Organisation Européenne et Méditerranéenne pour la Protection des Plantes
European and Mediterranean Plant Protection Organization

Normes OEPP
EPPO Standards

Diagnostics
Diagnostic

PM 7/50

Organisation Européenne et Méditerranéenne pour la Protection des Plantes
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Approval

EPPO Standards are approved by EPPO Council. The date of approval appears in each individual standard. In the terms of Article II of the IPPC, EPPO Standards are Regional Standards for the members of EPPO.

Review

EPPO Standards are subject to periodic review and amendment. The next review date for this EPPO Standard is decided by the EPPO Working Party on Phytosanitary Regulations.

Amendment record

Amendments will be issued as necessary, numbered and dated. The dates of amendment appear in each individual standard (as appropriate).

Distribution

EPPO Standards are distributed by the EPPO Secretariat to all EPPO member governments. Copies are available to any interested person under particular conditions upon request to the EPPO Secretariat.

Scope

EPPO Standards on Diagnostics are intended to be used by NPPOs in their capacity as bodies responsible for the application of phytosanitary measures. Standards on diagnostic protocols are concerned with the diagnosis of individual pests and describe different methods which can be used to detect and identify pests of phytosanitary concern for the EPPO region. General Standards on diagnostics are in preparation on: (1) the purpose of diagnostic protocols (which may differ according to the circumstances of their use); and (2) reporting and documentation of diagnoses.

In 1998, EPPO started a new programme to prepare diagnostic protocols for the regulated pests of the EPPO region (including the EU). The work is conducted by the EPPO Panel on Diagnostics and other specialist Panels. The objective of the programme is to develop an internationally agreed diagnostic protocol for each regulated pest. The protocols are based on the many years of experience of EPPO experts. The first drafts are prepared by an assigned expert author(s). They are written according to a 'common format and content of a diagnostic protocol' agreed by the Panel on Diagnostics, modified as necessary to fit individual pests. As a general rule, the protocol recommends a particular means of detection or identification which is considered to have advantages (of reliability, ease of use etc.) over other methods. Other methods may also be mentioned, giving their advantages/disadvantages. If a method not mentioned in the protocol is used, it should be justified.

The following general provisions apply to all EPPO Standards on Diagnostics:

- laboratory tests may involve the use of chemicals or apparatus which present a certain hazard. In all cases, local safety procedures should be strictly followed
- use of names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable
- laboratory procedures presented in the protocols may be adjusted to the standards of individual laboratories, provided that they are adequately validated or that proper positive and negative controls are included.

References

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EU (2000) Council Directive 2000/29/EC of 8 May 2000 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. *Official Journal of the European Communities* L169, 1–112.

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Definitions

Regulated pest: a quarantine pest or regulated non-quarantine pest.
Quarantine pest: a pest of potential economic importance to the area endangered thereby and not yet present there, or present but not widely distributed and being officially controlled.

Outline of requirements

EPPO Standards on Diagnostics provide all the information necessary for a named pest to be detected and positively identified by an expert (i.e. a specialist in entomologist, mycology, virology, bacteriology, etc.). Each protocol begins with some short general information on the pest (its appearance, relationship with other organisms, host range, effects on host, geographical distribution and its identity) and then gives details on the detection, identification, comparison with similar species, requirements for a positive diagnosis, list of institutes or individuals where further information on that organism can be obtained, references (on the diagnosis, detection/extraction method, test methods).

Existing EPPO Standards in this series

Forty-one EPPO standards on diagnostic protocols have already been approved and published. Each standard is

numbered in the style PM 7/4 (1), meaning an EPPO Standard on Phytosanitary Measures (PM), in series no. 7 (Diagnostic Protocols), in this case standard no. 4, first version. The existing standards are:

PM 7/1 (1) *Ceratocystis fagacearum*. *Bulletin OEPP/EPPO Bulletin* **31**, 41–44

PM 7/2 (1) *Tobacco ringspot nepovirus*. *Bulletin OEPP/EPPO Bulletin* **31**, 45–51

