), *Tomato apical stunt viroid* (TASVd; 1) and *Tomato chlorotic dwarf viroid* TCDVd; 2), from ornamental plants, and *Pepper chat fruit viroid* (PCFVd; 1) from pepper were used for all experiments.
Leaves of naturally infected plants were mechanically inoculated to young plants of potato cv. Nicola and tomato cv. Moneymaker in five replications. For *Brugmansia* spp. also roots were used as inoculum source because inoculation with leaf sap often was unsuccessful. All viroids isolates were successfully transmitted to both potato and tomato.
From all inoculated plants, leaf tissue was collected 6 weeks after inoculation for RT-PCR using primers amplifying the complete viroid genome. Uncloned PCR products were sequenced to determine the predominant pospiviroid genotype (= master sequence). After determination of the sequence, each genotype was inoculated to a series of potato and tomato plants and propagated for up to four passages. In total, 385 nucleotide sequences were determined, in which 17 mutations were identified. Generally, a specific mutation was only found in one of the inoculated plants and only consisted of a single substitution or deletion. After four series of inoculations, the number of different mutations varied between 0 and 4 per genotype. The PSTVd genotypes most frequently found in ornamental plants did not mutate, nor did the predominant genotypes of TASVd and PCFVd. Most mutations were encountered in PSTVd genotypes found in ornamentals only once and in one of two isolates of TCDVd. Also the predominant pospiviroid genomes in the original ornamental hosts had not mutated during the two years overlapping the period of the experiments, except for a single mutation in one genotype of PSTVd.

Discussion and main conclusions

The results presented in this investigation show that pospiviroid genotypes from various ornamentals and pepper are able to infect potato and tomato. This means that these often symptomless-infected ornamental plants pose a risk to economically important crops, which may result in serious diseases. The intriguing question remains why only such a limited number of pospiviroid infections in tomato, and even none in potato, have been reported during the last decade, despite the presence of high numbers of infected ornamental plants. This might be due to low chances for transmission - the inoculum source

often is not present in the immediate vicinity of tomato - or to poor transmission rates as observed for leaves of *Brugmansia spp*.
The substitution rate of predominant pospiviroid genotypes is low when pospiviroids are transferred between from ornamentals to potato and tomato. These results confirm the high stability of predominant pospiviroid genotypes. This implies that the predominant genotypes from naturally infected host plants may be used for phylogenetic analyses to trace sources of infection.
Deliverable D 2.4. *A report on viroid-host adaption of pospiviroid isolates from ornamental host plants* fulfilled.

Disseminations
The results were presented on an Ad-hoc EPPO meeting on tomato viroids in Ljubljana, 2009-10/28-29.
A manuscript presenting the results of this study has been accepted by Archives of Virology: Verhoeven J.Th.J. & Roenhorst J.W. (2010) High stability of original predominant pospiviroid genotypes upon mechanical inoculation from ornamentals to potato and tomato. (Archives of Virology, DOI: 10.1007/s00705-009-0572-9).

Work Package 3. Transmission pathways

Objectives of WP3
To examine new transmission pathways for PSTVd (as a model for pospiviroids). During the last few years infection of several species of ornamental plants with pospiviroids has been recorded and it has raised a concern of risk of transmission of pospiviroids from ornamentals to the important food crops tomato and potato. It is well known that pospiviroids are transmitted mechanically by sap. There are a few records of transmission by bumblebees (Antignus *et al.* 2007, Matsuura *et al.* 2010) and transmission by aphids through encapsidation in potato leaf roll virus (Querci *et al.* 1997). Until now no investigations of the possible role of thrips and honeybees as vectors for pospiviroids have been published. Thrips are interesting because they are well-known virus vectors, they feed by sucking and penetrating the plant cell wall exposing the cell for possible infection, and in certain periods they migrate in massive numbers, for examples during harvest of cereals, where invasion in glasshouses are seen. The western flower thrips is furthermore one of the insect species with the broadest plant host spectrum. Honeybees are not, like bumble bees, used commercial for pollination in tomato production, but honeybees forage on a lot of flowering ornamentals and might accidentally be locked in tomato glasshouses.

Task 3.1 Transmission by thrips

Summary
Experiments were carried out to examine whether PSTVd can be transmitted by the western flower thrips *Frankliniella occidentalis* and the onion thrips *Thrips tabaci* within ornamentals and from ornamentals to tomato by leaf sucking. Experiments comprised transmission from PSTVd infected *Solanum jasminoides* to non infected *S. jasminoides* and

tomato and the part with the Western flower thrips also included access to feed on pollen. No transmission of PSTVd was shown. Giving the thrips access to feed on pollen, PSTVd infected or non infected plant material neither contributed to viroids transmission.

Objectives of Task 3.1.

Pathways to be studied were transmission by leaf- and pollen feeding thrips and the aims were to elucidate whether PSTVd can be transmitted by the western flower thrips *Frankliniella occidentalis* and the onion thrips *Thrips tabaci* from infected ornamentals to non infected ornamentals and tomato.

Methods used and Results obtained

Methods

Experiments with *Frankliniella occidentalis*

For all experiments thrips were reared on bean plants by an established procedure. Attempts to rear on *Nicotiana glutinosa, Solanum jasminoides* and tomato failed.

As inoculum was used PSTVd isolate S1. The thrips were fed on PSTVd infected leaves of *Solanum jasminoides* and pollen from *Nicotiana glutinosa*.

Experimental design:

Per replicate 40 thrips were transferred to PSTVd infected plant material and each experiment included12 replicates with PSTVd infected material and 3 control replicates with non infected material.

The standard design was:

Thrips were inactivated and transferred from the rearing plant to an empty glass tube and starved for 2 h. Transfer to a petri dish with PSTVd infected plant tissue sucking for 24 h. Transfer to a non-infected receptor plant in a thrips tight container for 3 days. Insecticide sprayings in the container 3 times during 7 days to eliminate the thrips. Trasfer of the plant to a glasshouse for 5 weeks. Six weeks after possible transmission leaf samples were collected and tested for PSTVd by real-time PCR according to Boonham *et al.* 2004.

The following experiments were carried out where the thrips were allowed to feed on one or more PSTVd infected plant tissues and finally transferred to a non infected plant:

1. Infected *Solanum jasminoides* → non-infected tomato
2. Infected *Solanum jasminoides* → non-infected *S. jasminoides*
3. Infected pollen → infected tomato → non infected tomato
4. Non infected pollen → infected tomato → non infected tomato
5. Infected pollen → non infected tomato
6. Infected *Solanum jasminoides* → direct test of thrips

Experiments with *Thrips tabaci*

For all experiments thrips were reared on garden leek (*Allium porrum*) by an established procedure.

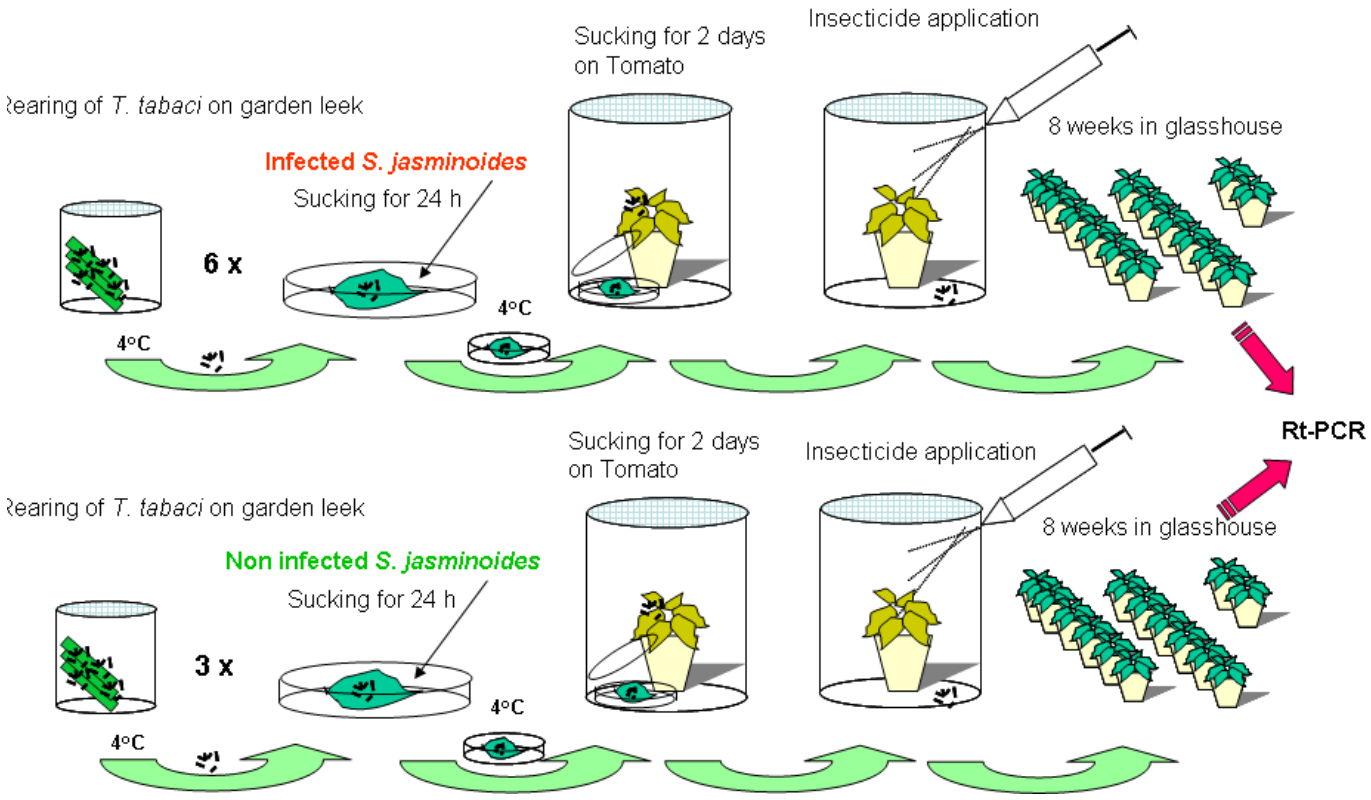
As inoculum PSTVd isolate S1 in *Solanum jasminoides* and B1 in *Brugmansia* sp. were used. The thrips were fed on PSTVd infected leaves of *Solanum jasminoides* and *Brugmansia* sp. The receptor plants were tomato from the cultivar “Rutgers”, which are described as a classical host for PSTVd (Matoušek *et al.* 2007).

Experimental design:

Per replicate 40 thrips were transferred to PSTVd infected plant material and each experiment included 6 replicates with PSTVd infected material and 3 control replicates with non infected material.

The standard design was:
Thrips were cooled at 4°C and transferred from the rearing plant material to a petri dish with PSTVd infected plant tissue sucking for 24 h. Transfer of the petri dish (again at 4°C) and release of the thrips to a non-infected receptor plant in a thrips tight container for 48 h. Insecticide spraying in the container was done to eliminate the thrips after anesthetising them with CO_2 for 3 min. Transfer of the plant to a glasshouse for 6-8 weeks. After incubation leaf samples were collected and after RNA extraction with a commercial kit (Qiagen, Hilden, Germany) following the manufacturers protocol, tested for PSTVd by conventional PCR according to Verhoeven *et al.* 2004 and Shamoul *et al.* 1997.
The thrips were also tested for PSTVd directly after feeding on the infected plant material.

The following scheme illustrates the experimental set up for a *Thrips tabaci* experiment and the same design with minor differences was used for the *Frankliniella occidentalis* experiments:



Results

Experiments with *Frankliniella occidentalis*

The results obtained are shown in Table 1.

Table 1. The results of real-time PCR test of infector plant leaves 6 weeks after start of possible transmission given as Ct values.

No.	Experiment	Ct values
1	Infected *S. jasminoides* → non-infected tomato	Negative
2	Infected *S. jasminoides* → non-infected *S. jasminoides*	Negative
3	Infected pollen → infected tomato → non infected tomato	Negative
4	Non infected pollen → infected tomato → non infected tomato	Negative
5	Infected pollen → non infected tomato	Negative
6	Infected *S. jasminoides* → direct test of thrips	35.50-38.66
7	All non infected controls	Negative
8	Positive control	18.70-18.85
9	Negative control	36.89 - negative

Experiments with *Thrips tabaci*

The results obtained are shown in Table 2.

Table 2. The results of conventional RT-PCR tests of infector plant leaves 8 weeks after start and of the thrips tested directly after feeding on the infected plant material.

No.	Experiment	Shamoul PCR	Pospi-PCR
10	Inf. *S. jasminoides* → non-inf. Tomato	n.d.	n.d.
11	Inf. *Brugmansia* sp. → non-inf. Tomato	n.d.	n.d.
12	*Thrips tabaci* from inf. *S. jasminoides*	(+)	n.d.
13	*Thrips tabaci* from inf. *Brugmansia* sp.	(+)	n.d.
14	non infected controls	n.d.	n.d.

n.d → not detected

Discussion of results and their reliability

The results clearly show that under the experimental conditions applied no transmission of PSTVd did occur. The results of the direct test of thrips 24 hours after they were allowed to suck on PSTVd infected leaf material indicates a slightly possible very weak response (experiment 6, 12 and 13). This might be referred to a positive signal from the thrips' intestines filled with plant sap or plant sap residues sticking to their mouthparts. Giving the thrips access to feed on pollen, PSTVd infected or non infected plant material (experiments 3, 4 and 5), did not contribute to viroids transmission as it has been recorded for transmission of pollen-associated tobacco streak ilarvirus virus by *Thrips tabaci* (Sdoodee &Teakle 2007).

Main conclusions

The results show that thrips do not vector PSTVd and most likely no other pospiviroids. This is to our knowledge the first record of this complex of problem. The results will contribute to a future assessment of the overall risk of having pospiviroids transmitted from ornamentals to tomato and potato. In fact EPPO has decided to recommend having a PRA of pospiviroids in tomato worked out.

Disseminations
The results were presented on an Ad-hoc EPPO meeting on tomato viroids in Ljubljana, 2009-10/28-29.
A joint scientific paper including transmission of PSTVd with thrips, bees and aphids is planned.
Deliverable D 3.1. *A report on whether thrips pose a risk for PSTVd transmission* fulfilled.

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Task 3.2 Transmission by bees

Summary
Experiments were carried out to examine whether honeybees and bumblebees can transmit PSTVd during their feeding/pollinating activities within ornamentals and from ornamentals to tomato. Experiments comprised transmission from PSTVd infected solanaeceous to non infected solanaeceous species and tomato. No transmission of PSTVd was shown.

Objectives of Task 3.2.
The bee studies focused on possibilities of transmission of PSTVd between viroid-infected and non-infected ornamentals and from ornamentals to tomato.

