EURL-Virology

(European Union Reference Laboratory for pests of plants on viruses, viroids and phytoplasmas)

TESTING OF BEGOMOVIRUSES CAPABLE OF INFECTING TOMATOES AND PLANTS OF THE FAMILY CUCURBITACEAE BY PCR

VALIDATION REPORT

The validation was performed by the National Institute of Biology, Department of Biotechnology and Systems Biology, Microbiology Unit, Laboratory for detection of viruses, viroids and phytoplasmas, Večna pot 111, Ljubljana, Slovenia, a partner of the European Union Reference Laboratory for pests of plants on viruses, viroids and phytoplasmas.

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1 Scope of validation

Detection of begomoviruses capable of infecting tomatoes and plants of the family Cucurbitaceae.

Method	PCRs with the sets of oligonucleotide primers according to: - Wyatt and Brown (1996) (EPPO PM7/152, Appendix 4) - Li et al. (2004) (EPPO PM7/152, Appendix 5) - Saison and Gentit (2015) (EPPO PM7/152, Appendix 6)
Harmful organism	Begomoviruses capable of infecting tomatoes and plants of the family Cucurbitaceae
Sample type	Plant material of tomato and plants of the family Cucurbitacea

2 Description of the method

2.1 DNA extraction

Plant material (~200 mg) is homogenized in 1 mL of lysis buffer (from a QuickPick™ SML Plant DNA kit, Bio-Nobile) using a tissue homogenizer (FastPrep®-24, MP Biochemicals). Total DNA is extracted using a QuickPick™ SML Plant DNA kit (Bio-Nobile) and a magnetic particle processor (e.g. KingFisher® mL, Thermo Scientific) as described by Mehle et al. (2013). Total DNA extract is eluted in 200 µL of elution buffer (from a QuickPick™ SML Plant DNA kit, Bio-Nobile). Undiluted DNA is used for testing.

2.2 Conventional PCRs

A. Conventional PCR adapted from Wyatt and Brown (1996)

Primers	Sequence	Amplicon size
AV494	5'- GCC YAT RTA YAG RAA GCC MAG -3'	580 bp
AC1048	5′- GGR TTD GAR GCA TGH GTA CAT G -3'	

Master Mix:

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	14.8	N.A.
Taq polymerase® buffer (*Invitrogen*)	10 x	2.5	1x
$MgCl_2$ (*Invitrogen*)	50 mM	1.0	2 mM
dNTPs (*Invitrogen*)	5 mM each	0.5	0.1 mM
Forward primer AV494	10 µM	2.0	0.8 µM
Reverse primer AC1048	10 µM	2.0	0.8 µM
Platinum Taq® DNA polymerase (*Invitrogen*)	10 U/µL	0.2	2 U
Subtotal		23.0	
DNA extract		2.0	
Total		25.0	

PCR conditions:
2 min at 94°C followed by 10 cycles (15 sec at 94°C, 20 sec at ramping 65°C -1°C to 56°C (annealing T down 1°C/cycle) and 30 sec at 72°C), 30 cycles (15 sec at 94°C, 20 sec at 55°C, 30 sec at 72°C) and 10 min at 72°C.

B. Conventional PCR adapted from Li *et al.* (2004)

Primers	Sequence	Amplicon size
SPG1	5'- CCC CKG TGC GWR AAT CCA T -3'	912 bp
SPG2	5′- ATC CVA AYW TYC AGG GAG CTA A -3'	

Master Mix:

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	22.76	N.A.
Taq polymerase® buffer (*Invitrogen*)	10 x	3.00	1x
$MgCl_2$ (*Invitrogen*)	50 mM	1.20	2 mM
dNTPs (*Invitrogen*)	5 mM each	0.60	0.1 mM
Forward primer SPG1	10 µM	0.60	0.2 µM
Reverse primer SPG2	10 µM	0.60	0.2 µM
Platinum Taq® DNA polymerase (*Invitrogen*)	10 U/µL	0.24	2.4 U
Subtotal		29.00	
DNA extract		1.00	
Total		30.00	

PCR conditions:
2 min at 94°C followed by 11 cycles (40 sec at 94°C, 40 sec at ramping 61°C +1°C to 71°C (annealing T up 1°C/cycle) and 90 sec at 72°C), 24 cycles (40 sec at 94°C, 40 sec at 60°C, 90 sec at 72°C) and 10 min at 72°C

C. Conventional PCR according to Saison and Gentit (2015)

Primers	Sequence	Amplicon size
Beg-CP-F	5′-GCC CAT GTA YMG RAA RCC-3′	580 bp or 950 bp
Beg-580-R	5′-GGR TTA GAR GCA TGM GTA CA-3′	

Master Mix:

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular grade water	N.A.	17.50	N.A.
Taq polymerase® buffer (*Invitrogen*)	10 x	2.50	1x
$MgCl_2$ (*Invitrogen*)	50 mM	0.80	1.6 mM
dNTPs (*Invitrogen*)	5 mM each	0.50	0.1 mM
Forward primer Beg-CP-F	10 µM	0.75	0.3 µM
Reverse primer Beg-580-R	10 µM	0.75	0.3 µM
Platinum Taq® DNA polymerase (*Invitrogen*)	10 U/µL	0.20	2 U
Subtotal		23.00	
DNA extract		2.00	
Total		25.00	