PM 7/3 (1) *Thrips palmi*. *Bulletin OEPP/EPPO Bulletin* **31**, 53–60

PM 7/4 (1) *Bursaphelenchus xylophilus*. *Bulletin OEPP/EPPO Bulletin* **31**, 61–69

PM 7/5 (1) *Nacobbus aberrans*. *Bulletin OEPP/EPPO Bulletin* **31**, 71–77

PM 7/6 (1) *Chrysanthemum stunt pospiviroid*. *Bulletin OEPP/EPPO Bulletin* **32**, 245–253

PM 7/7 (1) *Aleurocanthus spiniferus*. *Bulletin OEPP/EPPO Bulletin* **32**, 255–259

PM 7/8 (1) *Aleurocanthus woglumi*. *Bulletin OEPP/EPPO Bulletin* **32**, 261–265

PM 7/9 (1) *Cacoecimorpha pronubana*. *Bulletin OEPP/EPPO Bulletin* **32**, 267–275

PM 7/10 (1) *Cacyreus marshalli*. *Bulletin OEPP/EPPO Bulletin* **32**, 277–279

PM 7/11 (1) *Frankliniella occidentalis*. *Bulletin OEPP/EPPO Bulletin* **32**, 281–292

PM 7/12 (1) *Parasaissetia nigra*. *Bulletin OEPP/EPPO Bulletin* **32**, 293–298

PM 7/13 (1) *Trogoderma granarium*. *Bulletin OEPP/EPPO Bulletin* **32**, 299–310

PM 7/14 (1) *Ceratocystis fimbriata* f. sp. *platani*. *Bulletin OEPP/EPPO Bulletin* **33**, 249–256

PM 7/15 (1) *Ciborinia camelliae*. *Bulletin OEPP/EPPO Bulletin* **33**, 257–264

PM 7/16 (1) *Fusarium oxysporum* f. sp. *albedinis*. *Bulletin OEPP/EPPO Bulletin* **33**, 265–270

PM 7/17 (1) *Guignardia citricarpa*. *Bulletin OEPP/EPPO Bulletin* **33**, 271–280

PM 7/18 (1) *Monilinia fructicola*. *Bulletin OEPP/EPPO Bulletin* **33**, 281–288

PM 7/19 (1) *Helicoverpa armigera*. *Bulletin OEPP/EPPO Bulletin* **33**, 289–296

PM 7/20 (1) *Erwinia amylovora*. *Bulletin OEPP/EPPO Bulletin* **34**, 159–172

PM 7/21 (1) *Ralstonia solanacearum*. *Bulletin OEPP/EPPO Bulletin* **34**, 173–178

PM 7/22 (1) *Xanthomonas arboricola* pv. *corylina*. *Bulletin OEPP/EPPO Bulletin* **34**, 179–182

PM 7/23 (1) *Xanthomonas axonopodis* pv. *dieffenbachiae*. *Bulletin OEPP/EPPO Bulletin* **34**, 183–186

PM 7/24 (1) *Xylella fastidiosa*. *Bulletin OEPP/EPPO Bulletin* **34**, 187–192

PM 7/25 (1) *Glomerella acutata*. *Bulletin OEPP/EPPO Bulletin* **34**, 193–200

PM 7/26 (1) *Phytophthora cinnamomi*. *Bulletin OEPP/EPPO Bulletin* **34**, 201–208

PM 7/27 (1) *Puccinia horiana*. *Bulletin OEPP/EPPO Bulletin* **34**, 209–212

PM 7/28 (1) *Synchytrium endobioticum*. *Bulletin OEPP/EPPO Bulletin* **34**, 213–218

PM 7/29 (1) *Tilletia indica*. *Bulletin OEPP/EPPO Bulletin* **34**, 219–228

PM 7/30 (1) *Beet necrotic yellow vein benyvirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 229–238

PM 7/31 (1) *Citrus tristeza closterovirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 239–246

PM 7/32 (1) *Plum pox potyvirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 247–256

PM 7/33 (1) *Potato spindle tuber pospiviroid*. *Bulletin OEPP/EPPO Bulletin* **34**, 257–270

