EUPHRESCO Final Report

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Project Title
Validation of diagnostic methods for the detection and identification of whitefly transmitted viruses of regulatory or quarantine concern to the EU.

Project Duration:

Start date:	Autumn 2008
End date:	Autumn 2009

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Executive Summary

Introduction and main objectives

A project to validate diagnostic methods for the detection and identification of whitefly-transmitted viruses of regulatory or quarantine concern to the EU was initiated as a topic within the European Phytosanitary Research Coordination project: EUPHRESCO - Framework Six Programme Contract N°. 036212 (ERAC) web: www.euphresco.org, and funded by a non-competitive mechanism via the partner research groups of the project consortium. The project commenced in August 2008 for a twelve month period. The project participants were: Institute for Agricultural and Fisheries Research, Merelbeke, Belgium; Plant Protection Institute, Kostinbrod, Bulgaria; Agricultural Research Institute, Nicosia, Cyprus; State Phytosanitary Administration, Division of Diagnostics, Olomouc, Czech Republic; Laboratoire National de la Protection des Vegetaux, Angers, France; Benaki Phytopathological Institute, Athens, Greece; Plant Health and Environment Laboratory, MAF Biosecurity, New Zealand; Plantenziektenkundige Dienst, Wageningen, the Netherlands; and the Food and Environment Research Agency (formerly Central Science Laboratory), York, United Kingdom (the project co-ordinator).

Globally at least 114 virus species are known to be transmitted by whiteflies (family *Aleyrodidae*). *Bemisia tabaci* (Bt) transmits 111 of these species while *Trialeurodes vaporariorum* and *T. abutilonia* each transmit three species. *B. tabaci* and *T. vaporariorum* are present in some areas of the European–Mediterranean region. Of the whitefly-transmitted virus species, 90% belong to the genus *Begomovirus* (family *Geminiviridae*), 6% to the genus *Crinivirus* (family *Closteroviridae*), and the remaining 4% to the genera *Closterovirus* (family *Closteroviridae*), *Ipomovirus* (family *Potyviridae*) and *Carlavirus* (family *Flexiviridae*).

The whitefly-transmitted viruses considered within this project can all cause severe losses to the horticultural industries in the Mediterranean basin and are of quarantine concern to countries within and external to the EU. All are included on the EPPO A2 list, and comprise: *Cucurbit yellow stunting disorder virus* (CYSDV), genus *Crinivirus*; *Cucumber vein yellowing virus* (CVYV), genus *Ipomovirus*; *Tomato chlorosis virus* (ToCV) and *Tomato infectious chlorosis virus* (TICV), genus *Crinivirus*; and *Tomato yellow leaf curl virus* (TYLCV), genus *Begomovirus*. TYLCV is EC listed, and ToCV, TICV, CYSDV and CVYV are on the EPPO Action list. Of the viruses pertaining to this project which have severe affects on tomato crops, Bt transmits TYLCV in a persistent manner and *T. vaporariorum* transmits TICV and ToCV semi-persistently. ToCV is also transmitted semi-persistently by Bt and *T. abutilonia*. Severe losses in cucurbit crops are caused by CVYV and CYSDV; Bt transmits both viruses in a semi-persistent manner. Plant material that could carry Bt-transmitted viruses is subject to quarantine control falling within the scope of EC directive 2000/29/EC. The emergence of the polyphagous B biotype of *B. tabaci* has given whitefly-transmitted viruses a mode to infect new plant species. Bt has been intercepted on traded plant material, for example in a UK interception of Bt carrying TYLCV, and the presence of Bt on traded plant material facilitates global spread of whitefly-transmitted diseases. It is vitally important to equip National Plant Health Service laboratories with appropriate validated diagnostic methods for the detection and identification of whitefly-transmitted viruses of regulatory or quarantine concern to the EU so that any outbreak or interception can be dealt with rapidly and efficiently. The main aim of this collaborative project is the validation of real-time and conventional PCR diagnostic methods for a range of whitefly-transmitted viruses of concern to Europe.

Methods Summary

TaqMan and PCR work plans and protocols, and pro-formas for collection of information and validation data were designed for a pre-trial and main trial, and were distributed to all project

participants together with blind samples of healthy and infected plant material. A pre-trial was carried out using one virus assay (TYLCV) and one internal control assay, distributed by the project co-ordinator. For the main trial, six assays were provided: the internal control assay plus virus assays for TYLCV, ToCV, TICV, CVYV and CYSDV. The trials used a combination of published assays and previously unpublished assays designed by the co-ordinating laboratory.

Results and Conclusions

The pre-trial enabled a check for satisfactory nucleic acid extraction, detection and return of results, prior to commencement of the main trial. The project achieved its aims to produce method validation data for the detection and identification of the whitefly-transmitted viruses of regulatory or quarantine concern to the EU, and to promote the use of TaqMan real-time PCR methods by the project participants through technology transfer. The project demonstrated how this kind of co-operative project between countries could aid progress towards the production of validated diagnostic protocols.

The beneficiaries of this research are diagnostic laboratories and inspection services in member states; however, although the whitefly transmitted viruses are mainly of concern to Mediterranean horticulture, other countries within and outside Europe also have a concern with some of these viruses. The wider European and international community will benefit by the expected joint publication of the project results, and, where this work is taken forward in future, further developed into published European (EPPO) and international (IPPC) diagnostic protocols.

Validation of diagnostic methods for the detection and identification of whitefly transmitted viruses of regulatory or quarantine concern to the EU.

Objectives and tasks of the project, as stated in the work plan, with degree of achievement

A Project work plan was produced (see Appendix A). Objectives and tasks of the project are shown in Table 1.