PCR conditions:
3 min at 94°C followed by 35 cycles (30 sec at 94°C, 35 sec at 58°C, 30 sec at 72°C) and 7 min at 72°C

2.3 Sequencing of PCR products

Sanger sequencing of amplicons from the PCR tests is done to identify begomoviruses. Guidelines described in Appendix 7 of EPPO Standard PM7/129 is followed. The PCR amplicons are purified (DNA Gel Extraction kits; Millipore; or MinElute PCR Purification kits; Qiagen), according to the manufacturer instructions. The forward and reverse sequencing reactions for the purified PCR products are performed by Eurofins GATC, using the Sanger method. DNA sequences are aligned using the Vector NTI software or ClustalW (Mega7 package). The consensus sequences are compared with sequences from the GenBank database, using the BLAST algorithms (http://www.ncbi.nlm.nih.gov/blast).

Note: Sequencing of the PCR products is not part of the scope of validation

3 Validation procedure

Validation was performed according EPPO PM7/98.

3.1 Analytical specificity

'In-silico' comparison of primer sequences to sequences in the genomic libraries (sequences available at the NCBI nucleotide database on July 2020) has been done. Steps done for in-silico analysis are described briefly bellow:

- A list of all begomoviruses was compiled using the ICTV database (https://talk.ictvonline.org/taxonomy)
- A list of begomoviruses that can infect tomatoes and plants of the Cucurbitaceae family was compiled based on literature searches and searches of the NCBI Virus Database
- Reference sequences of begomoviruses that can infect tomatoes and plants of the Cucurbitaceae family (sequences of monopartites and DNA-A of bipartites) were collected using Entrez Direct tools, which provide access to NCBI's interlinked databases from a Unix terminal window
- Using the primer search algorithm from EMBOSS, a free open-source software analysis package designed specifically for the needs of molecular biology, we examined the probability of detecting the selected begomoviruses with each of the three PCR tests.

Tested targets (36 different targets tested with all three PCRs):

- Begomoviruses (isolates) included in the validation: isolate of squash leaf curl virus (SLCuV) obtained from Israel (Volcani center, ARO) and nine begomovirus isolates from DSMZ collection (chayote yellow mosaic virus, ChaYMV: PV-0843; tomato leaf curl New Delhi virus, ToLCNDV: PV-1109, PV-1111 and PV-1285; tomato yellow leaf curl Sardinia virus, TYLCSV: PV-0561; tomato yellow leaf curl Thailand virus, TYLCTHV: PV-0952; tomato yellow leaf curl virus, TYLCV: PV-0844 and PV-0560; watermelon chlorotic stunt virus, WmCSV: PV-0830).
- gBlocks representing fragments of viral sequences of the core region of the capsid protein (CP) gene and parts located on the open reading frame AC2 and AC1 included in the validation (in brackets accession number of NCBI sequence used for designing the gBlock): chayote enation yellow mosaic virus (KX259339); squash leaf curl China virus, SLCCNV (MN437657); luffa yellow mosaic virus, LYMV (NC_004824); croton yellow vein mosaic virus, CroYVMV (LN878119); squash leaf curl Yunnan virus, SLCuYV (MN563794); cucumber chlorotic leaf virus (MN013786); tomato golden leaf spot virus, ToGLSV (NC_021579); tomato bright yellow mosaic virus, ToBYMV (NC_038467); tomato leaf curl Sulawesi virus, ToLCSuV (NC_013413); coccinia mosaic Tamil Nadu virus, CMTNV (NC_024810). These gBlocks have been tested in concentrations about 1 million copies per reaction.
- Begomoviruses (isolates) included in the test performance study within Euphresco BegomoVal (2016-A-212): abutilon mosaic virus (AbMV, origin: the Netherlands, collection: ANSES), african cassava mosaic virus (ACMV, collection: DSMZ PV-0421), bean golden mosaic virus (BGMV, collection: DSMZ PV-0094), pepper golden mosaic virus (PepGMV, origin: Mexico, collection: ANSES), potato yellow mosaic virus (PYMV, origin: Martinique, collection: ANSES), tomato leaf curl New Delhi virus (10-2 dilution of ToLCNDV, origin: Spain, collection: ANSES), tomato mottle virus (ToMoV, origin: Florida USA, collection: ANSES), tomato severe rugose virus (ToSRV, origin: Brazil, collection: ANSES), Sri Lankan cassava mosaic virus (SLCMV, collection: DSMZ PV-0424), watermelon chlorotic stunt virus (WmCSV, collection: DSMZ PV-0830), chilli leaf curl virus (ChiLCV, origin: India, collection: ANSES), tomato yellow leaf curl virus (TYLCV, origin: Reunion Island and, Mauritius, collection: ANSES), 10-2 and 10-3 dilution of TYLCV (origin: New Caledonia, collection: ANSES), tomato leaf curl Comoros virus (ToLCYTV, origin: Mayotte, collection: ANSES), tomato yellow leaf curl Sardinia virus (TYLCSV, origin: Spain, collection: ANSES), tomato leaf curl Mali virus (ToLCMLV, origin: Senegal, collection: ANSES).