PM 7/34 (1) *Tomato spotted wilt tospovirus*. *Bulletin OEPP/EPPO Bulletin* **34**, 271–280

PM 7/35 (1) *Bemisia tabaci*. *Bulletin OEPP/EPPO Bulletin* **34**, 281–288

PM 7/36 (1) *Diabrotica virgifera*. *Bulletin OEPP/EPPO Bulletin* **34**, 289–294

PM 7/37 (1) *Thaumetopoea pityocampa*. *Bulletin OEPP/EPPO Bulletin* **34**, 295–298

PM 7/38 (1) *Unaspis citri*. *Bulletin OEPP/EPPO Bulletin* **34**, 299–302

PM 7/39 (1) *Aphelenchoides besseyi*. *Bulletin OEPP/EPPO Bulletin* **34**, 303–308

PM 7/40 (1) *Globodera rostochiensis* and *Globodera pallida*. *Bulletin OEPP/EPPO Bulletin* **34**, 309–314

PM 7/41 (1) *Meloidogyne chitwoodi* and *Meloidogyne fallax*. *Bulletin OEPP/EPPO Bulletin* **34**, 315–320

Some of the Standards of the present set result from a different drafting and consultation procedure. They are the output of the DIAGPRO Project of the Commission of the European Union (no. SMT 4-CT98-2252). This project involved four 'contractor' diagnostic laboratories (in England, Netherlands, Scotland, Spain) and 50 'inter-comparison' laboratories in many European countries (within and outside the European Union), which were involved in ring-testing the draft protocols. The DIAGPRO project was set up in full knowledge of the parallel activity of the EPPO Working Party on Phytosanitary Regulations in drafting diagnostic protocols, and covered regulated pests which were for that reason not included in the EPPO programme. The DIAGPRO protocols have been approved by the Council of EPPO as EPPO Standards in series PM 7. They will in future be subject to review by EPPO procedures, on the same terms as other members of the series.

European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes

Diagnostics[1]
Diagnostic

Tomato yellow leaf curl and *Tomato mottle begomoviruses*

Specific scope

This standard describes a diagnostic protocol for *Tomato yellow leaf curl begomovirus* (TYLCV) and *Tomato mottle begomovirus* (ToMoV).

Specific approval and amendment

This Standard was developed under the EU DIAGPRO Project (SMT 4-CT98-2252) by partnership of contractor laboratories and intercomparison laboratories in European countries. Approved as an EPPO Standard in 2004-09.

Introduction

EU countries regulate a number of viruses infecting tomato and other Solanaceous crops, transmitted by the whitefly *Bemisia tabaci*. Most of these are members of the genus *Begomovirus*. Two groups in particular are concerned: (1) *Tomato yellow leaf curl begomovirus* (TYLCV), present in certain European countries, together with various closely related viruses which were originally considered as strains but are now distinguished as species, and may or may not be present in European countries; (2) non-European viruses transmitted by *B. tabaci* (Brown & Bird, 1992), of which the named example in EU regulations is *Tomato mottle begomovirus* (still referred to in the phytosanitary texts as 'Florida tomato virus'); in recent years, a whole series of other non-European begomoviruses of *Solanaceae* has been described (Fauquet & Stanley, 2003; Fauquet *et al*., 2003). This Standard concentrates on the two named examples.

Tomato yellow leaf curl virus

Tomato yellow leaf curl virus (TYLCV) was first recorded as a whitefly-transmitted virus in tomato crops in Israel (Cohen & Harpaz, 1964) and later shown to be a member of the family *Geminiviridae*, genus *Begomovirus*. Together with related species which have recently been distinguished, it causes the most devastating virus disease complex of tomato in tropical and warm temperate regions of the world, where losses up to 100% are incurred (Moriones, 2000). Members of the TYLCV complex have been recorded in tomato crops in Spain, Italy, Portugal and France, and occur in most eastern Mediterranean countries (Idriss *et al*., 1997) and parts of sub-Saharan Africa, Asia, Australia, the Caribbean, and USA (Florida). In the EPPO region, two species are present (Moriones *et al*., 2000) according to the most recent nomenclature (Fauquet *et al*., 2000): *Tomato yellow leaf curl begomovirus* (TYLCV) (the first described species, originally known as the Israel strain (TYLCV-IL)), and *Tomato yellow leaf curl Sardinia begomovirus* (TYLCSV), originally known as the Sardinia strain (TYLCV-S). In this Standard, the two are mostly treated together, except at specific points where it is necessary to refer to them separately.