Table 1. Project objectives and time scale

What	Which participants	When	Degree of achievement
Collection and sample preparation of virus isolates for methods validation.	Project coordinators-CSL (Fera) (collection from PPS-NL, ARI-CY, Maf NZ)	August 08	achieved
Collection of methods participants have indicate they wish to include, and evaluation of participant assay requirements (i.e. primers/probes/method Fera needs to distribute)	Fera (assays in use from PPS-NL, MZE-CZ and LNPV-FR)	Sept 08	achieved
Pre-trial test.	All	Nov 08	achieved
Main trial-distribution of primers and probes, and methods.	Fera	Feb 09	achieved
Distribution of samples	Fera to All	Feb 09	achieved
Data collection	All	April 09	achieved
Data tabulation and statistical analyses/ draft report	Fera	June 09	achieved
E-mail 'round table' expert evaluation of results	All	July 09	achieved
Final report	Fera	Autumn 09	achieved

Data collection:
Isolates held by participants were tabulated using a form sent to all participants (Appendix B). Work plans and results sheet for both the pre-trial and main test trial (Appendix C) were distributed to all participants.

Virus Isolates:
Virus isolates used in the project are listed in Table 2.

Table 2: Virus isolates and origins

Sample designation	Type	Source	Participant
G	Healthy *Cucumis Sativus*	UK	Fera-UK
H	Healthy *Physalis wrightii*	UK	Fera-UK
I	Healthy *Lycopersicon esculentum*	UK	Fera-UK
Three	TYLCV	(non NZ origin)	MAF-NZ
Eight	TYLCV	Cyprus	ARI-CY
B	TYLCV	Spain	Fera-UK
Fourteen	TYLCV	The Netherlands	PPS-NL
E	TYLCV	Spain	Fera-UK
Two	ToCV	Spain	Fera-UK
Four	ToCV	Spain	Fera-UK
Six	ToCV *P. wrightii*	Spain	Fera-UK
Nine	ToCV	Spain	MAF-NZ
Ten	ToCV	Spain	Fera-UK
Eleven	TICV	(non NZ origin)	MAF-NZ
Twelve	TICV	Italy	Fera-UK
One	CVYV	Portugal	Fera-UK
C	CVYV	Spain	Fera-UK
Five	CVYV	Spain	Fera-UK
Seven	CYSDV	Cyprus	ARI-CY
Thirteen	CYSDV	Spain	Fera-UK
A	CYSDV	Spain	Fera-UK
D	CYSDV	Spain	Fera-UK

The samples were lyophilised, labelled with the sample designation, and distributed by the project co-ordinator.

The pre-trial

The aims of the pre-trial test were:

- To check the ability to run the main trial by the collection of data from all participants for one virus assay (TYLCV) and one internal control assay (COX).
- To check the ability of participants to extract nucleic acid, detect target, return results and to identify any problems. Participants without access to TaqMan real-time PCR technology undertook PCR validation.

The main trial

For the main trial, six targets were tested for using TaqMan real-time PCR and conventional PCR. The targets were COX (internal control assay,), TYLCV, ToCV, TICV, CVYV and CYSDV.

TaqMan and PCR assays

TaqMan assays (Table 3) and PCR assays (Table 4) consisted of previously published assays and unpublished assays provided by the co-ordinating laboratory. Aliquots of primers and probes were sent by the project co-ordinator to all participants.

Instructions were distributed to participants containing example reaction mixtures and cycling conditions for TaqMan real-time PCR and conventional PCR. Participants were instructed to use the methods provided or their own established methods, and record the methods used on the results sheet (Appendix C).

Table 3: TaqMan real-time PCR primers and probes

Target	Primer/probe	Sequence 5'-3'
TYLCV[1]	TYLCV-IS 1678F	TTC GTC TAG ATA TTC CCT ATA TGA GGA GGT A
	TYLCV-CONS 1701P (probe)	CCT GGA TTG CAG AGG AAG ATA GTG GGA ATT C
	TYLCV-CONS 1756R	GGC AAG CCC ATT CAA ATT AAA GG
ToCV[2]	ToCV 258F	GTC TGT TCC GGC TGA TTA CAA GT
	ToCV P (probe)	TGG GCA GAG ACT TTT CAT GCA GGC A
	ToCV 331R	AAT TGA AAC CCA AAG AGG AAC AAA
TICV[1]	TICV 370F	TGC AAA TCA GGG ACT TAA ATC AAA
	TICV 395P (probe)	TGT ACC CGC TGA CTA CAA GTG TGC GC
	TICV 453R	AAG AAT TGA GTA CAG ACC TTG CAA AC
CYSDV[3]	CYSDV F	GCT TAA TGT GGG AGA AGT TCT CCT A
	CYSDV P (probe)	CTC CGT GCG CTC GTT AGG TAC CGG
	CYSDV R	TCT GGA TAT AAC CTT CAG ACA CTC CTT
CVYV[3]	CVYV F	CGA AGG TCT CGA ATA AGC GTT C
	CVYV P (probe)	TAT TTA CGG TTT TAG TAA GAG TTT GGG ATC CGC AG
	CVYV R	GAA TAC CCC ACA CCG AAC TTC A
Plant cytochrome oxidase[4]	COX F	CGT CGC ATT CCA GAT TAT CCA
	COXSOL 1511T (probe)	AGG GCA TTC CAT CCA GCG TAA GCA
	COX RW	CAA CTA CGG ATA TAT AAG RRC CRR AAC TG

[1] Fera unpublished assay; [2] Morris et al. (2006); [3] Gil-Salas et al.(2007); [4] Tomlinson et al. (2005)