Tested non-targets:

- 5 non target viruses included in the test performance study within Euphresco BegomoVal (2016-A-212): banana bunchy top virus (BBTV, origin: Reunion Island, collection: ANSES), maize streak virus (MSV, origin: Reunion Island, collection: ANSES), pea necrotic yellow dwarf virus (PNYDV, origin: Austria, collection: ANSES), pepino mosaic virus (CH2 PepMV, collection: ANSES), tomato chlorosis virus (ToCV, collection: ANSES)
- Healthy tomato and zuchini plant material from DSMZ collection
- Tomato plant material (diferent cultivars): 35 samples analysed in 2021 (tested with Li et al. (2002) and with Wyatt and Brown (1996) or Saison and Gentit (2015))
- Plant material of the family Cucurbitacea: 25 samples (Cucumis sativus 3x, Cucumis melo 1x, Citrulus lanatis 1x, Cucurbita pepo 7x, Cucurbita pepo var. styriaca 3x, Cucurbita maxima 1x, Cucurbita moschata 2x, Cucurbita sp. 7x) analysed in 2021 (tested with Li et al. (2002) and with Wyatt and Brown (1996) or Saison and Gentit (2015))

3.2 Analytical sensitivity

To determine the analytical sensitivity of the PCR tests, the following experiments were carried out:

- Analysis of serial dilutions of lyophilized material of ChaYMV isolate from DSMZ collection (PV-0843) in zucchini leaf material
- Analysis of serial dilutions of lyophilized material of TYLCTHV isolate from DSMZ collection (PV-0952) in tomato leaf material
- Analysis of serial dilutions of lyophilized material of WmCSV isolate from DSMZ collection (PV-0830) in zucchini leaf material

The procedure for testing of the analytical sensitivity on serial dilutions of begomoviruses is schematically presented on Figure 1.

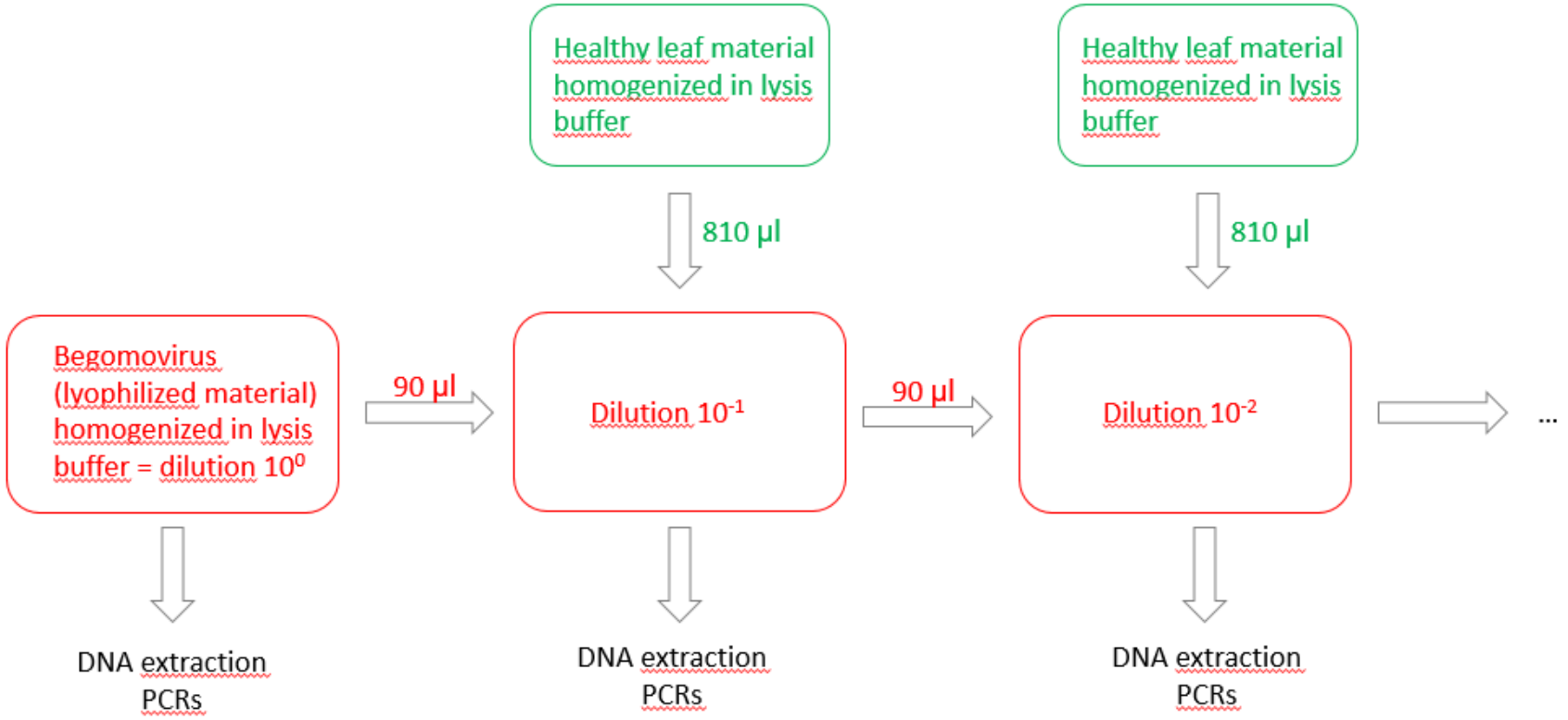

Figure 1: The procedure for testing of the analytical sensitivity on serial dilutions of begomoviruses. Three separate experiments were done using different begomoviruses (ChaYMV, TYLCTHV and WmCSV) and different healthy plant material (tomato leaves or zucchini leaves).