Methods used and Results obtained
Methods

Experiments with honeybees (*Apis mellifera*)

As inoculum was used PSTVd isolate S1.
The plant species included in the transmission trials comprised:
Nicotiana glutinosa: It is not a commercial grown ornamental, but it showed to be a very good host for PSTVd obtaining very high titter after mechanical inoculation and occurrence

of PSTVd could be shown in all organs of the flower including the pollen, except the gynoecium, and the flowers are very attractive to honeybees.
Brugmansia sp: Plants raised from stem cutting from an originally PSTVd infected plant obtained from a nursery were used.
Tomato: a bushy low growing type was chosen.
Experimental design:
All plants were at the flourishing stage during the experimental period. Mini glasshouses with room for 6 infected and 6 non-infected receptor plants were used. Two mini glasshouses with infected and non-infected plants were used per experiment. As control a mini house with 12 non-infected plants was included
A mini honeybee hive with 1500 imagines, with brood and without a queen was placed in each mini glasshouse. The honeybees were fed with sugar water. The bees were in the glasshouse for the first two weeks and then removed. Four to five weeks later the receptor plants were tested for PSTVd by real time PCR according to Boonham *et al.* 2004.
The honeybee activity was inspected daily during daytime, but no systematic recording was carried out.
The following experiments were carried out where honeybees were allowed to forage on PSTVd infected and non infected flourishing plants:

- Infected *Nicotaian glutinosa* and non infected *N. glutionosa*
- Infected *N. glutinosa* and non-infected tomato
- Infected *Brugmansia* sp. and non infected *Brugmansia* sp.

Experiments with bumblebees (*Bombus terrestris)*

PSTVd isolate S1 was used for inoculation. Young plants of *Petunia* cv. Surfinia Burgundy from a commercial nursery were used for inoculation. Petunias were chosen since bumblebees collect pollen on them and PSTVd has been detected on *Petunia* sp. in France in 2007, in Slovenia in 2008 and 2009 and on a consignment from Israel entering Germany in 2008.
A commercial bumblebee hive Natupol, Koppert (The Netherlands): colony with queen, workers, brood (pupae, eggs and larvae) and bag with sugar solution was placed in a net house together with 10 PSTVd positive *Petunia* sp. plants and 9 healthy tomato plants. All of the experimental plants were flowering throughout the whole experiment. The bumblebees were allowed to forage on the plants for 4 weeks. After 4 weeks more the receptor plants were tested for PSTVd by real time RT-PCR according to Boonham *et al.* 2004.

Results
Experiments with *honeybees*
The results obtained are shown in Table 1.

Table 1. The results of real-time PCR test of infector and receptor plant leaves and non infected controls 6-7 weeks after start of possible transmission given as Ct values.

No.	Experiment	Infector plant Ct value	Receptor plant Ct values
1	Infected *Nicotiana glutinosa* → non infected *N. glutionosa*	24.0-25.5	Negative
2	Infected *N. glutinosa* → non-infected tomato	23.9-27.0	38.2 - negative
3	Infected *Brugmansia* sp. → non infected *Brugmansia* sp.	18.8 – 20.7	Negative
4	All non infected controls	Negative	Negative

All the PSTVd infected infector-plants were clearly infected. No transmission of PSTVd was seen.

Experiment with bumblebees

No transmission of PSTVd was recorded in the experiment. All of the tested plants were negative, whereas the donor plants gave Ct values around 15,0 – 16,0.

Discussion of results and their reliability

N. glutinosa was very attractive to the honeybees and they foraged very actively and collected pollen from both infector and receptor plants. The negative transmission result indicates that PSTVd transmission by honeybees is very unlikely. The flourishing tomato plants were not attractive and very few honeybees were seen visiting the tomato plants. Even though *Brugmansia* produces very big flowers with a very big pollen production, only scarce foraging was seen in this species.

The results show that under the experimental conditions used no transmission of PSTVd with honeybees did occur. The risk of transmission from viroid infected ornamentals to tomato crops is probably negligible because the tomato flowers are unattractive to honeybees and the weight of a single honeybee is so low that mechanical damage to the plants with risk of sap transmission is much lower than for bumblebees.

The number of plants in the experiment of transmission by bumblebees was rather low, but the results indicate that PSTVd is not transmitted from ornamentals to tomatoes. This is in contrast with the transmission recorded for TASVd and TCDVd in tomato crops (Antignus *et al.* 2007, Matsuura *et al.* 2010). It is possible that pollination of healthy plants with infected pollen carried by bumblebees was the cause of TASVd and TCVd spread in tomato, whereas pollination does not occur between ornamental plants and tomato.

Main conclusions

The results show that PSTVd transmission with honeybees both among ornamentals with high pollen production and from ornamental to tomato is highly unlikely. This is to our knowledge the first record of viroid transmission trials with honeybees. The experiment with bumblebee transmission of PSTVd from ornamental to tomato did not confirm the transmission recorded for TASVd and TCDVd in tomato crops (Antignus *et al.* 2007, Matsuura *et al.* 2010).

The results will contribute to a future assessment of the overall risk of having pospiviroids transmitted from ornamentals to tomato and potato. In fact EPPO has decided to recommend having a PRA of pospiviroids in tomato worked out.

Disseminations
The results were presented on an Ad-hoc EPPO meeting on tomato viroids in Ljubljana, 2009-10/28-29.
A joint scientific paper including transmission of PSTVd with bees, thrips and aphids is planned.
Deliverable D 3.2. *A report on the risk that PSTVd can be transmitted from one plant species to another by honeybees and bumblebees* fulfilled.

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Task 3.3. Mechanical transmission through cultural practice

Summary
The effectiveness of mechanical transmission of *Potato spindle tuber viroid* (PSTVd) was studied within ornamentals and from ornamentals to potato and tomato. Three ways of transmission were successful i.e. inoculation with 'infected' plant sap diluted in water, by fingertips and razor blades. In many cases inoculation by contaminated fingertips was even successful two hours after viroid acquisition. In addition, even a single slash with a contaminated razor blade successfully transmitted PSTVd from ornamentals to tomato. The temperature, plant species to be inoculated and source of inoculum were found critical factors in transmission. An average temperature of 15 ^{o}C appeared critical for successful inoculation but inoculations were more successful at 20 and 25 ^{o}C. In addition, tomato was more susceptible to PSTVd than *Brugmansia suaveolens*, *Solanum jasminoides* and potato. Furthermore, *S. jasminoides* was a better source of inoculum than *B. suaveolens*. No transmission was obtained after repeated adding of inoculum on tomato roots. Based on these results it was concluded that PSTVd can be transmitted by contact and crop handling, which may also lead to transmission from ornamentals to other crops.

Objectives of Task 3.3
Recently, many pospiviroids have been identified in various ornamental plant species. In addition, evidence was found that PSTVd infections of tomato originated from vegetatively propagated ornamentals (Navarro et al., 2009; Verhoeven et al., 2010). How the PSTVd isolates had been transmitted from ornamentals to tomato, however, was still unknown. In task 3.3 the efficiency of PSTVd transmission by crop handling within ornamentals and from these crops to potato and tomato was studied.

Methods used and results obtained

PSTVd genotype B1 from *B. suaveolens* cv. Light Red and S1 from *S. jasminoides* were used as inoculum sources. Four different experiments were performed.

1) Potato cv. Nicola and tomato cv. Sheyenne plants were inoculated by diluted plant sap when they had 4-6 and 3-4 true leaves, respectively. Inocula were prepared by grinding 1 g of leaves from inoculum sources in water (milli-Q), and were mechanically rubbed on carborundum-dusted leaves of five potato and tomato plants. For each inoculum a 10-fold dilution series was prepared from 10^{-1} (1 g leaf tissue/10 ml) to 10^{-5}. Inoculated potato and tomato plants were grown in two groups at constant temperatures of 15 ^{o}C and 25 ^{o}C for 6 weeks.

Table 1. Diluted-sap inoculation of PSTVd from *Brugmansia suaveolens* and *Solanum jasminoides* to potato cv. Nicola and tomato cv. Sheyenne using 10-fold inoculum dilutions at 15 and 25 ^{o}C.

Inoculum from *Brugmansia suaveolens* (B1)					Inoculum from *Solanum jasminoides* (S1)			
Dilution*	Potato		Tomato		Potato		Tomato	
15 ^{o}C	I**	II	I	II	I	II	I	II
10^{-1}	0***	0	0	0	0	0	5	0
10^{-2}	0	0	0	0	0	0	4	0
10^{-3}	0	0	0	0	0	0	0	0
10^{-4}	0	0	0	0	0	0	0	0
10^{-5}	0	0	0	0	0	0	0	0
25 ^{o}C	I	II	I	II	I	II	I	II
10^{-1}	4	4	4	4	4	5	5	5
10^{-2}	0	0	0	0	0	1	5	5
10^{-3}	0	0	0	0	0	2	5	5
10^{-4}	0	0	0	0	0	0	5	3
10^{-5}	0	0	0	0	0	0	3	4

* Leaf sap was diluted in 10-fold steps; ** I = first replication; II = second replication; *** number of infected plants from 5

The inoculation of diluted sap was unsuccessful for nearly all inoculations at 15 ^{o}C. Only a few tomato plants became infected with PSTVd-S1 when the inoculum was diluted no more than 100-fold (Table 1). At 25 ^{o}C the inoculations were more successful, and even inoculum dilutions up to 10^{-5} resulted in infection when inoculating tomato with PSTVd-S1. Using *B. suaveolens* as source of infection, inoculations were only successful using a 10-fold sap dilution. In addition, potato was less susceptible to PSTVd-S1 than tomato.

2) Potato cv. Nicola and tomato cv. Sheyenne plants were inoculated by contaminated fingertips when they had 4-6 and 3-4 true leaves, respectively. Fingertips were contaminated by rubbing young leaves of the inoculum source between thumb, index and middle finger. One minute, 10 minutes and 2 hours after acquiring the viroid, a group of five potato or tomato plants was inoculated by smoothly rubbing non-carborundum-dusted leaves. Inoculated potato and tomato plants were grown in two groups at constant temperatures of 15 ^{o}C and 25 ^{o}C for 6 weeks.

At 15 ^{o}C only a single tomato plant became infected by PSTVd-S1 (Table 2). At 25 ^{o}C all tomato plants became infected by this isolate and 25 out of 30 plants by PSTVd-B1. Using the fingertip-inoculation method also showed that potato was less susceptible to PSTVd than tomato but using PSTVd-S1 still resulted in infections up to ten minutes after rubbing the sap of the infected plants onto the fingertips.

Table 2. Fingertip inoculation of PSTVd from *Brugmansia suaveolens* and *Solanum jasminoides* to potato cv. Nicola and tomato cv. Sheyenne using three different latency periods at 15 and 25 °C.

Inoculum from *Brugmansia suaveolens* (B1)					Inoculum from *Solanum jasminoides* (S1)			
Latency*	Potato		Tomato		Potato		Tomato	
15 °C	I**	II	I	II	I	II	I	II
1 min	0***	0	0	0	0	0	0	0
10 min	0	0	0	0	0	0	1	0
2 h	0	0	0	0	0	0	0	0
25 °C	I	II	I	II		II	I	II
1 min	0	0	5	5	0	3	5	5
10 min	0	0	1	5	0	5	5	5
2 h	0	0	5	4	0	0	5	5

* Period between viroid acquisition and inoculation; ** I = first replication; II = second replication; *** number of infected plants from 5

3) At 5 and 10 consecutive days, tomato cv. Sheyenne plants were inoculated by adding 2 ml of inoculum (0.5 g leaf tissue in 10 ml RNAse-free water) to the soil via a pipette. This way of inoculation, however, did not cause any infection, irrespective the viroid isolate or the frequency of inoculation.
4) Plants of *B. suaveolens* cv. Geel, *S. jasminoides* cv. Variegata and tomato cv. Moneymaker were inoculated with PSTVd-contaminated razor blades at plant heights between 10 and 20 cm. To acquire the viroid leaves and stems of each inoculum source was cut by a razor blade 8 to 10 times. For inoculation tops of a group of five healthy plants were cut. Per plant species the groups of five plants were inoculated once, twice or three times at a time interval of 2 weeks. Per group, viroid was only acquired before the inoculation of the first plant.
Inoculation by PSTVd-contaminated razor blades was very successful for tomato. Using PSTVd-S1 as inoculum 12 out of 15 tomato plants became infected after a single slash with a contaminated razor blade; 2 or 3 slashes resulted in infection of all inoculated plants (Table 3). Using PSTVd-B1 was a little less successful but still the majority of the inoculated tomato plants became infected after 2 and 3 slashes. In contrast to tomato, viroid spread within the ornamentals was very low: no infection was detected in plants of *S. jasminoides* cv. Variegata, and only a single plant of *B. suaveolens* cv. Geel became infected after three slash-inoculations with PSTVd-S1.

Table 3. Single, double and threefold razor-blade inoculation of PSTVd from *Brugmansia suaveolens* and *Solanum jasminoides* to *B. suaveolens* cv. Geel, *S. jasminoides* cv. Variegata and tomato cv. Moneymaker.

Inoculum from *Brugmansia suaveolens* (B1)				Inoculum from *Solanum jasminoides* (S1)		
Slashes	*B. suaveolens*	*S. jasminoides*	*S. lycopersicum*	*B. suaveolens*	*S. jasminoides*	*S. lycopersicum*
1	0*	0	4 (0-3)	0	0	12 (3-5)
2	0	0	11 (3-4)	0	0	15
3	0	0	9 (1-5)	1 (0-1)	0	15

* total number of infected plants of three replications (maximum 15); range within numbers of infected plants per replication between brackets

Discussion and main conclusions

The results of the transmission experiments showed that PSTVd is transmitted successfully by traditional mechanical inoculation and by contaminated fingertips and razor blades. The efficiency of transmission varied and appeared clearly influenced by temperature. At

15 °C only a few tomato plants became infected, whereas many plants became infected at 25 °C. The results also show that tomato is more susceptible to PSTVd than potato. The two last results explain that new PSTVd outbreaks have been reported from tomato occasionally but not from potato. Furthermore, *S. jasminoides* was a better source of inoculum than *B. suaveolens* cv. Light Red.
The fingertip inoculation appeared very successful for transmitting PSTVd from ornamentals to tomato: infectivity was even maintained after latency periods of 10 minutes and 2 hours. This would enable mechanical viroid transmission from ornamentals to potato and tomato, even if these plant species were not grown in each other's vicinity. In addition, the finding that PSTVd was transmitted very efficiently from ornamentals to tomato via contaminated razor blades indicates that contaminated knifes and shears form an actual way for viroid transmission. Furthermore, the successful transmission of PSTVd from ornamentals to tomato by contaminated fingertips and razor blades supports the conclusion that ornamentals formed the origin of PSTVd infections in tomato in the past (Verhoeven et al. 2010).
Deliverable D 3.3. *A report on risks of mechanically transmission of pospiviroids through cultural practices* delivered.