Table 4. Conventional PCR primers

Target	Primer	Sequence 5'-3'
TYLCV[1]	AV632 forward	CYG GTG TTG TKC GTT GTG TTA G
	AC1048 reverse	GGR TTD GAR GCA TGH GTA CAT G
ToCV[2]	ToCV 172 forward	GCT TCC GAA ACT CCG TCT TG
	ToCV 610 reverse	TGT CGA AAG TAC CGC CAC
TICV[3]	TICV 1 forward	ATG AGG TCT TTC ACA GTG G
	TICV 2 reverse	GTC CGA AAC TGA TTG AAC C
CYSDV[4]	410U forward	AGA GAC GGT AAG TAT
	410L reverse	TTG GGC ATG TGA CAT
Crinivirus[5]	3F forward	TTC GGT ACT ACT TTC WST ACT RTY AG
	R3A reverse	TCA AAI GTW CCK CCW CCR AAR TC
CVYV[6]	CVYV F forward	AGC TAG CGC GTA TGG GGT GAC
	CVYV R reverse	GCG CCG CAA GTG CAA ATA AAT
COX[5]	COX forward	CCG GCG ATG ATA GGT GGA
	COX reverse	GCC AGT ACC GGA AGT GA

[1] Martinez-Culebras et al. (2001); [2] Louro et al. (2000); [3] Li et al. (1998); [4] Celix et al. (1996); [5] Fera unpublished assay; [6] Cuadrado et al. (2001)

Table 5. Summary of TaqMan Methods used by participants

Partner	Extraction	Reagents	Instrument	Cycling
UK	Kingfisher RNA	ABI Gold	ABI 7900 HT	As project instructions
NZ	Invitek InviMag Plant DNA	Invitrogen qPCR Supermix-UDG/1 step kit	Corbett Rotor gene 3000 (software version 6.1)	48ºC for 30 min; 95ºC for 10 min; then 40 cycles of 95ºC for 15 s and 60ºC for 60 s.
CY	Qiagen Plant RNeasy	As generic project instructions but with Invitrogen Platinum Taq polymerase and Superscript 111 reverse transcriptase	Biorad CFX96	As project instructions
BE	Qiagen Plant mini RNeasy	Ambion –AB TaqMan AgPathID	ABI 7900 HT	As project instructions
GR	Ambion TRI	Invitrogen Superscript 111 Platinum one step q RT-PCR system	Eppendorf Masterplex Realplex S	As project instructions
FR	Epicentre Masterpure RNA purification	Qiagen Quantifast probe RT-PCR kit	Stratagene MX 3005P	Idem ringtest instructions
NL	RNA = Qiagen Plant mini Rneasy; DNA = BioNobile Quick Pick kit for Kingfisher extraction	ABI master mix reagents	ABI 7900 HT	48ºC for 30 min; 95ºC for 10 min; then 40 cycles of 95ºC for 15 s and 60ºC for 60 s.

Table 6. Summary of PCR methods used by participants

Partner	Extraction	Reagents	Instrument	Programme
BG	Qiagen Plant mini RNeasy	Common lab method	Thermocycler Auto-Q Sever (LKB)	Common lab method
CZ	Sigma Tri Reagent (main trial) or CTAB/ Qiagen DNeasy Plant mini (pre-trial)	Common lab method for use withTth DNA polymerase (Biotools)	Bioer Technology TC-XP Cycler	Common lab method
GR (Pre-trial only)	CTAB	Common lab method	No data	Common lab method

Results obtained

Pre-trial TaqMan real-time PCR results

TaqMan real-time PCR results are shown in terms of Ct value (cycle number at which fluorescence exceeded the threshold) and delta Rn (dRn, normalised end point fluorescence).

Table 7. COX TaqMan assay pre-trial results

	Sample A		Sample B		Negative control		Positive Control		water	
Partner (instrument used)	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn
Belgium (ABI 7900HT)	20	1.1	25	1.1	24	1.1	25	1.1	-	-
Cyprus (BioRad)	21		23		23		21		-	
France (Stratagene)	20		24		20		23		-	
Greece (Eppendorf)	20		23		25*		21		-	
Netherlands (ABI 7900HT)	21	1.1	26	1	25	1	22	1.1	-	-
NZ (Corbett Rotorgene)	15		19		18*		16		-	
UK (ABI 7900HT)	19	1.5	18	1.6	18	1.5	16	1.7	-	-

Table 8. TYLCV TaqMan assay pre-trial results

	Sample A		Sample B		Negative control		Positive Control		water	
Partner (instrument used)	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn
Belgium (ABI 7900HT)	12	1.1	26	1.1	-	-	15	1.1	-	-
Cyprus (BioRad)	14		35		36		17		-	
France (Stratagene)	15		37		-		16		-	
Greece (Eppendorf)	13		35		31		16		-	
Netherlands (ABI 7900HT)	18*	2.1*	-	-	-	-	22	2.1	-	-
NZ (Corbett Rotorgene)	11		-		-		14		-	
UK (ABI 7900HT)	11	1.9	-	-	-	-	13	2.5	-	-

Ct and dRn values are mean values for triplicate reactions (*mean for two replicates only)
delta Rn values are shown only for participants using the ABI 7900 HT instrument
- negative reaction (target not detected)

Pre-trial conventional PCR results

COX PCR results were positive for all samples.

Table 9. TYLCV PCR assay pre-trial results

Partner	Sample A	Sample B	Negative control	water	Positive control
Bulgaria	+	-	-	-	+
Czech Republic	+	-	-	-	+
Greece	+	-	-	-	+

+ positive reaction
- negative reaction (target not detected)

Main trial results

For anonymity, partner designations do not correspond to the previous list. Partners 1 and 2 used conventional PCR; partners 3-9 used TaqMan real-time PCR. Ct and dRn values are mean values for duplicate reactions (*result for only one reaction). delta Rn values are given only for partners using ABI instruments.