3.3 Analytical selectivity

As part of the evaluation of analytical sensitivity and analytical specificity, various healthy and begomovirus-infected plant species and cultivars were tested, and the results of these samples were also used to evaluate selectivity.

3.4 Analytical repeatability

Five replicates of DNA extracts with low concentrations of ChaYMV and TYLCTHV were tested in the same PCR run. The concentrations tested were the maximum dilutions that gave a positive result and were determined as part of the analytical sensitivity evaluation.

3.5 Analytical reproducibility

DSMZ (Germany) prepared several aliquots of freeze-dried leaf material. These were aliquots of begomovirus isolates from the DSMZ collection (ChaYMV PV-0843, ToLCNDV PV-1109, TYLCTHV PV-0952, TYLCV PV-0560, WmCSV PV-0830), healthy tomato leaf material, and healthy zucchini leaf material. Up to 9 aliquots of each material were included in the reproducibility evaluation. DNA extractions and PCRs have been performed by 4 different operators (ZK, NJ, TJ, JB) and with one of the following devices:

- GeneAmp PCR System 9700 - int. no. FITO 0223
- GeneAmp PCR System 9700 - int. no. FITO 0229
- T100 Thermal Cycler (gradient PCR) - int. no. FITO 0241
- PCR (deep well) Biorad C1000 - int. no. FITO 0246
- T100 Thermal Cycler (gradient PCR) - int. no. FITO 0253

4 Results of validation

4.1 Evaluation of DNA extraction

All samples of DNA extracted from begomovirus isolates produced PCR products. In addition, the performance of DNA extraction for each sample was verified by real-time PCR using a set of oligonucleotide primers and a probe for 18S rDNA. For most samples of tomato and plants of the family Cucurbitaceae, 18S rDNA resulted with Cq between 11 and 20. The undiluted samples D499/21 (sample of *Citrullus lanatus*), D602/21 (sample of *Cucurbita pepo*), and some isolates included in the reproducibility study gave a Cq for 18S rDNA > 25. Further testing confirmed that in all these cases the higher Cq values for 18S

rDNA were due to the presence of inhibitors (the tenfold diluted samples D499/21 and D602/21 gave a Cq value for 18S rDNA < 25; and all DNA extracted from the begomovirus isolates produced a PCR product).

4.2 Analytical specificity

- ***In-silico'* comparison of primer sequences to sequences in the genomic libraries**

In-silico analysis showed that at least two PCR tests are required for detection of all begomoviruses that can infect tomato and/or plants belonging to the family Cucurbitaceae:

- one PCR that target the core region of the capsid protein gene: Wyatt and Brown (1996) **or** Saison and Gentit (2015) **and**
- PCR that target the open reading frame AC2 and AC1: Li et al. (2002).

Reference sequences of begomoviruses that can infect tomatoes and plants of the *Cucurbitaceae* family are listed in Appendix 1.

Details about in-silico analysis that are available at NIB on the request:

- A list of all begomoviruses from the ICTV database that were available at the time of the *in-silico* analysis, as well as a list of begomoviruses that can infect tomato and plants of the Cucurbitaceae family
- The table with the number of mismatches with the primers of all three PCRs for all examined begomoviruses

Main observations:

- Number of begomoviruses capable of infecting tomatoes and/or plants of the family Cucurbitaceae (at the time of *in-silico* analysis): 212.
- Number of begomoviruses capable of infecting tomato and/or plants of the family Cucurbitaceae for which the nucleotide sequence of the target regions of the PCRs is known: 185 (Appendix 1).
- Based on *in-silico* analyses, 67 begomoviruses capable of infecting tomato and/or plants of the family Cucurbitaceae would be detected by all three PCRs, whereas 112

would be detected only by the PCR of Li et al. (2002) or only by PCRs targeting the core region of the capsid protein gene. This conclusion is based on the following observations:

  - Number of begomoviruses with 0 or up to 2 mismatches with the primers of all three PCRs: 67
  - Number of begomoviruses with 0 or up to 2 mismatches with the primers of Li et al. (2002) and >2 mismatches with the primers Wyatt and Brown (1996) and/ or Saison and Gentit (2015): 90
  - Number of begomoviruses with 0 or up to 2 mismatches with the primers of Wyatt and Brown (1996) and Saison and Gentit (2015) and >2 mismatches with the primers Li et al. (2002): 22

- Six begomoviruses have >2 mismatches with the primers of Li et al. (2002) and Saison and Gentit (2015) and/or Wyatt and Brown (1996). These are (in brackets number of mismatches are shown for all 3 PCRs in the following orders: Li et al. (2002)/ Saison and Gentit (2015)/ Wyatt and Brown (1996)):
  - coccinia mosaic Tamil Nadu virus (CMTNV; 3/ 3/ 3),
  - cucumber chlorotic leaf virus (4/ 1/ 3),
  - tomato bright yellow mosaic virus (ToBYMV; 3/ 4/ 4),
  - tomato golden leaf spot virus (ToGLSV; 4/ 3/ 3),
  - tomato leaf curl Sulawesi virus (ToLCSuV; 3/ 2/ 4),
  - watermelon chlorotic stunt virus (WmCSV; 5/ 6/ 6).