The main whitefly vector *Bemisia tabaci* transmits TYLC viruses in a persistent, circulative manner (Cohen *et al*., 1966). There are no reports of seed transmission in tomato and mechanical transmission does not occur in nature (Moriones *et al*., 2000). Four biotypes of *B. tabaci* are currently present in Europe, of which biotypes B and Q efficiently transmit TYLCV and TYLCSV. Global expansion of the B biotype of *B. tabaci*, also termed the silverleaf whitefly, and proposed as a separate species, *B. argentifolii* by Bellows *et al*. (1994) is associated with the emergence of TYLCV in Europe and the Western Hemisphere (Polston & Anderson, 1997). The B biotype of *B. tabaci* is highly fecund and has the ability to adapt to new host crops unlike established regional populations of *B. tabaci*. In Spain, TYLCV has been found to be the causal agent of a novel disease of phaseolus beans (Navas-Castillo *et al*., 1999; Sánchez-Campos *et al*., 1999) and also occurs in capsicums (Reina *et al*., 1999). TYLCV isolates are monopartite and consist of geminate, quasi-isometric particles, 20 nm in diameter and 30 nm in length (Brunt *et al*., 1990).

Tomato mottle begomovirus

The whitefly transmitted geminivirus *Tomato mottle begomovirus* (ToMoV) was first observed in 1989, and

[1]The Figures in this Standard marked 'Web Fig.' are published on the EPPO website www.eppo.org.

recorded by Simone *et al*. (1990) and Abouzid & Hiebert (1991), on tomato crops in Florida (USA). A serious outbreak occurred in the 1990/1991, associated with large outbreaks of *Bemisia tabaci* biotype B. It has since been recorded also in tomato in S. Carolina, Tennessee and Virginia, and on the weed *Solanum viarum*. ToMoV is mechanically transmissible. Symptoms on tomato are mild when compared with TYLCV or *Tomato golden mosaic begomovirus* (TGMV) (Abouzid *et al*., 1992). Experimental hosts include members of three solanaceous genera (*Lycopersicon*, *Nicotiana*, and *Physalis*) and *Phaseolus vulgaris*. The genome of ToMoV has been sequenced (Abouzid *et al*., 1992) and is bipartite.

Approach to detection and diagnosis

Serological methods have met limited success when used for whitefly-transmitted *Geminiviridae* (Harrison *et al*. 1991; Muniyappa *et al*., 1991) as antisera have proved difficult to produce. Relatively non-specific monoclonal antibodies have been produced, which detect TYLCV and ToMoV, but cross-react with a range of other *B. tabaci*-transmitted *Geminiviridae*. In the EPPO region, only TYLCV and TYLCSV currently occur, so field samples submitted for diagnosis are most likely to be these species. Serological tests on material from outside the EPPO region could also detect, but not distinguish, other species of the TYLC complex, and various other *Begomovirus* spp. including ToMoV. Molecular methods are then needed for diagnosis.

Identity

Name: *Tomato yellow leaf curl begomovirus*
Synonyms: *Tomato yellow leaf curl bigeminivirus*, *Tomato yellow leaf curl geminivirus*
Acronym: TYLCV
Taxonomic position: Viruses: *Geminiviridae*: *Begomovirus*
EPPO computer code: TYLCV0
Phytosanitary categorization: EPPO A2 list no. 182, EU Annex designation II/A2

Name: *Tomato mottle begomovirus*
Synonyms: Tomato mottle virus, Florida tomato virus
Acronym: ToMoV
Taxonomic position: Viruses: *Geminiviridae*: *Begomovirus*
EPPO computer code: TOMOV0
Phytosanitary categorization: EPPO A1 list no. 225, EU Annexes: listed as Florida tomato virus under non-European viruses transmitted by *Bemisia tabacci* in Annex I/A1