Disseminations
The results were presented on an Ad-hoc EPPO meeting on tomato viroids in Ljubljana, 2009-10/28-29.
A manuscript presenting the results of this study has been submitted to European Journal of Plant Pathology: Verhoeven J.Th.J., Hüner L., Virscek Marn M., Mavric Plesko I. and Roenhorst J.W. Role of mechanical transmission in spreading *Potato spindle tuber viroid* within ornamentals and to potato and tomato

References
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Verhoeven, J.Th.J., Jansen, C.C.C., Botermans , M. & Roenhorst, J.W. (2010) Epidemiological evidence that vegetatively propagated, solanaceous plant species act as sources of *Potato spindle tuber viroid* inoculum for tomato. Plant Pathology (in press). DOI 10.1111/j.1365-3059.2009.02173.x).

Work Package 4 – Diagnostics

Table of content

Appendix D. Protocol for test of pospiviroids by common RT-PCR

Summary
Methods that can detect a range of viroids in the pospiviroid genus have been developed and evaluated. The assays are based on conventional and real-time PCR and are for detecting the viroids from infected leaf material. These methods have been validated by a number of laboratories and comprise a reliable set of assays for the detection of PSTVd, TCDVd, CEVd, CLVd, TASVd and CSVd.

RNA extraction methods:

Three RNA extraction methods were evaluated. One kit method (Qiagen RNeasy Plant Mini Kit), a CTAB method and a semi-automated method based on the Kingfisher system. Slightly better results were obtained from RNA extracted using the Qiagen method but all methods gave comparable and reliable results.

Conventional PCR:

Conventional PCR primers ring-tested were the Pospi1 and Vid sets (Verhoeven *et al.* 2004).
Fifteen laboratories evaluated the conventional PCR assays.
The Pospi1 set of primers reliably detected PSTVd, TCDVd, CEVd, TASVd and CSVd.
The Vid set of primers reliably detected PSTVd, TCDVd and CLVd
Real-time PCR (Taqman):
Specific real-time PCR assays were designed using the Taqman technology (Applied Biosystems) assays were designed for CEVd, CLVd and TASVd. A generic assay was designed to detect 6 pospiviroids PSTVd, TCDVd, CEVd, CLVd, TASVd and CSVd.
Nine laboratories evaluated the real-time PCR assays.
One laboratory had possible problems of contamination. The following results are based on the remaining 8 laboratories.

- CLVd assay – All laboratories detected the CLVd sample, one laboratory detected a healthy control as positive. All other samples tested negative.
- CEVd assay – All laboratories detected the CEVd sample. 2 laboratories also detected the TASVd sample weakly and one laboratory detected the CSVd sample. All other samples tested negative.
- TASVd assay – All laboratories detected the TASVd sample. All other samples tested negative.
- The Generic assay – one laboratory had detection problems with this assay. The following results are based on the remaining 7 laboratories.
  All laboratories detected the PSTVd, TCDVd, CLVd and TASVd samples.
  6 out of 7 laboratories detected the CEVd and CSVd samples.

Conclusions:

The RT-PCR primer sets are a reliable and sensitive method to detect pospiviroids. Both Pospi1 and Vid sets are required to screen for all the viroids in the pospiviroid genus. Sequencing of products is necessary to identify the viroid present.
The CLVd and TASVd specific Taqman assays were found to be reliable.
The CEVd Taqman assay was reliable at detecting CEVd, however, cross-reaction with certain TASVd isolates may occur.
The generic Taqman assay reliably detected all 6 pospiviroid isolates.

Note these assays are designed for leaf material and not seed testing. Material should not be bulked together or samples otherwise diluted. These assays are designed to detect the maximum number of isolates of the viroids. The assays are based on the sequences available at the time; eg. TASVd had only 12 available sequences. There may be some isolates of the viroids that will not be detected by these assays.

Objectives of WP4. Diagnostics

Work package 4 was specifically concerned with testing known PCR primers that detect members of the pospivirus genus and the development of real-time assays. Specific real-time PCR assay already exist in the form of Taqman tests for CSVd and PSTVd that also detects TCDVd. This project aimed to develop real-time tests for TASVd, CEVd, CLVd and a generic assay that will detect all 6 viroids of concern, PSTVd CLVd, TCDVd, TASVd, CSVd and CEVd.

Task 1. Comparison of RNA extraction methods

Three methods were chosen for a comparison, Qiagen plant kit, a basic CTAB method (adapted from Lodhi *et al.*1994) and an automated method using the kingfisher machine (Mumford 2002). Details of these methods can be found in Appendix A. The material tested were fresh healthy leaf tissue from petunia and tomato and a dried tomato leaf that contained PSTVd.
The control Taqman assay cytochrome oxidase (COX) (Weller et al. 2000) was used with all three samples and the Taqman generic assay was also used with the PSTVd infected tomato.
In all cases there was found to be a slight advantage with the Qiagen RNeasy kit. However, all methods worked well to give RNA that could be amplified and the dried tomato leaf was found to be positive for viroid using the generic Taqman assay. A figure of these results can be found at the end of Appendix A.

Task 2. Conventional PCR Assays

Conventional PCR primers that were tested were the Pospi1 and Vid sets (Verhoeven *et al.* 2004). Together these sets detect all known viroids in the pospiviroid genus.
The Vid primer set is designed to the terminal left region of the viroids and gives a full-length genome product typically 360 nucleotides.

Vid-F 5’-TTCCTCGGAACTAAACTCGTG-3’
Vid-R 5’-CCAACTGCGGTTCCAAGGG-3’

The Pospi1 primer set is designed to the central conserved region of the viroid and gives a product typically 200 nucleotides.

Pospi-F 5’-GGGATCCCCGGGGAAA-3’
Pospi-R 5’-AGCTTCAGTTGTYTCCACCGGGT-3’

Ringtesting the conventional PCR primers

Laboratories that evaluation the conventional PCR assay.

Agricultural Institute of Slovenia	Slovenia
Agricultural Research Institute Cyprus	Cyprus
Austrian Agency for Health and Food Safety (AGES)	Austria
The Food and Environment Research Agency	UK
Finnish Food Safety Authority Evira	Finland
Laboratoire National de la Protection des Végétaux	France
MAF Biosecurity New Zealand	New Zealand
Naktuinbouw	The Netherlands
National Institute of Biology	Slovenia
National Reference Laboratory	The Netherlands
Plant Directorate	Denmark
Plant Protection and Inspection Services	Israel
Scottish Agriculture (SASA)	Scotland
State phytosanitary Adm Czech Republic	Czech Republic
Walloon Agricultural Research Centre	Belgium

The participants were provided with:

- A protocol (see Appendix D).
- A vial of freeze dried tomato leaves with PSTVd for practising in advance.
- 7 numbered vials with 6 different viroids and 1 non infected.*
- 1 positive (PSTVd) and 1 negative (non infected tomato) control.
- A result scheme to mail back to the organizer.

*Two out of 15 participants were only provided with 5 viroids because the organizer ran out of CEVd.

Rules for the test procedure: it was obligatory to use the two primer sets (Vid and Pospi1). The participants had free choice for the RNA extraction method and the RT-PCR. Two kits (Qiagen and Invitrogen) and assays were described in the protocol.
The procedure for the test was in short to run a RT-PCR with the two primer sets followed by sequencing of the PCR products and blasting for alignment.
The two primersets should detect the viroids in the following way:

Viroid	Pospi1	Vid
Potato spindle tuber viroid	x	x
Tomato chlorotic dwarf viroid	x	x
Citrus exocortis viroid	x	
Columnea latent viroid		x
Tomato apical stunt viroid	x	
Chrysanthemum stunt viroid	x	

The following results of the ringtest were obtained:

Number of participants	Correct diag-nosis	Partly correct diagnosis	Only PCR, no sequencing	No results returned
15	10	0	1	4

The conclusions of the results of the ringtest, of the remarks from the participants and of the DEP partners own experiences with the primersets were that the assay needs some local optimisation especially on the annealing temperatures, but that experienced laboratories can manage the protocol and obtain correct results, and finally that the Pospi1 and Vid primers are very strong and give reliable results.

Task 3. Taqman assays

Specific real-time PCR assays were designed using the Taqman technology (Applied Biosystems) assays were designed for CEVd, CLVd and TASVd. Specific Taqman assays for PSTVd, TCDVd and CSVd have been developed previously (PSTVd and TCDVd (Boonham *et al.* 2004) Eppo standard (Bulletin 34, 257-269), CSVd Eppo standard (Bulletin 32, 251-252)).
A generic Taqman assay was designed to detect 6 pospiviroids PSTVd, TCDVd, CEVd, CLVd, TASVd and CSVd

Assay Design

Table 1 shows the members of the pospiviroid genus in the first column and the number of sequences that were available from the NCBI database for those viroids in the second column. The assays were designed to detect the maximum number of isolates. The 3rd column of Table 1 shows the number of isolates of the viroid for which the generic pospiviroid assay should work and the final column shows the number of isolates for which the 3 specific assays that were designed should work based on sequence.
MPVd (*Mexican papita viroid*), TPMVd (*Tomato planta macho viroid*), IrVd (*Irsine viroid*) and PCFVd (Pepper chat fruit viroid) were included in the table to complete the picture for this genus. These viroids are not yet of concern to European tomato growers but that may change in the future. The generic assay should detect MPVd and TPMVd but will not detect IrVD and PCFVd.

Table 1. Pospiviroid sequences

	Seq.		Spec. assay
PSTVd	176	174	
TCDVd	13	13	
CSVd	34	34	
CEVd	210	204	208
TASVd	12	11	12
CLVd	36	32+	36
MPVd	10	10	
TPMVd	3	3	
IrVd	5	0	
PCFVd	2	0	

Ringtesting the Taqman Assays

Laboratories that evaluation the Taqman assays.

Agricultural Research Institute,	Cyprus
Aarhus University,	Denmark
Laboratoire national de la protection des végétaux,	France
Naktuinbouw,	Netherlands
Plantenziektenkundige Dienst,	Netherlands
MAF Biosecurity,	New Zealand
National Institute of Biology,	Slovenia
Agricultural Institute of Slovenia,	Slovenia
Food and Environment Research Agency,	UK

- Each partner tested the freeze-dried leaf material provided in the numbered vials for the conventional PCR test above.
- Each partner was given primers and probes for the assays.
- Each partner was given a suggested method to follow (Appendix B)
- Partners could use their own commonly used RNA extraction method, real-time reagents and real-time machines. A summary of what the partners used is in Appendix C.

The TASVd assay 2

The problems with designing this assay came from specificity, designing an assay that did not detect the closely related CEVd viroid.

TASVd-P2-228	5'- TCT TCG GCC CTC GCC CGR-3'
TASVd-F2-200	5'- CKG GTT TCC WTC CTC TCG C-3'
TASVd-R2-269	5'- CGG GTA GTC TCC AGA GAG AAG-3'

Ringtesting results: All partners detected the TASVd blind sample as positive with CT values in the range 9-20. Two partners included 2 additional samples that both tested positive. One partner who may have contamination problems detected TCDVd and CSVd as weak positives.
Conclusions: TASVd assay 2 is a reliable assay.

The CEVd Assay 2

The database had a very large number of sequences available (over 200) for this viroid. The more sequences available the better the assay should be. However, this also led to problems with designing due to the diversity of some of these isolates. This assay will probably not detect all known isolates of the viroid as indicated in Table 1.

CEVd-P2-337	5'- CCC TCG CCC GGA GCT TCT CTC TG-3'
CEVd-F2-304	5'- CTC CAC ATC CGR TCG TCG CTG A-3'
CEVd-R2-399	5'- TGG GGT TGA AGC TTC AGT TGT-3'

Ringtesting results: Although 2 partners were missing this sample for testing, all remaining partners detected the CEVd blind sample as positive with CT values in the range 17-22. One partner found CSVd sample to be a weak positive and one partner found TCDVd as a weak positive. Two partners detected the TASVd assay

Five partners included a total of 7 additional samples for testing that were known to contain CEVd and these all tested positive with the assay.
Conclusions: Since 2 partners that had no problems with other assays both detected TASVd as a weak positive, it is possible that this assay does cross-react with this viroid. This is further supported by the fact that TASVd and CEVd are closely related viroids. The CEVd assay is a reliable assay but may detect, as a weak positive, some TASVd isolates.

The CLVd Assay

This assay was designed with 2 forward primers to pick up the maximum number of isolates.

CLVd-P	5'-AGC GGT CTC AGG AGC CCC GG-3'
CLVd-F	5'-GGT TCA CAC CTG ACC CTG CAG-3'
CLVd-F2	5'-AAA CTC GTG GTT CCT GTG GTT-3'
CLVd-R	5'-CGC TCG GTC TGA GTT GCC-3'

Ringtesting results: All partners detected the CLVd blind sample as positive with CT values in the range 9-25. One partner found the healthy blind sample to be positive and one partner identified samples 6 and 7 (TASVd and CSVd) as weak positives.
Three partners included a total of 5 additional samples for testing that were known to contain CLVd and these all tested positive with the assay.
Conclusions: The CLVd assay appears to be a reliable assay.

The generic assay

This is the most complex of all the assays and contains 2 probes and 2 forward and reverse primers. There are also a large number of bases that are degenerate.

Pospi-P12	5'-CTY GWG GTT CCT GTG GTD CWC WCC TGA C-3'
Pospi-P22	5'-CTG TGG TGC TCA CCT GAC CCT GCA-3'
Pospi-F356a	5'-GGW GWA AGC CCK TGG AAC C-3'
Pospi-F356b	5'-GGT GTT TAG CCC TTG GAA CC-3'
Pospi-R96	5'-GGC TCC TGA GAC CGC TCC T-3'
Pospi-R102	5'-CCC GGG GAT CCC TGA AG-3'

Ringtesting results: One partner had detection problems with this assay. This partner also gave the highest CT values with the other assays. Excluding this partner there is good agreement with the remaining partners. Only one partner failed to detect the CEVd isolate and one partner failed to detect CSVd. Four partners included an additional 13 samples that included the host plants *Capsicum, Brugmansia* and *Surfinia* all were detected with this assay.
Conclusion: The generic assay is reliable for the detection of these viroids. However, as indicated from Table 1 this assay may have problems detecting some known isolates of some of the viroids. It is therefore not 100% effective. The assay had a large amount of degeneracy built into it to accommodate all the viroids and the problem that one lab had with this assay may indicate that the sensitivity of the assay may be limited. Good quality RNA is therefore essential when using this assay.