The possibility of detecting these 6 begomoviruses (as well as several others) was verified by laboratory testing of isolates or gBlocks (see below).

- **Analysis of a range of targets and relevant non-targets**

Testing of the range of targets (36 isolates) and non-targets (7 non-target viruses, 62 samples of tomato or plants of the family Cucurbitaceae) provided the data presented in Table 1.

This analysis showed that at least two PCR tests are required for detection of these 36 begomoviruses:

- one PCR that target the core region of the capsid protein gene: Wyatt and Brown (1996) **or** Saison and Gentit (2015) and
- PCR that target the open reading frame AC2 and AC1: Li et al. (2002).

With these PCRs we were able to detect also six begomoviruses that have >2 mismatches with the primers of Li et al. (2002) and Saison and Gentit (2015) and/or Wyatt and Brown (1996).

Table 1: Data about analytical specificity obtained by testing a range of targets and non-targets

	Inclusivity*	Exclusivity**
Li et al. (2004)	66.7% Test did not detect isolates of ToLCMLV, ACMV, ToMoV, TYLCV (Israel strain, isolate from Reunion Island and isolate from New Caledonia), SLCMV, SLCuV and WmCSV PV-0830 (isolate included in in-house validation and isolate from the test performance study), and gBlocks of CCLV, ToGLSV and ToLCSuV. All other isolates and gBlocks of begomoviruses included in the study were detected.	100%
Saison and Gentit (2015)	94.4% Test did not detect gBlocks of SLCCNV and LYMV, and the probability of detection of the isolate ToLCNDV PV1285 was 50% (half replicates positive and half negative). All other isolates and gBlocks of begomoviruses included in the study were detected.	100%
Wyatt and Brown (1996)	88.9% Test did not detect isolates of TYLCSV PV-0561 and ToLCNDV PV-1285, and gBlocks of SLCCNV and LYMV. All other isolates and gBlocks of begomoviruses included in the study were detected.	100%

*36 isolates of begomoviruses tested
**In some cases, nonspecific bends were generated and the absence of begomovirus was confirmed by sequencing of PCR products.

4.3 Analytical sensitivity

Analytical sensitivity was evaluated for three different begomovirus species (Table 2). As expected (see analytical specificity), the sensitivity of PCRs depends on the begomovirus species used. From the results presented in Table 2, we can conclude that when using at least two PCR assays (one PCR targeting the core region of the capsid protein gene and one

PCR targeting the AC2 and AC1 open reading frames), we can detect all begomoviruses only if they are present at relatively high concentrations, which is expected in symptomatic plant material. When testing asymptomatic plant material, where lower concentrations of begomoviruses are expected, all three PCRs must be used.

Table 2: PCR analytical sensitivity determined by testing the serial dilutions of three different begomoviruses in tomato (TYLCTHV) or zucchini (ChaYMV and WmCSV) leaf material.

Sample		Li et al. (2004)	Saison and Gentit (2015)	Wyatt and Brown (1996)
ChaYMV	undiluted	pos (+++)	pos (+)	pos (+++)
	10^{-1}	pos (++)	neg	pos (+++)
	10^{-2}	pos (+)	neg	pos (++)
	10^{-3}	neg	neg	pos (+)
	10^{-4}	neg	neg	neg
	10^{-5}	neg	neg	neg
	10^{-6}	neg	neg	neg
TYLCTHV	undiluted	pos (+++)	pos (++)	pos (+++)
	10^{-1}	pos (++)	pos (+)	pos (+++)
	10^{-2}	pos (+)	neg	pos (+++)
	10^{-3}	neg	neg	pos (++)
	10^{-4}	neg	neg	pos (+)
	10^{-5}	neg	neg	neg
	10^{-6}	neg	neg	neg
WmCSV	undiluted	neg	pos (+)	pos (+)
	10^{-1}	nt	pos (+)	neg
	10^{-2}	nt	neg	neg
	10^{-3}	nt	neg	neg
	10^{-4}	nt	neg	neg

nt-not tested; pos – band of the expected size (from strong (+++) to weak (+)); neg – no band

4.4 Analytical selectivity

No significant (important for diagnosis) influence of plant species (tomato/ plant of the family Cucurbitacea) or cultivar on test results was detected (see section Analytical specificity and Analytical sensitivity). DNA extracts from some plant materials may contain more inhibitors, but these can be monitored by real-time PCR for 18S rDNA, and in such cases testing of a higher dilution of DNA is required (see section DNA extraction).

4.5 Analytical repeatability

Repeatability for all three PCRs has been evaluated on ChaYMV and TYLCTHV DNA samples with relatively low concentration (for dilutions tested see Table 3). The repeatability of all three PCRs at the limit of detection is 100% (Table 3).