Detection

Symptoms

In tomato, symptoms caused by TYLC viruses vary depending on the growth stage at the time of initial infection, environmental conditions and the tomato cultivar; they include severe stunting, marked reduction in leaf size, upward cupping, chlorosis of leaf margins, mottling, flower abscission and significant yield reduction CSL (Morris, 2000). In phaseolus beans, TYLC symptoms include leaf thickening, leaf crumpling, upward curling of leaves, abnormal lateral shoot proliferation, deformation of pods and reduction in pod number. In capsicum, TYLC symptoms include interveinal and marginal leaf chlorosis, upward curling of leaf margins (or symptomless). In *Lisianthus*, TYLC symptoms include distortion of the growing tips, cup-shaped leaves, swelling of veins on the lower surface of the leaves, significant reduction in flower quality and stunting.

Symptoms of ToMoV in tomato include chlorotic mottling, leaf distortion and curling and stunting.

Sampling

For TAS-ELISA, appropriate sample selection is critical for serological detection of TYLC viruses and ToMoV. There is more detectable virus in freshly expanded young leaves present in the uppermost regions of the plant than in older plant parts. Younger leaf material should be selected for TAS-ELISA sample preparation as described in Appendix 1.

For PCR, detection of TYLC viruses and ToMoV is achieved using fresh or lyophilized leaf material. Leaf material should be derived from freshly expanded young leaves present in the uppermost regions of the plant and should be collected carefully to avoid cross contamination (by changing disposable gloves between samples, or preferably by punching a piece of leaf directly into an Eppendorf tube using the lid as a cutting implement). Recommended DNA extraction methods are described in Appendix 2.

Identification

The procedure for identification of TYLCV and ToMoV is summarized in the flow-diagram in Fig. 1.

Screening Tests

TAS-ELISA
In cases where TYLC viruses or ToMoV are suspected, a TAS-ELISA test (Appendix 1) can be performed to aid in presumptive diagnosis. However, final diagnosis cannot be achieved using ELISA alone, since currently available antisera may miss samples at early stages of infection, or cross-react with other ssDNA viruses. The antiserum supplied by Adgen (http://www.adgen.co.uk/) is effective as a universal screen, but cannot differentiate between TYLC viruses and ToMoV. The antisera supplied for TYLCV detection by DSMZ (DSMZ AS-0421/DSMZ AS-0546/2/DSMZ 0546/4 – http://www.dsmz.de/plvirus) can be used for the detection and differentiation of TYLCV and TYLCSV (present in Europe), but cannot effectively resolve mixed species infections. Known infected plants should be used as positive controls in ELISA tests, in addition to healthy leaf material of the host species as a negative control. A buffer-only control should also be included.