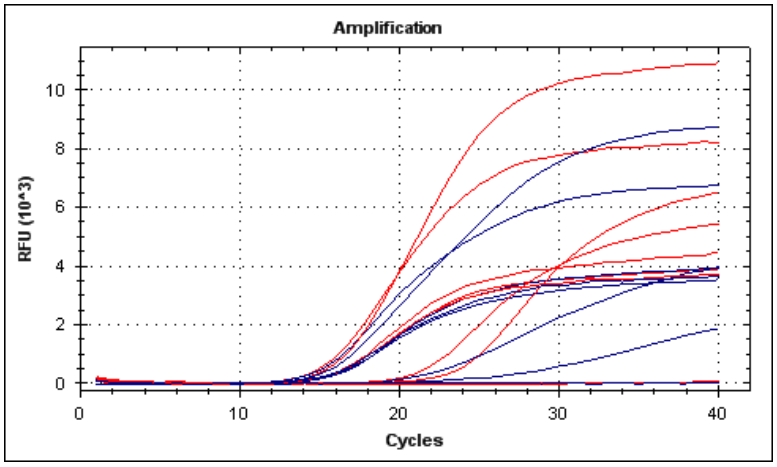
Multiplexing assays

Many laboratories mix more than one assay in the same tube. This is called multiplexing and has the advantage of reducing costs and time. A common use of multiplexing is to incorporate a control reaction with the test reaction. A control for the RNA/DNA extraction is an essential component of diagnostic laboratories routine analysis. Cytochrome oxidase or COX is a mitochondria gene; a Taqman assay for COX is in common use (Weller et al. 2000).

Below are the results of a multiplexing experiments performed by Ellis Meekes, Marcel Toonen and Rien Hooftman of Naktuinbouw (Netherlands). The red lines show the GenericTaqman result of the viroid assay in a singleplex assay. The blue lines show the results of the assay when COX is included in the reaction.

The CEVd, TASVd and CLVd assays appears to be unaffected by multiplexing.

Multiplexing The Generic assay



The generic assay above was tested with an isolate of each of the viroids and healthy controls. Most of the viroid samples gave a weaker positive result when in a multiplex reaction. In conclusion, multiplexing should not be performed with the generic assay and not undertaken by laboratory's for other assays unless that laboratory has performed their own validation, difficulty with multiplexing might also depend on the reagent-mix and the machine they are using.

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Appendix A

Examples of RNA extraction methods for leaf material

NOTE: Plant material can be fresh, frozen or freeze-dried.

1. QIAGEN RNeasy PLANT mini kit (cat. No. 74904)
From Qiagen, follow manufacturers instructions. Use RLT buffer. Beta mercaptoethanol can be omitted from the protocol providing samples are processed quickly. Process no more than 4 samples at one time.

2. CTAB RNA extraction method
Adapted from Lodhi et al. (1994) PMBR 12: 6-13.

1. Place tissue, 100-200 mg, in a suitable polythene bag and grind with a hand roller or automated machine.

2. Grind until the tissue forms a 'smooth paste'. Add 1-2 ml (10 vols) of buffer (See Below) and mix thoroughly using a hand roller.

3. Decant ground sap into a 1.5 ml microfuge tube and incubate sap at 65°C for 10-15 mins.

4. After incubation, centrifuge tubes at max speed in a microfuge for 5 min (at room temperature).

5. Remove 700 µl of clarified sap, place in a fresh microfuge and add an equal volume of chloroform:I.A.A. (24:1) and mix to emulsion by inverting the tube.

6. Centrifuge at max speed in a microfuge for 10 min (at room temperature).

7. Carefully remove upper (aqueous) layer and transfer to a fresh tube. Add an equal volume of chloroform:I.A.A., mix and spin as before (see 5 + 6).

8. Remove aqueous layer, taking extra care not to disturb interphase. Add an equal volume of 4 M LiCl. Mix well and incubate at 4°C overnight.

9. Spin for 25 min at 4°C at 12,500 rpm to pellet the RNA.

10. Decant off the salt/isopropanol and wash pellet by adding 400 µl 70% ethanol and spinning for 4 min.

11. Decant off the ethanol and dry the pellet to remove residual ethanol.

12. Resuspend pellet in 100 µl of molecular-biology grade water.

CTAB Buffer

To make 1 litre:

100 mM Tris-HCl, pH 8.0	12.12g (Tris base)
2% CTAB	20g
20 mM EDTA	7.44g
1.4 M NaCl	81.82g

1.0 % Na sulphite*
2.0 % PVP-40*
Dissolve the Tris and adjust pH to 8.0 (using HCl)
*PVP and NaSO added fresh to aliquot of stock buffer (containing first four reagents) immediately prior to extraction. Stock buffer can be autoclaved and stored at room temperature.

3. Automated Kingfisher robot (Labsystems)
Below is a guide for people who already use the kingfisher machines.

Machines: Either Kingfisher ml or Kingfisher 96.
Programs: Using pre-stored manufactures programs:Total RNA-ml-1 or TNA-96-Hot-Block 1

Kingfisher Buffer (CSL)	
Molecular grade water	750 ml
Absolute ethanol HPLC grade	250 ml
Guanadine hydrochloride	764.2g
Disodium EDTA dihyrate	7.4g
Polyvinylpyrolidine (PVP)	30 g
Citric Acid monohydrate	5.25g
Trisodium citrate	0.3g
Triton X 100	5 ml

Making buffer, start with PVP mix to a paste with a small amount of the water then gradually add the rest of the water. Add the other ingredients one by one. Final volume will be about 1.5 litres and pH should be between 2 and 2.8. Store in Fridge

Working kingfisher buffer

This is an aliquot of the buffer above with the addition of 2% TNAPP and 1% antifoam B (Sigma A6707).

TNAPP is tetrasodium pyrophosphate decahydrate (>99 % purity) make up a solution of 8.39% (w/v) ie 4.195g in 50mls of water pH to 10-10.9 with HCL, filter sterilise, store in a dark bottle at room temperature. Working kingfisher buffer is made up each time it is used.

1. Sample should be ground in approximately 2 mls of working buffer for 200mg of plant tissues. This can vary depending on tissue but the end product should be a liquid that can

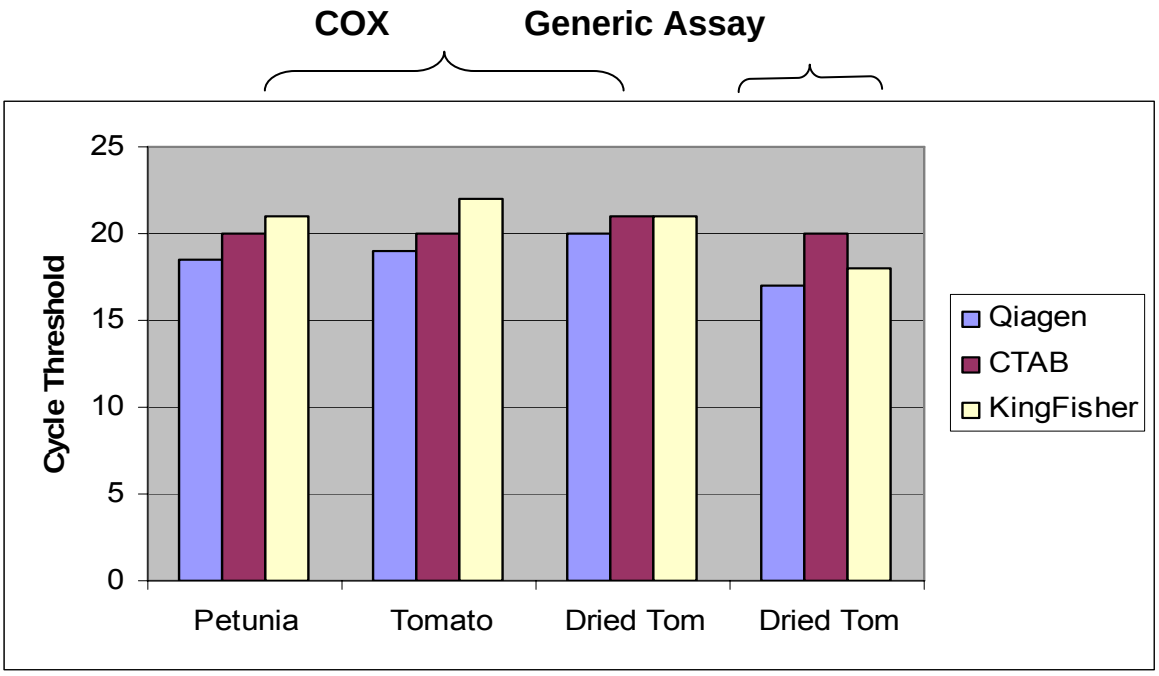
be easily poured into a 1.5ml centrifuge tube, if it is thick and slow moving add more buffer.

2. Centrifuge ground sample for 5min maximum speed. Remove 1ml of supernatant and add to first tube of kingfisher rack (A). Add 50 μl of vortexed Map solution A magnetic beads (Invitek/Thistle scientific).

3. Remaining positions in rack B = 1ml of straight Kingfisher buffer, C and D = 1ml of 70% ethanol and position E 200μl of molecular grade water.

4. The kingfisher ml will require an oven at 65°C for 5min at the pause step the kingfisher 96 has the heating step built in.

Taqman results (CT values) from the 3 methods of RNA extraction.



Appendix B

Detection of viroids with real-time PCR assays.

Real-time PCR is a method, which lends itself to high-though put allowing large numbers of samples to be processed quickly. It is becoming the method of choice for those laboratories that process large number of samples for which the main cost constraint is personnel.
Real-time PCR as a routine means of detection should only be undertaken by laboratories that have the necessary facilities to prevent contamination and by qualified personnel.
The following methods give an example only of RNA extraction method and real-time reagent; other methods can be used. The primers and probes for each assay have been designed with annealing temperatures to work with the real-time program stated in the methods.

Methods for detecting a generic pospiviroid assay and specific assays for CEVd, CLVd and TASVd using the Taqman real-time PCR technology.

RNA extraction

Extract RNA from leaf material using the Qiagen RNeasy plant kit (cat. No. 74904) following manufacturers instruction. The use of beta-mercaptoethanol is optional and can be omitted providing samples are processed quickly.

Control assay

Check the quality of the RNA to amplify in a Taqman reaction using an assay such as COX.

Cox F 5'-CGT CGC ATT CCA GAT TAT CCA-3'
Cox R 5'-CAA CTA CGG ATA TAT AAG RRC CRR AAC TG-3'
Cox P 5'-AGG GCA TTC CAT CCA GCG TAA GCA-3'
The probe is typically VIC-TAMRA labelled.

Real-time reagents

Applied biosystems TaqMan® 1000 RXN Gold with Buffer A Pack, Part No. 4304441
This supplies the buffer, Taq and $MgCl_2$ (25mM). In addition NTPs should be used at a concentration of 2.5mM, each primer at 7.5pmol/µl and each probe at 5.0pmol/µl. The reverse transcriptase (RT) is from Fermentas M-MuLV 200U/µl (cat. No. EP0441)

1µl of RNA should be added to the following for each reaction.

	1x in µl
Buffer A	2.5
$MgCl_2$	5.5
dNTP	2
Primer F	1
Primer R	1
Probe	0.5
TaqGold	0.125
RT	0.05
SDW	11.375
Total	24.00

Real-time program

Thermal cycling conditions:
48°C for 30 minutes, 95°C for 10 minutes. Followed by 40 cycles of: 95°C for 15 seconds, 60°C for 60 seconds.

CEVd Assay

CEVd-P2-337	5'- CCC TCG CCC GGA GCT TCT CTC TG
CEVd-F2-304	5'- CTC CAC ATC CGR TCG TCG CTG A
CEVd-R2-399	5'- TGG GGT TGA AGC TTC AGT TGT

CLVd Assay

CLVd-P	5'- AGC GGT CTC AGG AGC CCC GG
CLVd-F	5' GGT TCA CAC CTG ACC CTG CAG
CLVd-R	5' CGC TCG GTC TGA GTT GCC
+	
CLVd-F2	5'- AAA CTC GTG GTT CCT GTG GTT

TASVd Assay

TASVd-P2-228	5'- TCT TCG GCC CTC GCC CGR
TASVd-F2-200	5'- CKG GTT TCC WTC CTC TCG C
TASVd-R2-269	5'- CGG GTA GTC TCC AGA GAG AAG

Generic Assay

This assay is composed of 2 probes, 2 forward primers and 2 reverse primers. A mix of each of the two components is made to give one tube for each primer and the probe. The forward and reverse primers have each primer at a final concentration of 7.5pmol/μl (37.5μl of each and 425μl sdw). The probes have a final concentration of 5pmol/μl of each probe (25μl of each and 450μl of sdw).

Pospi-P12	5'-CTY GWG GTT CCT GTG GTD CWC WCC TGA C-3'
Pospi-P22	5'- CTG TGG TGC TCA CCT GAC CCT GCA-3'
Pospi-F356a	5'-GGW GWA AGC CCK TGG AAC C-3'
Pospi-F356b	5'-GGT GTT TAG CCC TTG GAA CC-3'
Pospi-R96	5'-GGC TCC TGA GAC CGC TCC T-3'
Pospi-R102	5'-CCC GGG GAT CCC TGA AG-3'

Appendix C

Methods of choice by the ring-testing partners.

Each letter represents one of the nine partners that participated in the ring-testing. Their letters shows the RNA extraction method, company reagents and real-time machines used by each partner.

RNA extraction method

B, C, D, E, F, G, H, I	(Qiagen RNeasy)
J	(Kingfisher)

Real-time reagent

C, D	(Qiagen)
E, F, G, I, J	(Applied Biosystems)
H	(Ambion)
B	(Invitrogen)

Real-time machines

G	(Corbet Rotor gene)
C, F, H, I, J	(Applied Biosystems 7900/7500)
B, E	(Biorad)
D	(Stratagene)

Appendix D

Protocol for test of pospiviroids by common RT-PCR

Based on the paper of Verhoeven, J.Th.J. , Jansen, C.C.C., Willemen, T.M., Kox, L.F.F., Owens, R.A. & Roenhorst, J.W. 2004. European Journal of Plant Pathology 110, 823-831.

RNA-extraction
Different methods are available.
We use the RNeasy Plant Mini Kit with RLT buffer and β-Mercaptoethanol added according to the protocol and elute RNA in 50 µl water.
Use max 100 mg fresh plant material per sample or 5 mg freeze dried plant material.
The plant material can be homogenized 1) in a mortar with liquid nitrogen or 2) in a plastic bag (like the ones used for ELISA sample preparation) with extraction buffer.

Primer pairs:
Two sets of primers are used:
Pospil and Vid.

Pospi1-RE: 5'-AGC TTC AGT TGT (T/A)TC CAC CGG GT-3'.
Pospi1-FW: 5'-GGG ATC CCC GGG GAA AC-3'.
Size of the expected PCR product: 197 bp.

Vid-RE: 5'-CCA ACT GCG GTT CCA AGG G-3'.
Vid-FW: 5'-TTC CTC GGA ACT AAA CTC GTG-3'.
Size of the expected PCR product: 359 bp.