Table 3: Parallel testing of dilutions of DNA samples at the detection limit:

Sample		Li et al. (2004)	Saison and Gentit (2015)	Wyatt and Brown (1996)
ChaYMV	dilution	10^{-2}	undiluted	10^{-3}
	replicate 1	pos	pos	pos
	replicate 2	pos	pos	pos
	replicate 3	pos	pos	pos
	replicate 4	pos	pos	pos
	replicate 5	pos	pos	pos
TYLCTHV	dilution	10^{-2}	10^{-1}	10^{-4}
	replicate 1	pos	pos	pos
	replicate 2	pos	pos	pos
	replicate 3	pos	pos	pos
	replicate 4	pos	pos	pos
	replicate 5	pos	pos	pos
Repeatability		**100%**	**100%**	**100%**

Note: The negative amplification controls were always negative.

4.6 Analytical reproducibility

Five target and two non-target samples were analysed in each PCR assay during the period 29.9.2020 – 25.1.2022. In total, these samples were tested by up to four different personnel, on up to nine different days, and with up to five different instruments. The negative amplification controls were always negative, and the positive amplification controls were always positive. The repeatability of all three PCRs for all samples was 100% (Table 4).

Table 4: Multiple testing of samples (performed on up to 9 different days (=number of runs), 4 different operators, and 5 different PCR instruments):

PCR	sample	Expected result	Number of runs	Number of positive results	Number of negative results	Reproducibility
Li et al. (2004)	ChaYMV	pos	9	9	0	100%
	ToLCNDV	pos	8	8	0	100%
	TYLCTHV	pos	9	9	0	100%
	TYLCV	pos	8	8	0	100%
	WmCSV	neg	9	0	9	100%
	Neg T	neg	8	0	8	100%
	Neg Z	neg	7	0	7	100%
Saison and Gentit (2015)	ChaYMV	pos	8	8	0	100%
	ToLCNDV	pos	7	7	0	100%
	TYLCTHV	pos	8	8	0	100%
	TYLCV	pos	7	7	0	100%
	WmCSV	pos	8	8	0	100%
	Neg T	neg	7	0	7	100%
	Neg Z	neg	7	0	7	100%
Wyatt and Brown (1996)	ChaYMV	pos	4	4	0	100%
	ToLCNDV	pos	3	3	0	100%
	TYLCTHV	pos	4	4	0	100%
	TYLCV	pos	3	3	0	100%
	WmCSV	pos	4	4	0	100%
	Neg T	neg	3	0	3	100%
	Neg Z	neg	3	0	3	100%

Note: The negative amplification controls were always negative and positive amplification controls were always positive.

5 Conclusions

Criteria		Results
Analytical sensitivity	Tested concentrations	Dilutions of ChaYMV, TYLCTHV and WmCSV in tomato or zucchini leaf material
	LOD	Maximum dilution of target DNA detected: ChaYMV: - Wyatt and Brown (1996): 10^{-3} - Li et al. (2004): 10^{-2} - Saison and Gentit (2015): undiluted TYLCTHV: - Wyatt and Brown (1996): 10^{-4} - Li et al. (2004): 10^{-2} - Saison and Gentit (2015): 10^{-1} WmCSV: - Wyatt and Brown (1996): undiluted - Li et al. (2004): no PCR product - Saison and Gentit (2015): 10^{-1}
Analytical specificity	Number of tested samples	No of targets tested: 36 No of non-targets tested (healthy plant material and other viruses): 69
	Percentage of accurate results	100% if two PCR tests are used: - one PCR that target the core region of the capsid protein gene: Wyatt and Brown (1996) or Saison and Gentit (2015) and - PCR that target the open reading frame AC2 and AC1: Li et al. (2002) Separate evaluation of each PCR: - Wyatt and Brown (1996): 96.2% - Li et al. (2004): 88.6% - Saison and Gentit (2015): 98.1%
	Percentage of false positives	- Wyatt and Brown (1996): 0% - Li et al. (2004): 0% - Saison and Gentit (2015): 0%
	Percentage of false negatives	0% if two PCR tests are used: - one PCR that target the core region of the capsid protein gene: Wyatt and Brown (1996) or Saison and Gentit (2015) and - PCR that target the open reading frame AC2 and AC1: Li et al. (2002) Separate evaluation of each PCR: - Wyatt and Brown (1996): 11.1% - Li et al. (2004): 33.3% - Saison and Gentit (2015): 5.6%
	'*In silico*' analysis	*In-silico* analysis showed that at least two PCR tests are required for detection of all begomoviruses that can infect tomato and/or plants belonging to the family Cucurbitaceae: - one PCR that target the core region of the capsid protein gene: Wyatt and Brown (1996) or Saison and Gentit (2015) and - PCR that target the open reading frame AC2 and AC1: Li et al. (2002).
Selectivity	Effect of sample on selectivity	There was no observed relevant impact of plant species or cultivars on the test results
Repeatability	nr. of parallels, percentage of identical results	Repeatability for all three PCRs is 100% in both tested samples (ChaYMV and TYLCTHV at the limit of detection)
Reproducibility	nr. of parallels, percentage of identical results	Percentage of identical results is 100% for all three PCRs No. of target samples tested: 5 No. of nontarget samples tested: 2 No. of operators: up to 4 No. of PCR instruments: up to 5 No. of different days: up to 9