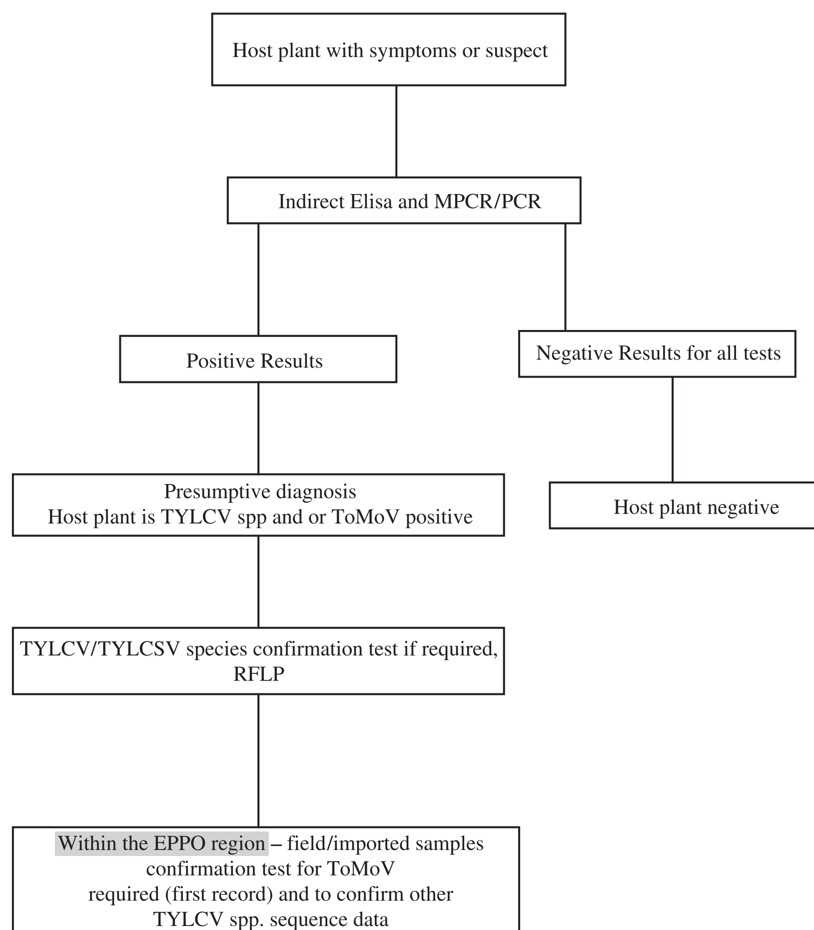

Fig. 1 Flow diagram for the identification of TYLCV and ToMoV.

PCR

For PCR, DNA is extracted from samples of plant material infected with TYLC viruses or other *Begomovirus* spp. according to the procedures described in Appendix 2. PCR for the detection of TYLCV/TYLCSV is carried out as described in Accotto *et al*. (2000) (Appendix 3). This confirms the diagnosis of TYLCV/TYLCSV, but also be a useful indicator of possible presence of other ssDNA virus. If a negative result is obtained with symptomatic material which gave a positive result in the screening text, or an atypical restriction digest pattern occurs using this method, the presence of another ssDNA virus such as ToMoV may be achieved by carrying out the PCR procedure of Deng *et al*. (1994) (Appendix 3), extended to ToMoV detection by Morris.

Various other methods have been experimented but are not included in this Standard. An alternative screening method for TYLCV/TYLCSV is dot-blot hybridization, but the method is limited in that it cannot reliably distinguish between the members of the TYLC complex because of some probe cross reactivity, and can be influenced by virus concentration in the sample. Probes for all TYLC viruses have not yet been documented (Accotto *et al*., 2000). Thus this method could be used as a general screen for TYLCV/TYLCSV within the EPPO region but, as discussed for ELISA, would have to be backed up by one of the PCR methods given in this protocol to prevent false negative or false positive results.

The degenerate primers of Polston *et al*. (1995) may be used for the detection of ToMoV followed by RFLP. Southern blotting for the detection of ToMoV (Murphy *et al*., 2000) is an alternative but of limited use since confirmation of ToMoV in Europe screened by any of the above methods currently also requires nucleotide sequence analysis. PCR extraction, PCR set-up, and post-PCR analysis should be performed in separate laboratory areas to avoid contamination. Filter tips and gloves should be used. A positive control should be included, and more than one negative control of the same species as the test plant. A master-mix control (no template in the reaction) blank is also required.

Confirmation

Confirmation of TYLCV or TYLCSV is achieved using RFLP (Accotto *et al*., 2000) as described in Appendix 3 or the PCR method of Martínez-Culebras *et al*. (2001) for the rapid discrimination of TYLCV and TYLCSV. PCR as described in Appendix 3 (Deng *et al*., 1994) is recommended to identify ToMoV, and all TYLC viruses, to be confirmed by sequencing. Within the EPPO region, confirmation of positive PCR tests for ToMoV would at present require sequence analysis (using

standard methodology) to confirm a first record. However, sequence analysis is not recommended as a method for routine diagnosis since it is time-consuming and costly. Where species-specific tools (primers, antisera) for molecular or serological identification are not available, it is the only method.