Table 10. COX internal control assays main trial results

	Sample designation																					
	G		**H**		**I**		**THREE**		**EIGHT**		**B**		**E**		**TWO**		**FOUR**		**SIX**		**NINE**		**TEN**		**ELEVEN**	
	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn
partner 1	POS		POS		POS		POS		POS		POS		POS		POS		POS		POS		POS		POS		POS	
partner 2	n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t	
partner 3	18	1.1	17	1.1	17	1.1	17	1.1	15	1.1	17	1.1	15	1.1	15	1.1	14	1.1	14	1.1	14	1.1	13	1.1	13	1.1
partner 4	19		17		17		17		16		18		16		19		19		16		17		16		15	
partner 5	24		21		22		23		20		20		21		23		23		22		20		20		20	
partner 6	17		15		18		20		18		17		18		20		20		14		19		20		19	
partner 7	23	0.7	22	0.8	21	0.8	21	0.9	19	1	20	0.9	20	0.9	22	0.8	22	0.8	22	0.9	16	1.2	16	1.3	15	1.2
partner 8	16		16		17		18		17		18		16		16		15		16		16		16		15	
partner 9	18	2.3	19	1.7	20	1.5	22	1	18	2.4	19	2.3	18	1.8	21	1.4	19	1.8	18	2	18	1.9	17	2	16	2.5

	Sample designation																					
	TWELVE		**ONE**		**FIVE**		**SEVEN**		**13**		**14**		**A**		**C**		**D**		**own plant**		**H_20**		**own positive**	
	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn
partner 1	n/t		POS		POS		POS		n/t		n/t		POS		POS		POS		POS		-		n/t	
partner 2	n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t	
partner 3	16	1.1	17	1.1	17	1.1	18	1.1	23	1.1	n/t	n/t	17	1.1	16	1	17	1.1	24	1.1	-	-	n/t	n/t
partner 4	18		15		14		20		23		n/t		18		18		17		19		-		19	
partner 5	21		23		24		23		27		21		23		22		22		20		-		n/t	n/t
partner 6	18		16		17		18		24		16		21		16		19		18		-		n/t	n/t
partner 7	-	-	23	0.7	24	0.6	26	0.5	24	0.7	19	1	21	0.9	21	0.9	22	0.8	22	0.9	-	-	n/t	n/t
partner 8	17		18		15		16		22		18		15		17		17		14		-		n/t	n/t
partner 9	24	0.7	17	1.9	18	1.8	17	2	30	0.4	n/t	n/t	18	1.9	18	2	17	2	19	1.5	-	-	n/t	n/t

n/t not tested; - negative result (target not detected)

Virus real-time PCR results

Positive samples are highlighted in red.

Table 11a. Tomato yellow leaf curl virus (TYLCV) assays main trial results

	Sample designation																					
	G		H		I		THREE		EIGHT		B		E		14		own plant		H_2O		own positive	
	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn
partner 1	-		-		-		+		+		+		+		+		-		-			
partner 2	-		-		-		+		+		+		+		+		-		-			
partner 3	25	1.2	28	1.2	29	1.2	11	1.2	10	1.2	11	1.2	9	1.2	12		28	1.2	-		8	1.2
partner 4	-		-		-		12		13		15		11		15		-		-		11	
partner 5	36		35		37		16		14		15		15		17		39*		-			
partner 6	-		-		-		18		15		13		14		19		36		-			
partner 7	36*		-		-		17	2.8	17	2.9	19	2.9	15	2.9	19	3	-		-			
partner 8	-		-		-		22		20		22		18		23		-		-			
partner 9	-		-		-		16	2.8	16	2.8	16	2.6	15	2.5	17	3	-		-			

Table 11b. Tomato infectious chlorosis virus (TICV) assays main trial results

	Sample designation																							
	G		H		I		TWO		FOUR		SIX		NINE		TEN		ELEVEN		TWELVE		own plant		H_20	
	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn
partner 1	n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t		n/t	
partner 2	+		-		+		+		+		+		+		-		-		-		-			
partner 3	-	-	-	-	-	-	-	-	-	-	-		29	1.1	32		9	1.1	15	1.2	-		-	
partner 4	-		-		-	-	-		-		-		32		-		13		19		-		-	
partner 5	-		-		-	-	-		-		-		35		-		17		20		-		-	
partner 6	-		-		-		-		-		-		32		39		14		18		-		-	
partner 7	-	-	-	-	-	-	-	-	-	-	-		32	1.5	37	0.3	12	1.9	19	1.6	-		-	
partner 8	-		-		-		-		-		-		-		-		11		16		-		-	
partner 9	-	-	-	-	-	-	-	-	-	-	-		34	0.4	-		13	1.8	22	2	-		-	

Table 11c. Tomato chlorosis virus (ToCV) assays main trial results

	Sample designation																							
	G		H		I		TWO		FOUR		SIX		NINE		TEN		ELEVEN		TWELVE		own plant		H_20	
	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn
partner 1	-		-		-		+		+		+		+		+		-		-		-		-	
partner 2	-		-		-		+		+		+		-		-		-		-		-		-	
partner 3	35		-		36		16	1.2	17	1.2	14	1.2	21	1.1	17	1.2	-		36		-		-	
partner 4	-		-		-		21		24		18		24		20		-		-		-		-	
partner 5	-		-		-		27		29		26		31		25		-		-		-		-	
partner 6	38		33		-		23		24		16		24		22		-		37		-		-	
partner 7	-		-		-		29	2	31	1.9	24	2.2	25	1.4	21	2.5	-		-		-		-	
partner 8	-		-		-		23		22		18		22		17		-		-		-		-	
partner 9	-		-		-		23	2	23	1.9	18	2	24	1.5	21	2.5	-		-		-		-	