6 Reference

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Appendix 1

Reference sequences of begomoviruses that can infect tomatoes and plants of the *Cucurbitaceae* family

BEGOMOVIRUS	Acc. No.
Ageratum enation virus	KP195264
Ageratum yellow vein China virus	MN201990
Ageratum yellow vein Hualian virus	NC_010812
Ageratum yellow vein virus	NC_027040
Bitter gourd mosaic virus	KX690627
Bitter gourd yellow mosaic virus	MH481856
Bitter gourd yellow vein virus	NC_024894
Cabbage leaf curl virus	MH248136
Chayote enation yellow mosaic virus	KX259339
Chayote yellow mosaic virus	KT454819
Chilli leaf curl India virus	KJ649706
Chilli leaf curl virus	KM921669
Chino del tomate Amazonas virus	NC_038443
Chino del tomate virus	AF226664
Cleome leaf crumple virus	MN337873
Coccinia mosaic Tamil Nadu virus	NC_024810
Coccinia mosaic Virudhunagar virus	KY860899
Cotton leaf curl Burewala virus	HM461862
Cotton leaf curl Gezira virus	MK947933
Cotton leaf curl Rajasthan virus	AM501481
Croton yellow vein mosaic virus	LN878119
Cucumber chlorotic leaf virus	MN013786
Eupatorium yellow vein virus	NC_043121
Euphorbia yellow mosaic virus	JX415192
Honeysuckle yellow vein virus	HM164545
Indian cassava mosaic virus	DQ780004
Kenaf leaf curl virus	HM448898
Luffa yellow mosaic virus	NC_004824
Malvastrum yellow vein Honghe virus	MN249688
Melon chlorotic leaf curl virus	KC153490
Melon chlorotic mosaic virus	NC_014380
Melon yellow mosaic virus	MH665365
Merremia mosaic virus	AY508991
Mungbean yellow mosaic India virus	MK757221
Papaya leaf curl China virus	EU874386
Papaya leaf curl virus	DQ629103
Pedilanthus leaf curl virus	NC_008299
Pepper golden mosaic virus	MN013410
Pepper huasteco yellow vein virus	JQ303122

BEGOMOVIRUS	Acc. No.
Pepper leaf curl Lahore virus	JN135234
Pepper leaf curl Yunnan virus	KU975395
Pepper yellow leaf curl Aceh virus	LC465991
Pepper yellow leaf curl Indonesia virus	LC542629
Pepper yellow vein Mali virus	MH778681
Potato yellow mosaic Trinidad virus	NC_004638
Potato yellow mosaic virus	MN006828
Rhynchosia golden mosaic Sinaloa virus	NC_038802
Sida micrantha mosaic virus	KC706535
Sida mosaic Sinaloa virus	KX440612
Sida yellow net virus	KU996356
Solanum mosaic Bolivia virus	KJ592721
Soybean chlorotic blotch virus	KT454815
Squash leaf curl China virus	MN437657
Squash leaf curl Philippines virus	NC_005845
Squash leaf curl virus	MG763920
Squash leaf curl Yunnan virus	MN563794
Squash mild leaf curl virus	NC_004645
Squash yellow mild mottle virus	NC_003865
Synedrella leaf curl virus	MN210357
Telfairia golden mosaic virus	NC_030403
Tobacco curly shoot virus	NC_003722
Tobacco leaf curl Japan virus	AB055008
Tobacco leaf curl Kochi virus	NC_004641
Tobacco leaf curl Pusa virus	KM383734
Tobacco leaf curl Thailand virus	NC_009553
Tobacco leaf curl virus	NC_029994
Tobacco leaf curl Yunnan virus	MK946452
Tomato bright yellow mosaic virus	NC_038467
Tomato chino La Paz virus	JN676150
Tomato chlorotic leaf curl virus	KY449277
Tomato chlorotic leaf distortion virus	NC_015962
Tomato chlorotic mottle Guyane virus	NC_038965
Tomato chlorotic mottle virus	KC706559
Tomato common mosaic virus	NC_010835
Tomato curly stunt virus	KM438733
Tomato dwarf leaf curl virus	AF035224
Tomato dwarf leaf virus	NC_016580
Tomato enation leaf curl virus	NC_026761
Tomato golden leaf distortion virus	NC_043122
Tomato golden leaf spot virus	NC_021579
Tomato golden mosaic virus	JF694488