RT-PCR
Two RT-PCR procedures are given using one-step RT-PCR kit from Qiagen and one-step RT-PCR kit from Invitrogen, respectively.

One-step RT-PCR kit from QIAGEN (Catalog no. 210212)
Use the procedure described in the protocol for the QIAGEN OneStep RT-PCR Kit and Q-Solution. Add 1 ul template RNA to the master mix.

RT-PCR reactions:
30 min at 50 °C (cDNA synthesis)
15 min at 95 °C (hot start activation of Taq polymerase)

35 cycles of:
30 s at 94 °C (denaturation)
60 s at 62 °C (annealing)
60 s at 72 °C (elongation)

7 min at 72 °C (extension)

∞ 4 °C (hold)

PCR product analysis
Electrophorese the PCR product in TAE buffer through a 1.8 % or 2% agarose gel containing ethidium bromide (5.0 µg ml^{-1}).
A 766 bp ladder (BioLabs) is used to estimate sizes of the PCR products.

One-step RT-PCR kit from Invitrogen (Superscript one-step RT-PCR system with Platinum Taq kit with article number 10928-042 (for 100 reactions) or 10928-034 (for 25 reactions).
RT-PCR reactions:
30 min at 43 °C (cDNA synthesis)
2 min at 94 °C (hot start activation of Taq polymerase)

15 cycles of:
30 s at 94 °C (denaturation)
1.5 min at 62 °C (annealing)
45 s at 72 °C (elongation)

30 cycles of:
30 s at 94 °C (denaturation)
45 s at 59 °C (annealing
45 s at 72C (elongation)

7 min at 72 °C (extension)

∞ 4 °C (hold)

PCR product analysis
Electrophoresis through a 2.0 % agarose gel in TAE buffer containing ethidium bromide (5.0 µg ml^{-1}).
A 1 kbp ladder (Invitrogen) is used to estimate sizes of the PCR products.

Precipitation of PCR product
The PCR products are purified using QIAGEN MinElute PCR Purification Kit according to the manufacture's handbook.
10 µl product is obtained. 1 µl is used for running a control on a 2 % agarose gel in TAE buffer containing ethidium bromide (5.0 µg ml^{-1}).
The remaining DNA is air-dried at room temperature.

Sequencing
The precipitated DNA and a requested amount of forward primer is mailed to a commercial firm that carry out sequencing, for example EurofinsDNA on the web-address: http://www.eurofinsdna.com/home.html.

Phylogenetic analysis
The resulting sequences can be BLASTed at the NBCI Genbank. Enter the following net address: http://blast.ncbi.nlm.nih.gov . Choose *Basic BLAST/nucleotide blast* and create multiple alignments of related sequences.

Work Package 5 - Seed test

Summary
This working group was concerned with the development of a protocol for detection of PSTVd in tomato seeds. For these studies, PSTVd infections in tomato plants were established and seeds taken from ripened fruits were analysed for presence of PSTVd using RT-PCR, real-time RT-PCR and growing-on tests followed by RT-PCR. The rate of seed infestation and the number of PSTVd infected plants arising from seeds carrying PSTVd were determined. Crucial parameters for viroid detection in seeds and the threshold for PSTVd detection in seed lots were studied.
The main conclusions obtained are: PSTVd is present in seeds of infected tomato and can lead to seedling infections.
Uncertainty exists whether seed infestation with PSTVd is also depending on tomato genotype and viroid strain!
The viroid cannot be inactivated by any of the routinely used seed treatments hence contaminated seeds are a potential source of PSTVd outbreaks.
From the infection experiments, the extend of natural viroid infestation cannot be deduced and realistically lies far beyond the recorded numbers.
PSTVd can be detected in seeds by RT-PCR and/or real-time RT-PCR. Seed testing of larger sample batches requires real-time RT-PCR. The threshold of reliable PSTVd detection is at < 1:1000 infected/healthy seeds.
Variable PSTVd concentrations in individual seeds and lower percentage of seed infections in commercial seed production render it difficult to evaluate a detection threshold and sample size. Future work should address how sub sampling can increase the likelihood of detecting PSTVd in large seed lots.

Objectives of WP5
To develop a protocol for detection of PSTVd in tomato seeds, including

- obtaining PSTVd infected tomato seeds.
- comparing methods for RNA extraction and exploring viroid cDNA amplification.
- evaluating RT-PCR and real time PCR detection methods.
- determining the infection rate of seed lots from PSTVd-infected tomato plants.

Task 5.1. Access to PSTVd infected seeds

Objectives of Task 5.1
To obtain PSTVd infected tomato seeds for the activities in WP5.

Methods and Results
The time consuming production of infected tomato seeds was initiated before experiments could be started. To produce and obtain seeds from PSTVd infected tomatoes presented an unexpected challenge for WP5 activities. While in a seed lot taken from PSTVd infected plants proved free of the viroid, a further challenge was to find the right growth stage of the tomato plant for the inoculation. A PSTVd isolate from *Solanum jasminoides* was used to establish PSTVd infections in tomatoes (var. Linda) using mechanical transmission or approach grafting for viroid inoculation. Infections at seedling stages severely reduced num-

bers and size of fruits with only few seeds produced per fruit. PSTVd inoculations of tomato made after the first flowering resulted in PSTVd infections in all plants. Normal sized fruits were produced and germination of seeds from infected plants was comparable to that of non infected tomatoes.

Main conclusions
Sufficient numbers of PSTVd infected tomato seeds were obtained to carry out the project activities.
Deliverable D 5.1. *PSTVd infected tomato seeds* was fulfilled but with delay.

Task 5.2 and 5.3. RNA extraction methods, viroid cDNA amplification, RT-PCR and real-time qPCR assays for viroid detection in seeds

Objectives of Task 5.2 and 5.3
To compare methods for RNA extraction, explore viroid cDNA amplification and to evaluate RT-PCR and real time PCR detection methods for seed detection.

Methods and Results
Several methods, laboratory recipes and commercial kits for RNA preparation from plants were compared. While laboratory recipes (CTAB, silica gel) proved were superior in some cases, a commercial kit (RNAeasy, Qiagen) was favoured for reproducibility and batch homogeneity guaranteed through the industrial QM process.
Reverse transcriptase PCR (RT-PCR) was done following accepted routines either using primer combinations described by Verhoeven *et al.* 2004 and Shamloul *et al.* 1997 or by quantitative RT-PCR using Taq Man assays as described by Boonham *et al.* 2004. These tests reliably detected PSTVd in RNA preparations from plant tissues as well as from seeds.
PSTVd detection by RT-PCR in RNA prepared from leaves from infected plants was generally straight forward and viroid detection was most reliable when young leaves from plants grown at high temperatures (>26 °C) were subjected to the assay.
Most crucial to detecting PSTVd in seeds was sample disruption and homogenization prior to RNA extraction. Ambiguities in detecting PSTVd in seeds were attributed to variable concentrations of PSTVd in seeds but also to processing of seed extracts. Homogenisation of single or only few seeds was done either using a pestle and mortar or a tissue lyser (Retsch/Qiagen). Processing of larger sample sizes (>100 seeds), however, required soaking of seeds and through blending. Best results were obtained using paddle blenders for food microbiologists (Stomacher, MiniMix), which by strong stirring and extrusion forces results in quite homogenous suspensions.
Activities of pre-amplification methods to enrich for target sequences were not initiated owed to the effort instead was put into solving the unpredictable problem with sample disruption and homogenization prior to RNA extraction described above.

Main conclusions
Several methods for RNA extraction from dry seeds can be used, but a commercial kit (RNAeasy, Qiagen) was favoured. PSTVd can be detected in seeds by RT-PCR and/or real-time RT-PCR. Seed testing of larger sample batches requires real-time RT-PCR.

However most crucial to detecting PSTVd in seeds is sample disruption and homogenization prior to RNA extraction.
Deliverable D 5.2. *A recommended protocol for extraction of PSTVd RNA from tomato seeds* fulfilled.

Task 5.4. Seed infection in PSTVd infected tomato

Objectives of Task 5.4
To determinate the infection rate of seed lots from PSTVd-infected tomato plants.

Methods and Results
Seed infestation and PSTVd infections in seedlings from infested seed. When single seeds were subjected to PSTVd testing, the viroid was found in all seeds from PSTVd infected plants. In growing-on tests with 3 x 100 seedlings from infested seeds, 5-7% seedling infections were determined. To investigate whether seedling infections arose from PSTVd seed coat contamination and subsequent introduction of PSTVd by wounding, several seed treatments were compared:
100 seeds from a seed lot from PTSVd infected tomatoes were treated in 30 ml solution at room temperature with agitation.

A 4 % Menno Florades for 60 min (Menno Chemie)
B Na_3PO_4 (10%) for 180 min (Cordoba-Selles et al. 2007, Plant Disease)
C 3 % v/v Pectinex (Novozyme), 0.1 N HCl) 24 hours (modified after Cordoba-Selles *et al.* 2007)
D no treatment

None of the treatments reduced the number of seedling infections arising from PSTVd infected seeds and similar to untreated seeds, PSTVd was found in 5-7% of seedlings. To prove the efficacy of the treatments, tomato seeds were incubated for several hours in sap from PSTVd infected *Physalis floridana* plants and left to dry. Thus incrustinated PSTVd was completely washed off by any of the treatments.
Hence it can be assumed that PSTVd infections in seedling arise either from PSTVd penetrated deeply into the seed coat or, from PSTVd invasion into the embryo for which proof however is pending.

Threshold for PSTVd detection in seed lots. To determine optimum sample size for reliable detection of PSTVd in seed lots, seeds from non infected tomato plants were spiked with seeds from PSTVd infected plants and subjected to seed testing by work package partners. There was no difference in detection of 10 infected seeds blended either in 90 or 990 seeds while highly variable results were obtained testing individual seeds pooled with 1000 healthy seeds. In this serial experiment, PSTVd was either not detected or, detection was reached with high (ct) values. This showed that PSTVd infestation/contaminations of seeds are very variable. PSTVd in highly contaminated seeds can be detected even in a 1:1000 mix, while low PSTVd concentrations in seeds are not detected. Hence a threshold for reliable detection of PSTVd by real-time RT-PCR is at 1000 seeds and sub sampling has to be considered to improve reliability of PSTVd testing.

Main conclusions

PSTVd is present in seeds of infected tomato and can lead to seedling infections.
Uncertainty exists whether seed infestation with PSTVd is also depending on tomato genotype and viroid strain!
The viroid cannot be inactivated by any of the routinely used seed treatments hence contaminated seeds are a probable source of PSTVd outbreaks.
From the infection experiments, the extend of natural viroid infestation cannot be deduced and realistically lies far beyond the recorded numbers.
PSTVd can be detected in seeds by RT-PCR and/or real-time RT-PCR. Seed testing of larger sample batches requires real-time RT-PCR. The threshold of reliable PSTVd detection is at < 1:1000 infected/healthy seeds.
Variable PSTVd concentrations in individual seeds and lower percentage of seed infections in commercial seed production render it difficult to evaluate a detection threshold and sample size. Future work should address how sub sampling can increase the likelihood of detecting PSTVd in large seed lots.
Deliverable D 5.3. *A report on the infection rate of seeds from PSTVd infected tomato plants* fulfilled.

Disseminations

The results were presented on an Ad-hoc EPPO meeting on tomato viroids in Ljubljana, 2009-10/28-29.

References

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Córdoba-Sellés, M., García-Rández, A., Alfaro-Fernández, A., & Jordá-Gutiérrez, C. 2007. Seed Transmission of Pepino mosaic virus and Efficacy of Tomato Seed Disinfection Treatments. Plant Disease 91, 1250-1254.
Shamoul, A. M., Hadidi, A., Zhu, S. F., Singh, P. R. & Sagredo, B. 1997. Sensitive detection of potato spindle tuber viroid using RT-PCR and identification of a viroid variant naturally infecting pepino plants. *Canadian Journal of Plant Pathology* 19, 89-96.
Verhoeven, J.Th.J., Jansen, C.C.C., Willemen, T.M., Kox, L.F.F., Owens, R.A. and Roenhorst, J.W. 2004. Natural infections of tomato by *Citrus exocortis viroid*, *Columnea latent viroid*, *Potato spindle tuber viroid* and *Tomato chlorotic dwarf viroid*. European Journal of Plant Pathology 110: 823-831.

Appendixes

Appendix 1. Research Consortium Agreement

Version [4 final] j. []

CO-OPERATION AGREEMENT

Made between the undersigned,

Aarhus University
The Faculty of Agricultural Sciences
Blichers Allé 20, P.O. Box 50
DK-8830 Tjele
CVR: 57607556
Hereinafter called DJF

and

The Department for Environment, Food & Rural Affairs, Nobel House, 17 Smith Square, London SW1P 3JR
acting through the Central Science Laboratory,
Sand Hutton, York YO41 1LZ, UK
VAT:
Hereinafter called CSL

and

Plant Protection Service
P.O. Box 9102, 6700 HC Wageningen
The Netherlands
VAT:
Hereinafter called PPS

and

Agricultural Institute of Slovenia
Hacquetova 17, 1001 Ljubljana, Slovenia
VAT: SI 23887729
Hereinafter called AIS

and

INRA Equipe de Virologie, UMR
GDPPIBVM, Campus INRA
78 Avenue Edouard Bourleaux
33883 Villenave d'Ornon cedex, France
VAT:
Hereinafter called INRA.

and

Austrian Agency for Health and Food Safety (AGES)
Institute of Plant Health
Dept. for Phytopathology, Fruit Production & Viticulture
Spargelfeldstr. 191, 1226 Vienna, Austria
VAT:
Hereinafter called AGES

and

German Collection of Microorganisms and Cell Cultures, DSMZ, Plant Virus Department
c/o JKI, Messeweg 11/12, 38104 Braunschweig, Germany
VAT:
Hereinafter called DSMZ

and

Laboratoire National de la Protection des Végétaux
7 Rue Jean Dixmeras
49044 Angers Cedex 01, France
VAT:
Hereinafter called LNPV

hereinafter called collectively the Parties/Partners or Party/Partner,

concerning the Project:

Detection and Epidemiology of Pospiviroids (DEP)
which will be implemented under a subsidy granted under the EUPHRESCO, administered by the EUPHRESCO Call Secretariat, C/o Austrian Agency for Health and Food Safety (AGES), Institute of Plant Health, Spagelfeldstr. 191, 1226 Vienna, Austria.

1. Project Title
English: **Detection and Epidemiology of Pospiviroids**

Short version:DEP

hereinafter called the Project.