Table 11d. Cucumber vein yellowing virus (CVYV) assays main trial results

	Sample designation																							
	G		H		I		ONE		FIVE		SEVEN		13		A		C		D		own plant		H_20	
	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn
partner 1	+		-		-		+		+		+		n/t		-		+		+		-		-	
partner 2	-		-		-		+		+		+		-		-		+		+		-		-	
partner 3	32	1.2	30	1.2	32	1.2	15	1.2	15	1.2	18	1.2	-		-		16	1.2	22	1.2	36		-	
partner 4	-		-		-		15		15		20		-		-		19		21		-		-	
partner 5	-		-		-		21		23		23		-		-		23		31		-		-	
partner 6	-		-		-		16		17		17		-		32		19		26		-		-	
partner 7	-		-		-		18	1.9	20	1.8	22	1.7	-		-		21	1.9	24	1.9	-		-	
partner 8	-		-		-		19		18		19		-		-		20		26		-		-	
partner 9	-		-		-		16	2	18	2.2	18	2	-		-		19	2.1	23	1.8	-		-	

Table 11e. Cucurbit yellow stunting disorder virus (CYSDV) assays main trial results

	Sample designation																									
	G		H		I		ONE		FIVE		SEVEN		13		A		C		D		own plant		H_20		own positive	
	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn	Ct	dRn
partner 1	-		-		-		-		-		+		n/t		+		+		+		-		-		n/t	
partner 2	-		-		-		-		-		+		-		+		+		+		-		-		n/t	
partner 3	31		30		32		32		32		18	1.4	23	1.4	16	1.4	17	1.4	18	1.4	-		-			
partner 4	-		-		-		-		-		23		29		19		22		24		-		-		18	
partner 5	-		-		-		-		-		24		29		21		26		24		-		-			
partner 6	-		-		-		38		36		19		24		15		20		19		-		-			
partner 7	-		-		-		-		-		25	2.7	26	2.5	19	2.9	24	2.8	26	2.6	-		-			
partner 8	-		-		-		-		-		17		21		14		19		17		-		-			
partner 9	-		-		-		-		-		20	2.5	-		19	2.7	22	2	20	2.3	-		-			

For sample identity see Table 2.

Discussion of results and their reliability

Different nucleic acid extraction methods (CTAB, Invitek Plant mini kit, Qiagen Plant mini kit and Kingfisher automated extraction method) were used by partners. All methods were successful in giving amplifiable nucleic acid as shown by the control COX assays. All of the real-time PCR instruments used by the project partners were found to be fit for purpose.

Pre-trial

Sample A and the positive control were different isolates of TYLCV. All partners were able to use the assay to detect the virus. Sample B was identical to the negative control and in a number of labs one or both of these samples was being recorded as a weak positive. All partners returned their results in the required time-scale.

Pre-trial PCR results
Results for both participants using PCR were in agreement. The COX PCR gave a clear product with all extractions. The TYLCV PCR primers identified correctly the known positives and not the healthy material.

Pre-trial real-time PCR results
In the pre-trial, all partners who used TaqMan detectected TYLCV in positive samples. In some cases a weak positive (Ct values in the mid 30s) was recorded for the healthy tomato sample. One participant obtained the same result using their own assay. There are two possibilities for this result: contamination or a problem with assay. Contamination, possibly from the positive sample A during extraction, is a possibility. TaqMan is more sensitive than PCR such that a sample giving a Ct value in the mid 30s would be unlikely to give a visible PCR product. Hence the less sensitive PCR would not detect this level of contamination if it occurred during extraction or during dried leaf preparations. The negative plant material was processed on a different day, in a different lab and by a different person, and partners own negative plant samples were also giving this weak positive result. Therefore, contamination at the dried sample stage appears unlikely. Phylogenetic analysis of the tomato genome did not identify any sequence that could be causing cross-reactivity with tomato DNA. However, in an alternative approach an RNA extraction method was used to detect the expressed viral RNA instead of the viral DNA. This method was found by the project co-ordinator to reduce the false positive results being produced by the assay, suggesting that the observed problem is due to interaction between this assay and plant DNA. This assay was re-ordered for the main trial with new samples for testing.

The pre-trial was a success in that it enabled a check to be carried out for one virus assay (TYLCV) and one internal control (COX). This produced satisfactory nucleic acid extraction, TaqMan detection, and dissemination of results by all partners. The main trial to identify 5 viruses was prepared along the same guidelines as the pre-trial.

Main trial

The COX internal control results indicated that there were no problems with nucleic acid extraction of samples, or with the TaqMan or PCR machines used by the

partners. All five virus TaqMan assays consistently gave clearly identifiable positive Ct values for the infected samples.

Contamination
One partner experienced contamination (partner 3) as shown by the healthy control TaqMan data for most of the assays. A second partner recorded two false positives for the healthy controls using the ToCV assay, and two partners recorded weak false positives for the healthy controls using the TYLCV assay.

TYLCV
The main trial consisted of 5 positive and 3 negative samples. As in the pre-trial, some partners found this assay to give a weak false positive result with healthy material. These results may represent contamination for these partners, however it is possible that the false positive results reflect the persistence of the problem identified in the pre-trial. The TYLCV assay has been used successfully at Fera for a number of years to detect the virus in whitefly, rather than plants. Due to uncertainty in the results obtained for this assay, it is recommended that a new assay targeting a different area of the virus genome should be designed and evaluated.

TICV and ToCV
The same set of 10 samples was used for both these assays. Of these, 2 samples were infected with TICV, 5 were infected with ToCV, and 3 samples were healthy. Both TaqMan assays appeared to be specific, with most partners recording negative results for the healthy controls and the partners own controls. TICV and ToCV were correctly detected by all partners. However, previously unknown mixed infections became apparent from the results. Results for sample 9 indicate the presence of TICV as well as ToCV, and in some cases sample 10 (ToCV) gave a weak positive result for TICV. Mixed infections were not entirely surprising since these viruses are often kept together in collections and are not routinely tested for secondary infections. Samples other than 9 and 10 gave only the expected results, suggesting mixed infection in samples 9 and 10 rather than any cross-reactivity in these 2 assays. In the context of possible future work to produce diagnostic protocols for the regulation of these pathogens such results may be expected to require further confirmation by sequencing.