The results of TAS-ELISA and PCR screening tests for initial identification require confirmation. Where RFLP results positive for TYLCV or TYLCSV are obtained using the method of Accotto *et al*. (2000), the sample can be confirmed as positive. When negative results are achieved using the PCR method of Accotto *et al*. (2000), or if RFLP using this method produces a digestion pattern which is not indicative of TYLCSV or TYLCV, confirmation of the negative result or diagnosis of another ssDNA virus such as ToMoV should be achieved by the PCR procedure of Deng *et al*. (1994), followed by sequencing. Where the PCR result thus obtained is negative, the sample can be confirmed as negative. Where the PCR result is positive, preliminary identification of ToMoV could be determined by PCR amplicon size, followed by sequencing. Confirmation of ToMoV requires sequence analysis both in samples derived from imports to the EPPO region and in field samples from EPPO countries to confirm a first record of a 'new' *Begomovirus* in the region.

Reporting and documentation

Guidelines on reporting and documentation are given in EPPO Standard PM7/– (in preparation).

Further information

Further information on these organisms can be obtained from Virology teams PLHC and PLHB, Central Science Laboratory, Sand Hutton, York Y041 1LZ (UK). E-mail: jane.morris@csl.gov.uk.

Acknowledgements

This protocol was originally drafted by J. Morris, Central Science Laboratory, York (GB).

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Appendix 1

ELISA test

Preparation of the sample for ELISA testing

Weigh approximately 1 g of the infected plant material. Place each sample in a suitable polythene bag for processing. Add the

correct volume of extraction buffer (Macintosh *et al*., 1992) and homogenize the sample using a Homex 6 machine (Bioreba) or using a wallpaper seam roller, or similar. Pipette 100 µL of homogeneous sample into a pair of wells on the microtitre plate for testing. Conserve the remainder of the extract at 4°C until testing is completed.

TAS-ELISA test

This ELISA, based on the method of Thomas *et al*. (1986), employs *African cassava mosaic virus* (ACMV) polyclonal antiserum as the trapping antibody, and mouse monoclonal antisera for detection. Microtitre plates (Nunc Maxisorp Immunoplate) are used. Known infected plants are used as positive controls, together with healthy plants of the same species as the test plants as a negative control.

Add the crude ACMV polyclonal antibody at the recommended dilution (10^{-3}) to the coating buffer. Pipette the solution into the microtitre plates, 100 µL per well. Incubate for 3 h at 33°C. Flick out the contents of the wells. Wash the wells three times with PBS-Tween with 3 min soaks between washes. Blot dry on absorbent paper. Add sample homogenate at 100 µL per well, using two wells per test sample. Incubate at 4°C overnight. Flick out the contents of the wells. Wash the wells four times with PBS-Tween with 3 min soaks between washes. Blot dry on absorbent paper. Add TYLCV/ToMoV monoclonal antibody SCR 23 at the recommended dilution in dried milk buffer at 100 µL per well. Incubate for 2 h at 33°C. Flick out the contents of wells. Wash the wells four times with PBS-Tween with 3-min soaks between washes. Blot dry on absorbent paper. Prepare alkaline phosphatase conjugate at appropriate dilution in dried milk buffer. Add 100 µL to each well. Incubate for 2 h at 33°C. Flick out the contents of the wells. Wash the wells three times with PBS-Tween with 3-min soaks between washes. Blot dry on absorbent paper. Prepare alkaline phosphatase substrate solution. Add 100 µL to each well. Incubate at ambient temperature for 1 h. Read absorbance at 405 nm.

Interpretation of the ELISA test

The ELISA test is negative if the absorbance of the sample is less than 2 times the absorbance of the healthy control. The ELISA test is positive if the absorbance of the sample is equal or greater than 2 times the absorbance of the healthy control.