2. Project Objective

The Parties agree to enter into co-operation on the Project as described in the application of 7th May 2008 and with clarification points of 15th July 2008 from the parties for subsidy for research and development pilot projects under EUPHRESCO and confirmation of approval of funding from the Call secretariat of 3rd October 2008.
The aim of the project is to provide new knowledge of the epidemiology and diagnosis of *Potato spindle tuber viroid* (PSTVd), *Citrus exocortis viroid* (CEVd), *Columnea latent viroid* (CLVd), *Chrysanthemum stunt viroid* (CSVd), *Tomato apical stunt viroid* (TASVd) and *Tomato chlorotic dwarf viroid* (TCDVd) with major efforts targeting (1) the risk of transmission of pospiviroids from ornamental plants to crops of tomato and potato and (2) the development and validation of diagnostic laboratory manuals to be presented to national and EC plant health authorities for surveillance and seed-testing purposes.

3. Finances

Financial support for the above Project has been granted by the national funding organisations involved in the EUPHRESCO pilot call titled Epidemiology and diagnosis of Potato spindle tuber viroid and other pospiviroids of plant health concern in relation to Solanacea.

The Project Coordinator responsible for the Project vis-à-vis EUPHRESCO is DJF represented by Steen Lykke Nielsen

All costs connected with the implementation of the Project must be paid by each of the Parties in accordance with approved budgets. Each of the Parties must prepare activity accounts for their respective sub-budgets. The total amount appointed in the project budget will be deemed to constitute as the maximum commitment of each party.

The accounts must be prepared in accordance with the current rules in force for the national funding organisations.
According to the contract conditions in “Applicants’ Guide for the EUPHRESCO Pilot Call with “virtual common pot” funding for transnational research projects” (Annex 1) the overall principle is that changes to the budget cannot occur during the contract. However, minor

changes are permitted, but must be agreed on between the specific consortium partner and its national funding organisation. The Coordinator must be informed immediately.

4. Organisation

The Coordinator and the Work Package (WP) Leaders make up a Steering Group. The Steering Group will have the overall technical responsibility and will also be responsible for ensuring that the deliverables of the project are delivered in time. This implies that the Project at all times will meet its objective, and that the time schedule set out for the Project is complied with. Any decision on major changes in the course of the Project shall only be made unanimously by the Steering Group.

The Steering Group shall consist of:

Steen Lykke Nielsen, DJF Coordinator and WP1 and WP2 leader.
J.T.J. Verhoeven, PPS, WP3 leader.
Rick Mumford, CSL, WP4 leader.
Stephan Winter, DSMZ, WP5 leader.

However, it is the responsibility of all consortium partners to live up to and fulfil their part of the DEP-project as described in the project application.
In the contract conditions in "Applicants' Guide for the EUPHRESCO Pilot Call with "virtual common pot" funding for transnational research projects" (Annex 1) is stated "changes to the composition of research consortia ….cannot occur during the contract. If a research Partner drops out of a consortium, the problem has to be solved by the consortium" in line with the Consortium agreement.

5. Reporting

It is the responsibility of the Coordinator to work out a short note on the progress and activity plan for the last 5 months from the midway project meeting of the Steering Group. The note will be discussed by all partners of the Consortium. The note must be presented to the Call Steering Committee about the UPHRESCO Pilot Call: DEP.

No later than 2 months after completion of the Project, the Coordinator in collaboration with all Consortium Partners must present a final report on the research results achieved. The report must be submitted to the Call Steering Committee about the UPHRESCO Pilot Call: DEP, and the whole report or parts of it must be published on the web side of EUPHRESCO. The report must also be offered to The Standing Committee for Plant Health in EU.
Separate diagnostic protocols, which are the outcome of activities in WP4 and WP5, must be prepared by WP4 and WP5 leaders and submitted to EPPO by the Coordinator on behalf of the Consortium.

6. Confidentiality

Confidential Information means any of whatever nature or form that is disclosed information by one Party (the Disclosing Party) to another Party (the Receiving Party) in connection with the Project, which

- is clearly marked "Confidential"; or
- if disclosed orally, was at the time of disclosure marked as "Confidential" and within 30 (thirty) calendar days changed to a physical form and marked "Confidential" by the Disclosing Party.

Each Party is under the obligation to treat the Confidential Information received in relation to the Project as if it was its own Confidential Information.

The duty of confidentiality does not apply to any information which:

- was at the time of disclosure to the Receiving Party readily accessible or in the public domain, i.e. published or otherwise generally available to the public; or
- has after disclosure to the Receiving Party been published or become generally available to the public otherwise than through any act or omission on the part of the Receiving Party; or
- was already in the possession of the Receiving Party, without any restrictions on disclosure, prior to or at the time of disclosure to the Receiving Party; or
- was rightfully acquired or received from others without any undertaking of a duty of confidentiality; or
- was learned or developed independently of the work under the Cooperation Agreement by the Receiving Party; or
- was disclosed by the Receiving Party with the Disclosing Party's prior written approval; or
- consists of information obtained through general and publicly funded research performed at public institutions.
- is required to be disclosed by the Receiving Party pursuant to any court order or statutory duty or other legal requirement.

Any duty undertaken according to this condition will terminate three (3) years after completing of the final Project report unless otherwise stated in the individual stipulations of this Cooperation Agreement.

The Parties are obligated to enjoin on all persons assigned to the Project the same duty of confidentiality stipulations as the duties applying to the Parties.

The duty of confidentiality further comprises Parties/persons, who have withdrawn from the Cooperation Agreement howsoever caused.

This section will be construed in respect of the provision of the "Applicants' Guide for the EUPHRESCO Pilot Call with "virtual common pot" funding for transnational research projects" stating that all Project Results should be made available to the public and of section 8 concerning Publication.

7. Intellectual Property Rights/ exploitation

According to an agreement between the Coordinator and all partners, no partner has claimed intellectual property rights to any products that will be the outcome of the project. However,

the infectious cDNA clones delivered from WP2 will be distributed only for non-commercial use under a material transfer agreement set up by INRA.

8. Publication

All publications must observe the common rules for authorship set by the Vancouver Requirements. Further more, according the"Applicants' Guide for the EUPHRESCO Pilot Call with "virtual common pot" funding for transnational research projects": When the Partners give information concerning the Project and its results in scientific publications, in any information material or at seminars or in connection with other public relation activities it should be clearly stated that the Project has been financially supported by EUPHRESCO and the national funding organisation(s).

9. Liability

Any claim for damages between the Parties for any loss, material or personal damages originating as a result of carrying out the work described herein this Agreement must be settled according to the Belgian general law of damages. The Parties cannot claim damages for loss of profit or indirect losses.

The Parties will not be liable to each other if the project does not lead to the expected results, or if the results cannot be exploited, or if the time schedule cannot be kept due to unforeseen problems.

If one or more Parties are exploiting the Project Results commercially, it is such Party's own responsibility, and the other Parties cannot claim against each other with reference to the law on product and professional liability.

10. Disputes

Any disputes or differences arising from the implementation of this Cooperation Agreement, which cannot be settled amicably at executive level between the Parties, must be brought before a Belgian court of law and decided finally according to Belgian law.

11. Accession, Termination and breach of agreement

This Agreement shall come into force retroactively as from 31. October 2008 – after the Parties have duly signed the Cooperation Agreement. The Cooperation Agreement will remain in full force and effect until any one of the following events occurs:

- The Project is accomplished and completed as described in the Project description and appurtenant Annexes, and the final report is approved by EUPHRESCO's Call Steering Committee about EUPHRESCO Pilot Call: DEP.

- The Parties all agree to terminate the Project howsoever caused.

- A Party may leave the Project if the other parties all agree and the EUPHRESCO's Call Steering Committee about EUPHRESCO Pilot Call: DEP approves it

After any conclusion of the Project, the Parties will have no obligations to each other, except for such conditions as may be expressly stipulated in this Cooperation Agreement and in the DEP project description.

If a Party commits a material breach of any obligation imposed by the present Cooperation Agreement, the other Parties may give notice of termination of the Cooperation Agreement to the Party in question. However, such termination is only available when the defaulting Party has not– within 14 days after receiving a demand to do so –taken steps to remedy the default. Notice thereof must be sent by registered post, and the Project Coordinator shall notify the EUPHRESCO's Call Steering Committee about EUPHRESCO Pilot Call: DEP. The confidentiality provisions will survive any such breach.

This Cooperation Agreement is executed in 8(eight) counterparties, (one for each Party), each of which will be deemed an original, but all of which will constitute one and the same instrument.

Annexes:

Annex 1: "Applicants' Guide for the EUPHRESCO Pilot Call with "virtual common pot" funding for transnational research projects"

Annex 2: Application for the project: Detection and Epidemiology of Pospiviroids (DEP) of 7th May 2008.

Annex 3: Clarification of points asked by the Call Steering Committee about the EUPHRESCO Pilot Call - Detection and Epidemiology of Pospiviroids (DEP) of 15th July 2008.

Signatures and date (supplemented with name and title in block letters)

Co-operation Agreement regarding the EUPHRESCO Pilot Project:
Detection and Epidemiology of Pospiviroids

Authorised to sign on behalf of DJF:

Signature: _____ Date: /

Head of Research Group Steen Lykke Nielsen
Department of Integrated Pest Management, Faculty of Agricultural Sciences, Aarhus University

Signature: _____ Date: /

Head of Department of Integrated Pest Management, Faculty of Agricultural Sciences, Aarhus University

Signatures and date (supplemented with name and title in block letters)

Co-operation Agreement regarding the EUPHRESCO Pilot Project: Detection and Epidemiology of Pospiviroids

Authorised to sign on behalf of ……..:

Signature: _____ Date: /

[Name and title], Partner

Signature: _____ Date: /

[Name and title], Partner

Appendix 2. Minutes of DEP kick off meeting

Minutes of DEP kick off meeting 4th to 5th November 2008 in Aarhus University, Research Centre Flakkebjerg

Participants: Thierry Candres (TC), Ko Verhoeven (Ko), Mojca V. Marn (MVM), Richard Gottberge (RG), Wendy Monger (WM), Martina Paape (MP) (substitute for Stephan Winter), Valeri M-Demilly (VMD) and Steen Lykke Nielsen (SLN).

Notes taken by Steen Lykke Nielsen

Programme. Se the full programme in Annex 1.

WP1. Coordination by Coordinator Steen Lykke Nielsen
Status for signing of the national contract. All partner but Austria and Germany have a signed approved contract. (Stephan Winter informed in a mail received after the meeting that the German contract has been signed).
Overview of the project description and the riders added during the clarification of points asked by the Call Steering Committee. SLN went through the clarification of points asked by the Call Steering Committee. Encapsulation has been omitted in the project description. However, it was suggested to look for PLRV in *Solanum jasminoides* and other solanaeceous ornamentals in the survey.
Status for the research consortium agreement. Each partner received a copy of a Research Consortium Agreement worked out by SLN and were asked to have it approved at their Institute. Suggestions for changes in the text should be mailed to SLN, who will make corrections and mail a new version to all partners to sign it. A deadline was not settled, but SLN has afterwards set **a deadline for corrections to 30th November 2008** latest. SLN mail an electronic version to all partners.
Affiliation, participation and /or synchronization of external research groups. Very positively quite a lot of scientists have applied to be affiliated to DEP. The Consortium is positive and it was decided that SLN contact these persons and ask them to contact the WP leaders of the specific topics they are interested to be affiliated to. WM proposed to use the CSL standard for cooperation agreement with non partners. (Wendy, will you please mail me the CSL standard to the partners in the DEP-consortium).

WP2. Host range studies and interactions. Coordinator Steen Lykke Nielsen

A. *Overview of the viroid isolates available and decision of which ones to include in the project collection and agreement of how to exchange material between partners.* Ko, MP and SLN presented a list of the isolates of the six pospiviroids available at their Institutes. TC, MVM, RG, VMD and WM will mail their lists to SLN, who will put together all the lists in a common list set up like KO's list including name of viroid, no. of isolate, year, original host, sequenced: yes/no, accession no., present as nucleic acid or in live host. It was decided that the isolates shared in the DEP-project only are shared between the 8 partners and are not used for commercial use, and before passed on to non-partners, acceptance must be obtained. Ko said that his experience with inoculation of PSTVd was that pure water was better than a standard inoculation buffer.
B. *Plans for the surveys in The Netherlands, France and Denmark.* SLN expressed a little concern about Danish growers might be unwilling to have their plants included in the survey, because of the implications if quarantine viroids are detected in their

glasshouses. Ko and TC did not shared this concern for The Netherlands and France respectively. MVM informed that Slovenia also will carry out a survey for pospiviroids in ornamentals and the results can be included in the final report. It was decided that both positive **and negative** results shall be recorded and included in the report. Ko suggested to include test for potato leaf roll virus in *Solanum jasminoides* and other solanaceous species to obtain results that might contribute to assessment of the probability that encapsulation can be a possibility.

C. *Plans for collection of solanaceous weeds.* Will be carried out in The Netherlands, Denmark and France.
D. *Establishment of infectious cDNA clones of the six viroids.* TC informed that the infectious clones are for use for reference and inoculation of weeds. Which isolates to be included? No decision was made.
E. *Transmission of viroids from ornamental to tomato/potato and sequence analysis.* Ko informed abut the planned activities.

WP3. Transmission pathways Coordinator Ko Verhoeven

A. *Introduction to the WP and the specific tasks.* It was decided that all partners involved in the transmission activity shall use the same PSTVd-isolate. It was decided to use the PSTVd isolate S1 which is isolated from *Solanum jasminoides* by Ko as the primary PSTVd isolate and the PSTVd-isolate isolated from *Brugmansia* sp. by Ko as the secondary isolate. Where flowering ornamental is included in the trial it was decided to use PSTVd-infested *Solanum jasminoides* if possible (for example if it is sufficient attractive for bees to forage on. Else another ornamental must be included). Ko can supply other partners with stem cuttings of PSTVd-infested *Solanum jasminoides*. All interested partners should immediately obtain a Letter of Authority allowing Ko to mail the stem cuttings.
   Ko listed 3 kind of possible transmissions by bees and thrips: 1) By pollination (between the same plant species); 2) Mechanical transmission via the insects (the viroid is carried directly on the insect); 3) Mechanically transmission by the insects via viroid-infected pollen transferred by the insects.
B. *Transmission by thrips. Coordination of the plans for partner 6 (Thrips tabaci) and partner 1 (Frankliniella occidentalis). Which ornamental to be included? Can sufficient tomato pollen be obtained?* It was decided that for the *Frankliniella occidentalis* part, the plan about thrips feeding on pollen should be changed to that thrips feed on pollen from ornamental and afterwards suck on tomato (and not the opposite as written in the project description). RG, Ko and Annie Enkegaard from Denmark met separately and discussed in details the thrips experiments. When the plans have been adjusted, they shall be sent to Ko and the other partners in the thrips part.
A. *Transmission by bees. Coordination of the plans for partner 4 (bumblebees) and partner 1 (honeybees). Which ornamental to be included? Can sufficient tomato pollen be obtained?* In the project description it is not possible to distinguish between mechanical and pollen transmission. It should be changed. Maybe it could be made as Petri dish assays. MVM will include PSTVd infested *Petunia* sp. as ornamental plant, because MVM has found PSTVd-infested *Petunia* in Slovenia. SLN considered to include *Petunia* and *Nicotiana glutinosa* as pollen producing ornamentals, but first it must be elucidated whether PSTVd infects the pollen of these plants. TC doubted that *N. glutinosa* is a good host for PSTVd at all. As mentioned above it was decided first to test whether *S. jasminoides* can be used as the ornamental. Anyway, the ornamental to be included must primary be inoculated with the PSTVd-isolate S1 as mentioned above. MVM, SLN and Per Kryger from Denmark met separately and discussed in de-

tails the bee experiments. When the plans have been adjusted, they shall be sent to Ko and the other partners in the bee part.