CVYV and CYSDV
The same 10 samples was used for both these assays, of which 3 samples were infected with CVYV, 4 samples were infected with CYSDV, and 3 samples were healthy. Healthy samples G, H and I, and the partners' own controls tested negative with both assays (with the exception of partner 3). Both viruses were detected by all partners in samples C, D and 7. Mixed infected were indicated by both PCR and TaqMan. Since these assays target different positions on the virus genome, it appears that both viruses were present in these samples.

PCR
Two participants without access to a real-time PCR instrument used published PCR assays instead of the TaqMan assays used by the other partners. Inclusion of these laboratories had the beneficial effect of promoting the use of TaqMan technology in these areas. This resulted in PCR validation data for these assays. This is especially pertinent where current EPPO diagnostic methods incorporating PCR methods do not exist (TICV, ToCV and CYSDV). There was good participant agreement for the results of each of the assays ToCV, TYLCV, CVYV and CYSDV results. One participant returned data for the TICV assay; however some contamination of the healthy controls was apparent, thus the TICV assay data could not be analysed further.

Little has been published regarding procedures for the collection of validation data; therefore, the pro-formas designed for this project and a flow chart of the procedure used is included as Appendices.

Main conclusions

The achieved project deliverable was the generation of validated TaqMan methods for four whitefly-transmitted viruses: TICV, ToCV, CVYV and CYSDV. A need for further work on the TYLCV TaqMan assay has been identified.

Expected benefits and usability of results (technology transfer)

The establishment of a validated set of methods for the identification of whitefly-transmitted viruses in this project is important for their detection, diagnosis and management. Joint publication of the results of the project is expected to ensue from this work. The project has enabled technology transfer to all participants with regard to the project deliverable in the form of validation data for the assays employed. The assays comprised published assays and some unpublished assays developed at Fera. Technology transfer is valuable in providing the project participants with validated assays for the detection of the whitefly-transmitted viruses in the project. The results obtained in the form of validation data have shown that all of the TaqMan assays used in this project were fit for purpose for their respective virus sequences. Some further work is required by the assay originator (Fera) to further investigate the specificity of the TYLCV assay with regard to plant DNA. The Project has promoted the use of TaqMan real-time PCR methods by use and discussion of the methods by the project participants. One EU member state participant who did not use TaqMan before the project has begun using TaqMan technology as a result of their involvement in the project. The validation data delivered in the project is also a necessary step to progress the production of validated diagnostic protocols, including proficiency testing, beyond the duration of this project.

Implication for stakeholders and policy

The project has achieved its aims, which were:

- To produce validated methods for the detection and identification of the whitefly transmitted viruses of regulatory or quarantine concern to the EU.
- To promote the use of TaqMan real-time PCR.

The project also demonstrated how this kind of colaboration between countries could progress the validation of diagnostic methods towards the production of universal diagnostic protocols.

The beneficiaries of this research product are diagnostic laboratories and inspection services in member states. Although the whitefly transmitted viruses are mainly of concern to Mediterranean horticulture, other countries within and outside Europe also have a concern with some of these viruses, some of which are EC listed (TYLCV), EPPO listed (CYSDV, CVYV, ToCV, TYLCV, TICV), or on the EPPO Action list (e.g. ToCV, TICV CYSDV, CVYV). The wider European and international community will potentially benefit beyond the expected joint publication of the project results where this work is taken forward and further developed into EPPO and IPPC diagnostic protocols.

Recommendations for future work

Future work to further promote and disseminate the project results at relevant scientific meetings and via the EUPHRESCO co-ordinators is recommended. The

joint publication of the results of this project, towards producing and publishing EPPO/IPPC approved diagnostic protocols, is recommended.

Papers, other publications and dissemination activities

The project has promoted the use of TaqMan real-time PCR methods, and the co-operative nature of this type of project by the publication and dissemination of the project aims and activities to date (Morris *et al*, 2009. Euphresco Phytosanitary ERA-NET, 2009). The expected joint publication of the validation data produced will disseminate the results of the work to the EU and global scientific community.

Morris, J., Monger, W.A. and Boonham, N. (2009). Validation of diagnostic methods for the detection and identification of whitefly transmitted viruses of regulatory or quarantine concern to the EU. Advances in Plant Virology Conference 1st–3rd April, Harrogate, Yorkshire. (AAB proceedings 2009 Abstract and poster).

Euphresco Phytosanitary ERA-NET. Euphresco non-competitive projects Euphresco International Workshop, Braunschweig, Germany, 26-27 May 2009 (Poster).

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Pro-formas for Information/Validation data collection designed in the project

Appendix A: Project work plan

Appendix B: Pro-forma to collect initial project information

Appendix C: Pre-trial test work plan and results pro forma; main trial work plan and results pro-forma

Appendix D: Nucleic acid extraction method examples

Appendix A: work plan

1. Collection and sample preparation of virus isolates (FERA). Collection and preparation of virus isolates for methods validation from all partners which have material available, also collection and preparation by FERA of virus isolates from other EU sources where available.

2. Collection by FERA of PCR or TaqMan assay methods that participants currently routinely use and would like to include in the methods validation, (from all participants who have indicated this - PPS-NL, MZE-CZ and LNPV-FR) (and from this produce an assessment by FERA of participant requirements ie which assay per virus FERA to send to all).

3. Pre-trial test by all labs. Aim – to check ability to run main trial. Collection of data from all participants for one virus/assay to be distributed to all by FERA, to check nucleic acid extraction and detection/return of results/identify any problems.

4. Production and distribution to all participants by organizers via e-mail of a Pro-forma for results data. (PCR+/, TaqMan Ct values).