BEGOMOVIRUS	Acc. No.
Tomato golden mottle virus	NC_008058
Tomato golden vein virus	NC_038807
Tomato interveinal chlorosis virus	NC_038469
Tomato interveinal chlorosis virus-2	MK087038
Tomato latent virus	NC_038963
Tomato leaf curl Anjouan virus	NC_038894
Tomato leaf curl Antsiranana virus	AM701766
Tomato leaf curl Arusha virus	NC_009030
Tomato leaf curl Bangalore virus	KP164856
Tomato leaf curl Bangladesh virus	KM383758
Tomato leaf curl Barka virus	NC_024116
Tomato leaf curl Burkina Faso virus	NC_033779
Tomato leaf curl Cameroon virus	KT382327
Tomato leaf curl Cebu virus	NC_010439
Tomato leaf curl China virus	NC_005320
Tomato leaf curl Comoros virus	NC_028114
Tomato leaf curl Cotabato virus	NC_010441
Tomato leaf curl Diana virus	NC_038896
Tomato leaf curl Gandhinagar virus	NC_023034
Tomato leaf curl Ghana virus	NC_010313
Tomato leaf curl Guangdong virus	NC_008373
Tomato leaf curl Guangxi virus	NC_008329
Tomato leaf curl Gujarat virus	HM625838
Tomato leaf curl Hainan virus	NC_013102
Tomato leaf curl Hanoi virus	NC_015124
Tomato leaf curl Hsinchu virus	DQ866131
Tomato leaf curl Joydebpur virus	NC_007723
Tomato leaf curl Karnataka virus	MN020537
Tomato leaf curl Karnataka virus 2	KF551578
Tomato leaf curl Karnataka virus 3	KF551585
Tomato leaf curl Kerala virus	NC_011135
Tomato leaf curl Kumasi virus	NC_011096
Tomato leaf curl Kunene virus	MT045996
Tomato leaf curl Liwa virus	NC_023312
Tomato leaf curl Madagascar virus	NC_006874
Tomato leaf curl Mahe virus	MH410152
Tomato leaf curl Mali virus	MH794642
Tomato leaf curl Mayotte virus	NC_006876
Tomato leaf curl Mindanao virus	NC_010440
Tomato leaf curl Moheli virus	NC_038897
Tomato leaf curl Mumbai virus	MH577037
Tomato leaf curl Namakely virus	NC_038899

BEGOMOVIRUS	Acc. No.
Tomato leaf curl New Delhi virus	NC_004611
Tomato leaf curl New Delhi virus 2	NC_038470
Tomato leaf curl New Delhi virus 4	NC_038471
Tomato leaf curl New Delhi virus 5	EF450316
Tomato leaf curl Nigeria virus	NC_012206
Tomato leaf curl Oman virus	NC_014542
Tomato leaf curl Palampur virus	NC_010840
Tomato leaf curl Patna virus	NC_012492
Tomato leaf curl Philippines virus	EU487035
Tomato leaf curl Pune virus	KP178732
Tomato leaf curl purple vein virus	NC_035481
Tomato leaf curl Rajasthan virus	NC_038472
Tomato leaf curl Ranchi virus	NC_016965
Tomato leaf curl Seychelles virus	NC_009031
Tomato leaf curl Sinaloa virus	NC_009606
Tomato leaf curl Sri Lanka virus	NC_004647
Tomato leaf curl Sudan virus	KT728748
Tomato leaf curl Sulawesi virus	NC_013413
Tomato leaf curl Taiwan virus	KP195718
Tomato leaf curl Togo virus	HE659517
Tomato leaf curl Toliara virus	NC_038901
Tomato leaf curl Uganda virus	NC_038473
Tomato leaf curl Vietnam virus	GQ338765
Tomato leaf curl virus	JX416176
Tomato leaf deformation virus	NC_014510
Tomato leaf distortion virus	NC_038474
Tomato mild mosaic virus	NC_010833
Tomato mild yellow leaf curl Aragua virus	NC_009490
Tomato mosaic Havana virus	NC_003867
Tomato mosaic Trujillo virus	KY449275
Tomato mosaic leaf curl virus	NC_005850
Tomato mottle leaf curl virus	NC_032006
Tomato mottle Taino virus	NC_001828
Tomato mottle wrinkle virus	NC_025265
Tomato rugose mosaic virus	NC_002555
Tomato rugose yellow leaf curl virus	NC_020257
Tomato severe leaf curl Kalakada virus	KP195267
Tomato severe leaf curl virus	MH678589
Tomato severe rugose virus	KC706617
Tomato twisted leaf virus	MK440292
Tomato vein clearing leaf deformation virus	MK423208
Tomato yellow leaf curl Axarquia virus	NC_025680

BEGOMOVIRUS	Acc. No.
Tomato yellow leaf curl China virus	NC_004044
Tomato yellow leaf curl Guangdong virus	NC_008374
Tomato yellow leaf curl Indonesia virus	NC_008267
Tomato yellow leaf curl Kanchanaburi virus	MK946453
Tomato yellow leaf curl Malaga virus	JN859135
Tomato yellow leaf curl Mali virus	NC_027215
Tomato yellow leaf curl Sardinia virus	JN859134
Tomato yellow leaf curl Saudi virus	NC_022229
Tomato yellow leaf curl Shuangbai virus	NC_029995
Tomato yellow leaf curl Thailand virus	KX943294
Tomato yellow leaf curl Vietnam virus	NC_009548
Tomato yellow leaf curl virus	MK908813
Tomato yellow leaf curl Yunnan virus	NC_021341
Tomato yellow leaf deformation dwarf virus	MN145942
Tomato yellow leaf distortion virus	NC_011348
Tomato yellow margin leaf curl virus	NC_005852
Tomato yellow mottle virus	NC_019946
Tomato yellow spot virus	KC706628
Tomato yellow vein streak virus	KJ413253
Watermelon chlorotic stunt virus	NC_003708