B. *Mechanical transmission through cultural practice. Which ornamental to be included?* Ko informed abut the planned activities.

WP4. Diagnostics. Coordinator Rick Mumford/Wendy Monger.

A. *Introduction to the WP and the specific tasks.* WM introduced the activities.
B. *Status for which assays are already available.* Some work on diagnostic of some of the pospiviroids has already been carried out in other UK projects and these results will be published independently of DEP.
C. *Overview of which assays to develop during the project.* SLN is responsible for the common RT-PCR. WM is responsible for development of a generic TaqMan assay and specific assays for CEVd, TASVd and CLVd. Concerning extraction methods the experience already obtained by WM is that all methods work very well. WM will in the protocol describe the extraction method in general and give 3 examples: King Fisher, C-TAB and ?? (RNAeasy??). **Wendy, please correct!** For developing of the assays WM wants to obtain all pospiviroids available except for PSTVd only isolates, which have been sequenced. PSTVd will be propagated in tomato.
D. *Validation by ringtesting. Time schedule. Who will participate?: Partners in DEP. Other laboratories. Statistical analysis of the results.* In the project description is stated that the common RT-PCR protocol first shall be ringtested among the DEP partners and afterwards ringtested among non-partners. The Q-RT-PCR protocols (both the generic and the 3 specific) shall only be ringtested among the DEP partners. The purpose of the ringtests is to test the robustness of the protocols. The kit for the ringtest should include a protocol, samples and primers, and for the Q-RT-PCR also the probe. WM will also include an internal control. SLN will consider that for the common RT-PCR. The same isolates will be used to ringtest the common and the generic Q-RT-PCR.
   A long discussion was made on how comprehensive the generic ringtests (common and Q-RT-PCR) should be. It was decided not to include dilution series (but it was recommended not to use infected leaf material directly, but dilute it). The test sample material should be freeze dried leaf material. The ringtests should include the pospiviroids in different host plants. The ringtest must at least include all 6 pospiviroids plus Iresine viroid, but should only include isolates, which sequences are known to secure no mutations in the primer and probe sites. WM and SLN agree on which isolates to include and who will propagate which.

WP5. Seed test. Coordinator Stephan Winter.

A. *Introduction to the WP and the specific tasks including a time table.* MP introduced the plans of the seed test. Some work on seed testing has already been carried out in other German projects and these results will be published independently of DEP.
B. *Overview of PSTVd-infected seeds available.* Germany does not have many PSTVd infected tomato seeds available. RG has 50 g of PSTVd infected tomato seeds (the S1 isolates from *S. jasminoides*). The German and Danish partner is right now trying to produce more PSTVd infected tomato seeds. SLN uses the mild isolate of PSTVd obtained from Stephan.
C. *RNA-extraction. Which methods to compare?* All methods tested until now see to work fine. DSMZ prefer RNAeasy. Stephan/Martina finds out whether they need a validation test for the extraction.

D. *Amplification before RT-PCR and RT-PCR.* It was noted that rolling circle amplifications implies a serous risk of lab contamination with products.
E. *Infection rate of seed lots. A plan for the trial.* It is important to be able to distinguish between true seed infection and surface contamination, so some seeds should be surface sterilised. The results of the activity: infection rate of seed lots should be included in the final protocol of seed test.
F. *Other matters.* The final protocol of the seed test shall be validated among all 8 partners. There was a long discussion of sampling size. Sampling size should be included in the final protocol. It should be a table connecting the infection rate with how many seeds to be tested to obtain for example 5 % confidence interval. It was recommended to look in the EPPO protocol for test of seeds.
Procedure for cleaning seeds: all partners should use the same procedure. It was decided to use the method normally used by tomato seed producers including low pH and pectinase (a protocol for this tomato seed cleaning procedure is included as Annex 2).
A description of the pre treatment of the seeds before the RNA extraction should be worked out by MP and mailed to the partners.
Stephan is asked to inform the other partners of WP5 how Stephan wants them to be involved in the activities.
SLN informed that a part of the first draft of the seed WP concerning threshold of pooling seeds has been deleted from the final project description.

Dissemination of results. SLN will contact Colin Jeffries and ask which results Colin needs for revision of the EPPO Standard of PSTVd.
EPPO will be offered all protocols, but EPPO cannot use the protocols directly, unless worked into an EPPO Standard. However this is not a part of the DEP-project.
ISTA will be offered the protocol of seed testing for validation.
Concerning the 3 protocols for specific diagnose of the 3 viroids not included in DEP. Wendy and Rick decide if they want these 3 protocols included in the final report as a kind of total package.
TC's infectious cDNA clones will be deposited in DSMZ.
Writing and publishing the results in scientific papers will be discussed at the final DEP meeting.

WP1. Coordination.

A. *Midterm meeting of the WP coordinators. Can this be held as video-link meeting?* It will be aimed at to hold the midterm meeting as a video link meeting. CSL and SLN have the facilities for that. Ko and Stephan find out. If not possible a telephone meeting will be considered. As the last priority a one day meeting in an airport as for example Shiphole will be considered.
B. *Final meeting. Appointment of date and place.* The final meeting will be held in the end of October 2009 as a one and a half day meeting beginning in the morning the first day. MVM has subsequently informed that the final meeting can be held at her Institute in Ljubljana, Slovenia.

Annex 1. Programme of the meeting.

Program Kick off meeting on the EUPHRESCO project Detection and Epidemiology of Pospiviroids (DEP) 4th – 5th November 2008 in Research Centre Flakkebjerg, Denmark.

Participants: Thierry Candres, Ko Verhoeven, Mojca V. Marn, Richard Gottberge, Wendy Monger, Martina Paape, Valeri M-Demilly and Steen Lykke Nielsen.

Monday 3rd November 2008: Arrival at Hotel Frederik d. II, DK-4200 Slagelse.

Tuesday 4th November 2008
08.30-09.00 Pick up at Hotel Frederik d. II and transport to Research Centre Flakkebjerg

Presentation of work package 1-5, experimental plans and time tables.

09.00-09.10. Welcome by **Steen**
09.10-10.00. WP1. Coordination by Coordinator Steen Lykke Nielsen

A. Status for signing of the national contract.
B. Overview of the project description and the riders added during the clarification of points asked by the Call Steering Committee.
C. Status for the research consortium agreement.
D. Affiliation, participation and /or synchronization of external research groups.
E. Dissemination: Postponed to Wednesday morning.

10.00-11.30. WP2. Host range studies and interactions. Coordinator Steen Lykke Nielsen

F. Introduction to the WP and the specific tasks including a time table. **Steen.**
G. Overview of the viroid isolates available and decision of which ones to include in the project collection and agreement of how to exchange material between partners. **All.**
H. Plans for the surveys in The Netherlands, France and Denmark. **Ko, Thierry and Steen.**
I. Plans for collection of solanaceous weeds. **Ko, Thierry and Steen**.
J. Establishment of infectious cDNA clones of the six viroids. **Thierry.**
K. Transmission of viroids from ornamental to tomato/potato and sequence analysis. **Ko.**

11.30-12.30.WP3. Transmission pathways Coordinator Ko Verhoeven

C. Introduction to the WP and the specific tasks (**Ko**).
D. Transmission by thrips. Coordination of the plans for partner 6 (Thrips tabaci) and partner 1 (Frankliniella occidentalis). Which ornamental to be included? Can sufficient tomato pollen be obtained? **Richard and Steen**.
E. Transmission by bees. Coordination of the plans for partner 4 (bumblebees) and partner 1 (honeybees). Which ornamental to be included? Can sufficient tomato pollen be obtained? **Mojca** and **Steen**.
F. Mechanical transmission through cultural practice. Which ornamental to be included? **Ko**.

12.30-13.30. Lunch.

13.30-14.00. Continuation of WP3.

14.00 – 15.00. WP4. Diagnostics. Coordinator Rick Mumford.

E. Introduction to the WP and the specific tasks. **Wendy**
F. Status for which assays are already available. Introduction by **Wendy**.
G. Overview of which assays to develop during the project. Introduction by **Wendy**.
H. Validation by ringtesting. Time schedule. Who will participate?: Partners in DEP. Other laboratories. Statistical analysis of the results **Steen**.

15.00-15.30. Coffe brake.

15.30-16.00. Continuation of WP4.

16.00-17.30. WP5. Seed test. Coordinator Stephan Winter.

G. Introduction to the WP and the specific tasks including a time table. **Martina**.
H. Overview of PSTVd-infected seeds available. **All**.
I. RNA-extraction. Which methods to compare? **Martina**.
J. Amplification before RT-PCR and RT-PCR. **Martina**.
K. Infection rate of seed lots. A plan for the trial. **Martina**.

17.30. End of the day. Transport back to the hotel.

19.00. Dinner at Hotel Frederik d. II

Wednesday 5th November 2008

08.30-09.00. Check out at Hotel Frederik d. II and transport to Research Centre Flakkebjerg.

09.00-09.30: Dissemination of results. **Steen**

09.30-11.00. Follow up on matters from yesterday. **All.**

11.00-11.15. WP1. Coordination. **Steen.**

C. Midterm meeting of the WP coordinators. Can this be held as video-link meeting? Appointment of date.
D. Final meeting. Appointment of date and place

11.15-11.30. Concluding remarks. **Steen.**

11.30-12.30. Guided tour at Research Centre Flakkebjerg. **Steen.**

12.30-13.00. Lunch

13.00-13.30 Transport to Slagelse Railway Station.

13.54 Train departure.

15.15 Arrival at Copenhagen Airport.

You are all asked to bring a list of the isolates of the six pospiviroids you have in your national collection.

Appendix 3. Midterm report

May 15, 2009

EUPHRESCO Pilot project: Detection and Epidemiology of Pospiviroids (DEP).
Midterm report.

WP1 - Project Management and Coordination.

A kick off meeting was held in Denmark 4th to 5th November 2008.
A midterm telephone meeting was held with participation of the WP-leaders May 15th, 2009.
The final project meeting has been scheduled to 27th to 28th October 2009 at the Agricultural Institute of Slovenia in Ljubljana.
A joint meeting on tomato viroids in cooperation with EPPO by Vice Director Francoise Petter in continuation of the final project meeting is under preparation.
Conclusion WP1: the milestones until now have been achieved.

WP2 - Host range studies and host-viroid interactions.

Task 2.1 Establishment of a collection of isolates of the six pospiviroids: The project partners have obtained access to all partners' collections of isolates of the six viroids. Isolates have been distributed among the partners.

Task 2.2 Surveying for new host plants of pospiviroids: Partner 1: has obtained an agreement with the Danish Plant Directorate to obtain plant samples collected by the inspectors of the Plant Directorate during their inspections of glasshouse and nursery crops in the period May – September 2009. An agreement has been obtained with the Botanical Garden of Aarhus and the Botanical Garden of Copenhagen to admission to collect plant samples of *Solanaceae* and *Gesneriaceae* from the Botanical Gardens' collections. The sampling will take place in May to July 2009.
Partner 3: 55 weed samples (2 *Datura stramonium*, 10 *Solanum dulcamara* and 43 *Solanum nigrum*) have been tested for pospiviroids; all negative.
91 ornamental plant samples (including *Physalis peruviana*) have been tested for pospiviroids; IrVd-1 was identified in *Celosia plumose*, and PSTVd in *Brugmansia* spp., *Physalis peruviana* and *Solanum jasminoides*. More samples will be tested.
Partner 5: Contacts have been made with a technical institute providing advice to nurseries to gain access to samples of possible host plants. An agreement has also been reached with the Plant Protection Service to have access to the samples that will be collected during the annual survey of nurseries for PSTVd presence. These samples will be evaluated for the presence of other viroids.
Approximately 25 samples of potential host plants have already been tested, all negative so far.

Task 2.3 Production of infectious cDNA clones of the six targeted pospiviroids:
Task 2.3 Production of infectious cDNA clones of the six targeted pospiviroids
PCR amplification of full-length genomes has been performed for PSTVd, CSVd, TASVd and TCDVd and the corresponding cDNAs sequenced and cloned in a suitable plasmid vector so as to generate infectious constructs. Tests to validate the in-

fectivity of the constructs are ongoing. The next steps are to generate similar constructs for the last two viroids (CEVd and CLVd), to complete the infectivity assays and to then use the constructs to perform artificial inoculation trials for CEVd, CLVd, TASVd and TCDVd on solanaceous weeds (Task 2.2)

Task 2.4 Transmission of viroids from ornamentals to tomato and potato: 11 pospiviroid isolates from ornamentals (1x CEVd; 7x PSTVd; 1x TASVd and 2x TCDVd) and one isolate of PCFVd from pepper were successfully transmitted to potato and tomato by mechanical inoculation.
Task 2.5 Sequence analysis: Isolates of WP 2.4 were sequenced before and after transmission to potato and tomato plants. In total 348 sequences have been determined; a few are still to come. The sequences still need to be analysed.

Conclusions WP2: Task 2.1 and 2.3 are fulfilled. Tasks 2.2, 2.4 and 2.5 are running according to the plans.

WP3 – Transmission pathways

Task 3.1 Transmission by thrips: Partner 1 and 6 have prepared detailed experimental plans, and infectious plants for the experiments have been established. PSTVd infected pollen production has been established. The rearing of partner 1's stock of the thrip's *Frankliniella occidentalis* on the selected ornamental PSTVd-host plants has not yet been successful. A new thrip's stock has been obtained. However, if the rearing on the ornamentals does not turn successfully, the period of thrips sucking/staying on the ornamental will be diminished from days to minutes, still imitating real life situation.

Task 3.2 Transmission by bees: Partner 1: Detailed experimental plans have been prepared. Infectious and non-infectious plants of three ornamentals have been established. Presence of PSTVd in pollen of two of the ornamentals has been verified. Honey bees have been ordered. The experiments are planned to start ultimo May when inflorescence has started.
Partner 4: *Petunia* has been selected as PSTVd infected ornamental for bumblebee transmission experiment. PSTVd has been detected in leaves, calyx, petals and anthers of infected *Petunias.* The transmission experiments will be performed when infected *Petunia* and healthy tomato plants will start flowering.