5. Distribution by FERA of primers and probes (one assay per virus) to all participants. (All other reagents/consumables etc to be supplied by each participant).

6. Distribution of virus samples for method validation to all participants for identification in the form of lyophilized samples, or RNA preps or clones (dependent on form isolates acquired in at 1.).

7. Methods validation results on Pro-forma from all partners to be sent to FERA.

8. Analyses of data.

9. Project e-mail ‘round table’ with expert evaluation of results.

10. Project Report delivered to the EUPHRESCO Project Office (euphresco@fera.gov.uk)

Appendix B: Pro-forma produced to gather initial project information

EUPHRESCO Whitefly transmitted virus project

The primary aim of the project is to validate real-time and conventional PCR diagnostic methods for a range of whitefly-transmitted viruses of concern to Europe.

Viruses to test for:

- *Tomato yellow leaf curl virus* (TYLCV)
- *Cucumber vein yellowing virus* (CVYV)
- *Curcubit yellow stunt disorder virus* (CYSDV)
- *Tomato infectious chlorosis virus* (TiCV)
- *Tomato chlorosis virus* (ToCV)

Central Science Laboratory (CSL) has developed real time PCR assays based on TaqMan chemistry and some PCR primers for the viruses, FERA also has some material to test for all the viruses.

Ring testing would involve receiving from FERA either plant material, RNA extracts or plasmids for the whitefly-transmitted viruses and aliquots of the primers/probes.

Please fill in the following form and return:

Would you like to participate in the ring testing? **YES/NO**

Do you have facilities to do PCR testing? **YES/NO**

Do you have facilities to do TaqMan testing? **YES/NO**

Do you have PCR or TaqMan assays (or other) that you routinely use and would like to include in the ringtesting? **YES/NO**

What plant material do you have that could be included in the testing?

Virus	Isolates available (numbers)	Material available (type/amount)
TYLCV		
CVYV		
CYSDV		
TiCV		
ToCV		

Any other comments ?

Appendix C: Pre-trial work plan (as distributed to all participants)

Aim: To check ability to run main trial by the collection of data from all participants for one virus assay and one internal control assay, to be distributed to all by FERA, to check nucleic acid extraction and TaqMan detection/and return of results, and to identify any problems.

- 4 freeze dried vials of plant leaves will be supplied by FERA to all participants this will include 2 blind samples A and B and a positive and negative control. (NB: A Master mix only control of sterile distilled water to be supplied by participant)
- A CTAB method for virus extraction will be distributed by FERA as an example of a nucleic acid extraction method. Participants may use their own protocols.
- A virus assay, and an internal control assay (COX) will be employed. FERA will distribute virus specific TaqMan primers and FAM labelled probe, and an internal control also FAM labelled -COX assay to all participants. Each assay to be run in simplex. The primers and probes supplied by FERA are to be used in conjunction with the molecular assay reagents/methodology participants currently use. (ie the primers and probes required should work with any TaqMan method participants currently use).
- A number of replicate wells are required: Three replicate TaqMan plate wells per sample extract should be employed for the virus assay and internal control assay respectively.
- Participants without TaqMan technology will be supplied with PCR primers for the virus assay and an internal control assay. Please send any gel photo and explanatory key.

Please fill in and return the excel sheet supplied, by e-mail to FERA project co-ordinator.

Contents of kit (distributed to all participants by project co-ordinator):

Samples to be tested:
A Blind sample
B Blind sample
Negative Control
Positive Control
H_2O Control

- Work Plan
- Nucleic acid extraction method example
- Four lyophilised vial containing leaf material of each sample
- 2 sets of PCR primers (and probes for TaqMan) ready to use - not diluted prior to use

An example nucleic acid extraction method was provided to all project participants as described by EPPO (2005). Diagnostic Protocols for regulated Pests: Tomato yellow leaf curl virus and Tomato mottle virus EPPO Bulletin 35, 319-325, (Appendix D).

TaqMan Instructions supplied to participants

TaqMan master mix example

Primers and probe supplied at a working concentration 7.5pmol/μl for each primer and 5.0pmol/μl for each probe. Fera use 1μl of primer and 0.5μl of probe in each 25μl reaction.

Applied Biosystems Gold kit

	x1 well MasterMix μl
Buffer	2.5
$MgCl_2$	5.5
dNtp's	2
Primer F	1
Primer R	1
Probe	0.5
TaqGold	0.125
SDW	11.375
Total	24.00

Prepare sufficient master mix for each of the assays (main trial: 2 wells for each sample plus 2 wells for the water control): (pre-trial: 3 wells per each of the 4 samples plus water control x 2 assays).

In this example add 1μl of sample per well.

Thermal cycling conditions: (Note that the primers and probe are designed to work best at 60°C. Both are Fam-Tamra probes.

The standard conditions are:
50°C for 2 minutes
95°C for 10 minutes

Followed by 40 cycles of:
95°C for 15 seconds
60°C for 60 seconds

Return of results was requested on the excel sheet provided by e-mail to Fera, and returns were received by the due date as per the project work plan.

PCR instructions supplied to participants

Primers - provided ready for use, supplied at working concentration of 10pmol/μl to add directly to master mix as detailed below.

Fera use one-step master mix bought from ABgene and add 1μl of each primer to a 25μl reaction.

PCR reaction components (kept on ice)
Final concentrations**:**
50mM KCL
10mM Tris –HCL
0.025 mg BSA
2.5 mM $MgCl_2$
80μM each of dNTP's
1.0μM primer forward
1.0μM primer reverse
1 U Taq DNA polymerase

Thermo-cycling conditions:
2 min 95°C
Then 35 cycles of:
30sec at 95°C
30sec at 54°C
1 min at 72°C.
Final step of 5min at 72°C.

For the main trial instructions to participants (Appendix C) included that assays should be RT-PCR with cDNA made in either a one or two-step approach.