Task 3.3 Mechanical transmission through cultural practice: Partner 3: Mechanical inoculation of PSTVd from *Brugmansia suaveolens* and *Solanum jasmonides* to tomato has been performed using different inoculum concentrations when inoculated after grinding leaves in water (milli Q) and different latency periods when inoculated directly via contaminated fingers. All experiments were performed at both 15 and 25 ^{o}C. Transmission experiments by tools (knives) are scheduled for the next months.
Partner 4: Experiments with transmission of two different isolates of PSTVd, two different inoculum concentrations in the water supply and two different periods of irrigation to tomato plants have been performed.

Conclusions WP3: All tasks are running according to the plans. The experimental plans for *Frankliniella occidentalis* will be corrected according to the observed behavior of the thrips on the ornamental host plants.

WP 4 – Identification methods and diagnostic protocols

Task 4.1 Development of real time RT-PCR:

Partner 2 has carried out the following activities:

RNA extraction methods have been written up to cover the needs of different labs. This includes a cheap CTAB method, a kit method and an automated method for kingfisher extractions.
The real time assays developed in this project use the Taqman technology (Applied Biosystems). Real time assays for these 3 viroids have been developed. They have been used at FERA to show specific identification of the viroids they were designed for.
A generic real time assay has been developed that will detect, CEVd, CLVd. TASVd, PSTVd, TCDVd and CSVd.
The assays will be tested on a range of viroid isolates by partner 2, 4 and 8.
A generic protocol for detection of viroids using real-time RT-PCR will be prepared during the next months.

Partner 8 will in May test the specific primers and probe of each pospiviroid CEVd, CLVd and TASVd and primers and probe for the generic real time PCR obtained from partner 2. Partner 8 will be using for extraction method EPICENTRE Master-Pure™ RNA Purification kit and for the amplification method we shall use QIAGEN Quantifast Probe RT-PRC + ROX Vial Kit.

Partner 4 has prepared CEVd, CLVd and TASVd infected plants for the test of specific primers and probe and for the test of the test of primers and probe for the generic real time PCR obtained from partner 2.

Task 4.2. Preparing a protocol for common RT-PCR for screening for pospiviroids:

The protocol consists of two parts. A common RT-PCR and a sequence analysis of uncloned PCR products. The common RT-PCR has been tested successfully on isolates of all six pospiviroids. However, the sequencing part causes problems. Therefore the protocol has not yet been finished.

Task 4.3 Validation by ringtesting: Seven non-partners have shown interest in participating in the ringtest. Freeze dried leaf-samples of viroids for the ringtest have been prepared for the test. The ringtest has been delayed because the sequencing part of the protocol of 4.2 causes problems.
Validation of the generic Q-RT-PCR test: initial testing prior to a full-scale ringtest will be started in May.

Conclusions WP4:
4.1. Running according to the plan.
4.2. Delayed because of technical problem with part of the protocol.
4.3. The milestone for the ringtest of common RT-PCR has not yet been fulfilled. It is estimated that it still will be possible to fulfill it in due time.

Work package 5 – Seed test

5.1 Access to PSTVd infected seeds
Seeds from several PSTVd infected tomato batches are now available and will be made available for comparative testing during May.

5.2 RNA extraction methods and target amplification:
In series of repetitive tests to prove the reliability of methods for extraction of RNA from tomato seeds, several commercial products were compared reaching similar results.
The limiting factor to RNA extraction from seeds however was the homogenization of the seeds and variation in RNA yields and subsequent target amplification was most probably due to incomplete breakage of seed materials. Hence methods for physical breakage of seeds (e.g. stomacher) will be compared to reach a complete exposure and extraction of RNA.

5.3 RT-PCR and real-time qPCR assays for viroid detection in seeds:
The real time PCR assay using primers described by Boonham et al 2004 revealed good results in detecting PSTVd in RNA extracted from seeds. Differences, however, were found in the performance of thermal cyclers detecting PSTVd.

5.4 Seed infection in PSTVd infected tomato
Seed testing for presence of PSTVd was done revealing highly variable detection results. This was most probably due to either incomplete homogenization of seeds or PSTVd present on seed coat only. Distinction between true seed infection (that is presence of PSTVd in embryo) and external contamination of seed (that is PSTVd adhering to the seed coat), will be addressed in future work

Conclusions WP5:
5.1. The milestone has been achieved.
5.2. Seed homogenization for adequate RNA template preparation need to be addressed further.
5.3. Is running according to the plan.
5.4. Different surface sterilization and seed treatment methods will be tested to resolve seed infections from PSTVd on seeds and to standardize the seed surface treatment. Test of plants of germinated seeds involving several partners will be done according to the project plan.

15th May 2009
Steen Lykke Nielsen
Project leader

Appendix 4. Minutes of DEP's final meeting

Minutes of DEP final meeting 27th to 28th October 2009 at Agricultural Institute of Slovenia, Hacquetova 17, SI 1000 Ljubljana

Participants: Thierry Candresse, Ko Verhoeven, Mojca V. Marn, Irena Mavric Plesco, Richard Gottberger, Wendy Monger, Stephan Winter, Valérie M-Demilly, Xavier Tassus and Steen Lykke Nielsen

Notes taken by Steen Lykke Nielsen

Programme. Se the full programme in Annex 1

The results of WP2-WP5 were presented and discussed according to the programme. Overall all tasks have been fulfilled and the results will be published in the final report.

Special comments and decisions were taken for the following topics:

Host range studies: Only hosts where occurrence of viroids has been confirmed by retesting (by repeated RT-PCR and sequencing or inoculation to a host plant and a PCR test) will be regarded as new hosts.

Detection and diagnostics: The advantage of the different methods should be listed.

Re Final report and dissemination of the results.
Each WP leader is responsible for collecting the contributions from the participating partners and writing the final report together for the specific WP. Each WP leader forwards a standard format for the report to the other participants in the WP. Steen gathers all contributions to the final report.
Dimensioning of the final report web-version: Each WP-task should be restricted to one page plus annexes.
The protocol of the common RT-PCR will be included as an annex. The protocol has proven very robust by testing by the partners and in the ringtest. Remarks on need of adjusting the annealing temperature must be included. The protocol should be published in EPPO Bulletin.
Publishing of the protocols of the three specific assays and the generic assay of WP4 on the Web-based version of the final report will collide with the wish of publishing them in a scientific journal first. Therefore the four protocols will only be included in the full final report.
The results of the seed test should be discussed with Colin Jeffries whether to include them in the EPPO standard of PSTVd.
Publication of results in scientific journals:
Ko is already working on a paper on the mechanical transmission of viroids.
The host range studies can be published as new disease reports. No conclusive decision was taken whether to publish in common or separately.
It was decided to publish the vector transmission results including thrips, bees and aphids in a common paper by Richard, Mojca, Stephan and Steen.

The seed results: No decision was taken.

Discussion of possibilities for future cooperation on viroids
In the EUPHRESCO frame, but it is not possible to continue with the present consortium.
Seed transmission is still an interesting objective. Maybe a consortium including ISTA, seed companies and some DEP partners. Or a project on seed transmission in tomato seed of several pests including viroids.

Continuation of the DEP final meeting in an EPPO ad-hoc meeting on tomato viroids:
The DEP final meeting was immediately after the closure continued in EPPO ad-hoc meeting on tomato viroids 28th-29th October 2009 in the same localities at Agricultural Institute of Slovenia. All DEP participants participated in this meeting besides other scientists.

Annex 1.
Programme Final meeting in the EUPHRESCO project Detection and Epidemiology of Pospiviroids (DEP) 27th – 28th November 2009 at Agricultural Institute of Slovenia, Hacquetova 17, SI 1000 Ljubljana, .

Participants: Thierry Candresse, Ko Verhoeven, Mojca V. Marn, Richard Gottberger, Wendy Monger, Stephan Winter, Valérie M-Demilly, Xavier Tassus and Steen Lykke Nielsen.

Tuesday 27th October 2009.
Presentation of results of work package 1-5.

09.00-09.10. Welcome by Mojca V. Marn and introduction to Agricultural Institute of Slovenia
09.10-10.20. Introduction to the programme of the meeting by **Mojca** and **Steen.**

10.20-11.30. WP2. Host range studies and interactions. Coordinator Steen Lykke Nielsen

L. Results of the surveys in The Netherlands, France and Denmark. **Ko, Thierry and Steen.**
M. Establishment of infectious cDNA clones of the six viroids. **Thierry.**
N. Results of transmission of viroids from ornamental to tomato/potato and sequence analysis. **Ko.**

11.30-12.30.WP3. Transmission pathways. Coordinator Ko Verhoeven

G. Transmission by thrips. Results of transmission experiments with *Thrips tabaci*. **Richard.**
H. Transmission by thrips. Results of transmission experiments with *Frankliniella occidentalis*. **Steen**.
I. Transmission by bees. Results of transmission experiments with bumblebees. **Mojca.**
J. Transmission by bees. Results of transmission experiments with honeybees. **Steen**.
K. Results of mechanical transmission through cultural practice. **Ko**.

12.30-13.30. Lunch.

13.30-14.00. Continuation of WP3.

14.00 – 15.00. WP4. Diagnostics. Coordinator Wendy Monger.

I. Selection of the best RNA extraction method. **Wendy.**
J. Results of design of specific real time PCR assays for CEVd, CLVd and TASVd and a generic popiviroid assay. **Wendy**.
K. Results of validation tests of the TaqMan assays.**Valerie and Mojca.**
L. Results of the ringtest of the TaqMan assays. **Wendy.**
M. Results of the ringtest of a protocol for common RT-PCR for screening for pospiviroids. **Steen**.

15.00-15.30. Coffe brake.

15.30-16.00. Continuation of WP4.

16.00-17.30. WP5. Seed test. Coordinator Stephan Winter.

L. Procedure for cleaning/disinfecting tomato seeds before viroids test procedure. **Stephan.**
M. Selection of the best RNA extraction method and cDNA amplification. **Stephan.**
N. Presentation of the selected protocol for Q-PCR detection of PSTVd in tomato seeds. **Stephan.**
O. Sensitivity of the Q-PCR seed test. **Stephan, Richard and Steen.**
P. Infection rates of tomato seeds. **Stephan and Steen.**

Wednesday 28th October 2009.

09.00-09.30: Follow up on matters from yesterday. **All.**

09.30-11.00. Dissemination of the results. **All.**

A. Requirement for the final report and the version for the EUPHRESCO web-side. **Steen.**
B. Plan for dissemination of the diagnostic protocols to plant health authorities (national, EPPO, Standing Committee of Plant Health)
C. Strategy for common publishing of the results.

11.00-11.15. WP1. Coordination. **Steen.**

Cost statements.
Time table for finalising the DEP project

11.15-11.30. Discussion of possibilities for future cooperation on viroids. **All.**

11.30. Concluding remarks. **Steen.**

11.30-13.00. Lunch

13.00-18.0 EPPO ad hoc meeting on tomato viroids.

Appendix 5. Protocol for test of pospiviroids by common RT-PCR

Protocol for test of pospiviroids by common RT-PCR

Based on the paper of Verhoeven, J.Th.J. , Jansen, C.C.C., Willemen, T.M., Kox, L.F.F., Owens, R.A. & Roenhorst, J.W. 2004. European Journal of Plant Pathology 110, 823-831.

RNA-extraction
Different methods are available.
We use the RNeasy Plant Mini Kit with RLT buffer and β-Mercaptoethanol added according to the protocol and elute RNA in 50 µl water.
Use max 100 mg fresh plant material per sample or 5 mg freeze dried plant material.
The plant material can be homogenized 1) in a mortar with liquid nitrogen or 2) in a plastic bag (like the ones used for ELISA sample preparation) with extraction buffer.

Primer pairs:
Two sets of primers are used:
Pospil and Vid.

Pospi1-RE: 5'-AGC TTC AGT TGT (T/A)TC CAC CGG GT-3'.
Pospi1-FW: 5'-GGG ATC CCC GGG GAA AC-3'.
Size of the expected PCR product: 197 bp.

Vid-RE: 5'-CCA ACT GCG GTT CCA AGG G-3'.
Vid-FW: 5'-TTC CTC GGA ACT AAA CTC GTG-3'.
Size of the expected PCR product: 359 bp.

RT-PCR
Two RT-PCR procedures are given using one-step RT-PCR kit from Qiagen and one-step RT-PCR kit from Invitrogen, respectively.

One-step RT-PCR kit from QIAGEN (Catalog no. 210212)
Use the procedure described in the protocol for the QIAGEN OneStep RT-PCR Kit and Q-Solution. Add 1 ul template RNA to the master mix.

RT-PCR reactions:
30 min at 50 °C (cDNA synthesis)
15 min at 95 °C (hot start activation of Taq polymerase)

35 cycles of:
30 s at 94 °C (denaturation)
60 s at 62 °C (annealing)
60 s at 72 °C (elongation)

7 min at 72 °C (extension)

∞ 4 °C (hold)

PCR product analysis
Electrophorese the PCR product in TAE buffer through a 1.8 % or 2% agarose gel containing ethidium bromide (5.0 µg ml^{-1}).
A 766 bp ladder (BioLabs) is used to estimate sizes of the PCR products.

One-step RT-PCR kit from Invitrogen (Superscript one-step RT-PCR system with Platinum Taq kit with article number 10928-042 (for 100 reactions) or 10928-034 (for 25 reactions).
RT-PCR reactions:
30 min at 43 °C (cDNA synthesis)
2 min at 94 °C (hot start activation of Taq polymerase)

15 cycles of:
30 s at 94 °C (denaturation)
1.5 min at 62 °C (annealing)
45 s at 72 °C (elongation)

30 cycles of:
30 s at 94 °C (denaturation)
45 s at 59 °C (annealing
45 s at 72C (elongation)

7 min at 72 °C (extension)

∞ 4 °C (hold)

PCR product analysis
Electrophoresis through a 2.0 % agarose gel in TAE buffer containing ethidium bromide (5.0 µg ml^{-1}).
A 1 kbp ladder (Invitrogen) is used to estimate sizes of the PCR products.

Precipitation of PCR product
The PCR products are purified using QIAGEN MinElute PCR Purification Kit according to the manufacture's handbook.
10 µl product is obtained. 1 µl is used for running a control on a 2 % agarose gel in TAE buffer containing ethidium bromide (5.0 µg ml^{-1}).
The remaining DNA is air-dried at room temperature.

Sequencing
The precipitated DNA and a requested amount of forward primer is mailed to a commercial firm that carry out sequencing, for example EurofinsDNA on the web-address: http://www.eurofinsdna.com/home.html.

Phylogenetic analysis
The resulting sequences can be BLASTed at the NBCI Genbank. Enter the following net address: http://blast.ncbi.nlm.nih.gov . Choose *Basic BLAST/nucleotide blast* and create multiple alignments of related sequences.