Analysis of PCR product:

The PCR fragments were detected by agarose gel electrophoresis and stained with ethidium bromide: A 2% agarose gel was prepared. After running products the gel was removed and and stained in ethidium bromide solution (0.5mg/ml-^{1}) for 45 min. The amplified DNA fragments were visualised by UV trans-illumination. The gel was photographed to provide a permanent record, saved to disc and emailed gel photos received by the project co-ordinator.
Interpretation of the PCR test result:

The COX internal primer test produced a product of approx 600bp. The TYLCV primers produced a product of 335bp

The resulting excel sheet, together with any gel photo and explanatory key were returned by e- mail to Fera by the due date. Additional guidance for RNA extraction methods (Appendix D), were produced by the project co-ordinator for the main trial with regard to the RNA viruses, and distributed to all participants.

Main test work plan

Aim: To validate PCR and Taqman assays for the whitefly transmitted viruses of concern to Europe

Viruses to test for:
Tomato yellow leaf curl virus (TYLCV)
Cucumber vein yellowing virus (CVYV)
Curcubit yellow stunt disorder virus (CYSDV)
Tomato infectious chlorosis virus (TiCV)
Tomato chlorosis virus (ToCV)

- Majority of samples are supplied as freeze dried vials of plant leaves, 5 samples have been supplied as dried RNA pellets in eppendorf tubes. The excel sheet provided indicates which sample should be tested with each assay.

- The freeze-dried plant material should have their RNA extracted by a method presently in use by the participant's laboratory. (NOTE extract RNA from the TYLCV samples not DNA as was previously used). The dried RNA pellet material supplied in eppendorf tubes should first be centrifuged to ensure the pellet is at the bottom of the tube and 12ul of sterile water used to resuspend. (NB: A Master mix only control of sterile distilled water to be supplied by participant and a own plant control of tomato leaf or similar.)

- Fera will distribute virus specific TaqMan primers and FAM labelled probe and an internal control also FAM labelled - COX assay to all participants. The primers and probes supplied by Fera are to be used in conjunction with the molecular assay reagents/methodology participants currently use. (ie the primers and probes required should work with any TaqMan method participants currently use).

- Two replicate wells are required for each sample. (Note this is different from pre-testing, which was 3).

- Participants without TaqMan technology will be supplied with PCR primers for the virus assays and an internal COX control assay. All PCR assays should be RT-PCR with cDNA made in either a one or two-step approach. Positive and negative results should be recorded on the results excel sheet. Please send any gel photo and explanatory key. In addition to the individual assays for the above viruses a set of primers have been supplied called Crini. These can be used after the specific assays have been finished with any remaining samples. Samples to be tested with these generic primers are on the excel sheet.

- Please fill in and return the excel sheet supplied, by e-mail to the Fera project co-ordinator.

Appendix D

Nucleic acid extraction method example (pre-trial)

NB: Gloves should always be worn to prevent contamination.

1. Grind ~200mg of plant material into a fine powder (freeze-dried material can be ground to a powder in a pestle and mortar with or without liquid nitrogen). This was written for fresh material freeze dried weighs a lot less, use about half the vial of material supplied.
2. Add 2-3ml of CTAB extraction buffer (see below). Homogenise the sample.
3. Decant ground sap into a 1.5ml microfuge tube. Incubate for 10-15 minutes at 65°C.
4. Centrifuge for 5 minutes at 13,000 rpm.
5. Remove 700μl of supernatant into a new microfuge tube.
6. Extract with an equal volume (700μl) of chloroform: IAA (24:1). Vortex at low speed or invert tube to mix. Centrifuge at 13,000 rpm at room temperature for 10 min. Collect the upper aqueous layer without disturbing the interphase and transfer the contents to another eppendorf.

6a. Repeat chloroform extraction step 6.

7. Precipitate with 0.5 vols of 5M sodium chloride and an equal volume of ice-cold isopropanol. Incubate at -20°C for at least 1 hours.
8. Pellet the DNA by centrifugation at 13,000 rpm for 10-min. Wash precipitate by addition of 500μl of 70% ethanol and centrifuge for 2 min at 13,000rpm. Carefully discard ethanol. Dry the pellet, either leave to air dry or use speedy vac. Re-suspend pellet in 100μl of sterile water.

Extraction buffer

2% CTAB
100 mM Tris-HCl pH 8.0
20 mM EDTA
1.4 M NaCl
1% Na sulphite
2.0% PVP-40
Mix first 4 reagents. Make up to 1 litre with distilled water. (Store solution at ambient temperature.)
*Add PVP and Sodium sulphite fresh to aliquot of stock buffer. (This will keep for ~2weeks)]

RNA extraction method examples (main trial)

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

1. QIAGEN RNeasy PLANT mini kit (cat. No. 74904)
   From Qiagen, follow manufacturers instructions.

2. CTAB RNA EXTRACTION METHOD
   Adapted from Lodhi *et al*. (1994) *PMBR* **12**: 6-13.

   1. Place tissue, 100-200 mg, in 10 x 15 cm 500 gauge polythene bag and grind. Pre-freezing (at -80°C or in liq. N_2) may help with the grinding of some tissues.
   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. Resuspend the pellet in 200 μl of TE buffer-(Appendix 1) containing 1% SDS. After the pellet is resuspended add 100 μl of 5 M NaCl and 300 μl of ice cold iso-propanol. Mix well and incubate at -20°C ± 10ºC for 25 min ±5min.
   11. Centrifuge for 10 min at max speed to pellet nucleic acid.
   12. Decant off the salt/isopropanol and wash pellet by adding 400 μl 70% ethanol and spinning for 4 min.
   13. Decant off the ethanol and dry the pellet to remove residual ethanol.
   14. Resuspend pellet in 100 μl of molecular-biology grade water.

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