Materials used for TAS-ELISA

- Extraction buffer for tissue maceration (Macintosh *et al*., 1992): 0.05 M Tris-HCl pH 8.5, 6.05 g; 0.06 M Na_2SO_3, 7.56 g. Make up to just below 1 L with deionized distilled water, adjust pH to 8.5 with HCl and make up to 1 L with additional water
- Carbonate coating buffer pH 9.6: Na_2CO_3, 1.59 g: $NaHCO_3$, 2.93 g; distilled water, 1 L. Dissolve the ingredients and check pH. Store solution at 4°C
- 10 × Phosphate-buffered saline (PBS) 1 × = pH 7.2: NaCl, 80 g; KH_2PO_4, 2 g; $Na_2HPO_4 \cdot 12H_2O$, 11.5 g; KCl, 2 g; distilled water, 1 L. Dissolve all ingredients and check pH. Dilute to 1 × for use
- Phosphate-buffered saline Tween (PBS-T): 10 × PBS, 100 mL; 10% Tween 20, 5 mL; distilled water, 895 mL. Mix ingredients well
- Antibody buffer: PBS-T, 100 mL; 5% dried milk powder, 5 g. Prepare on day of use
- Substrate buffer (diethanolamine buffer 1 M): diethanolamine, 95 mL; distilled water, 800 mL. Mix and adjust to pH 9.8 with concentrated HCl. Make up to 1 L with distilled water. Add 0.203 g of $MgCl_2$. Store solution at 4°C
- 2 (p-nitrophenyl phosphate) phosphatase substrate: dissolve two 5 mg tablets (Sigma 104) per 10 mL of substrate solution
- TYLCV-detection kits incorporating the detecting monoclonal antisera SCR23 within test kit 1072-05 were obtained from Adgen (Auchincruive, Scotland).

Appendix 2

DNA extraction methods

DNA extraction method 1 is recommended for PCR, with extraction method 2 supplied as an alternative. The leaf squash method of Atzmon *et al*. (1998) is also an alternative, but details are not provided.

DNA extraction method 1

The method of Accotto *et al*. (2000) can be used for TYLCV and ToMoV DNA extraction. Leaf material can easily be collected using an Eppendorf tube without touching the leaf with fingers, thus avoiding cross contamination. The method can be carried out using either 10 mM β-mercaptoethanol in the extraction buffer or 10 mM citric acid. Grind 0.15 g of leaf material to a fine powder in liquid nitrogen using either a pestle and mortar, or a plastic grinding bag and wallpaper roller. Ensure the tissue does not thaw once frozen. Add 500 µL of extraction buffer (100 mM Tris-HCl pH 8, 50 mM EDTA, 500 mM NaCl, 1% SDS and 10 mM β-mercaptoethanol or citric acid 10 mM). Mix tubes by vigorous shaking and then incubate at 65°C for 5 min. Add 150 µL of 5 M potassium acetate and shake tubes vigorously to mix, then incubate at 0°C for 10 min. This step removes most proteins and polysaccharides as a complex with the insoluble potassium dodecyl sulphate precipitate. Tubes are then spun at 13 000 rev min^{-1} for 10 min and the supernatant (about 500 µL) withdrawn to a tube containing 350 µL of cold isopropanol, then mixed well and centrifuged at 13 000 rev min^{-1} for 10 min. Wash precipitate by addition of 500 µL of 70% ethanol and centrifuge for 5 min at 13 000 rev min^{-1}. Carefully discard ethanol and desiccate for 15 min in a vacuum chamber or thoroughly air-dry. Resuspend pellets in 100 µL of 10 mM Tris, 1 mM EDTA pH 8.0.

DNA extraction method 2

For the method of Lohdi *et al*. (1994), add 200 mg of plant material to liquid nitrogen. Add 2–3 mL of CTAB extraction

buffer (Appendix 4). Homogenize the sample using a wooden/plastic wallpaper roller or similar. Decant ground sap into a 1.5 mL microfuge tube. Incubate for 10–15 min at 65°C. Centrifuge for 5 min at 13 000 rev min^{-1}. Remove 700 µL of supernatant into a new microfuge tube. Extract with an equal volume (700 µL) of chloroform: IAA (24 : 1). Vortex at low speed for 2 s. Centrifuge at 13 000 rev min^{-1} at ambient temperature for 10 min. Collect the upper aqueous layer and transfer the contents to another Eppendorf. Repeat. Precipitate with 0.5 volume of 5 M sodium chloride and an equal volume of ice-cold isopropanol. Incubate at –20°C overnight. Pellet the DNA by centrifugation at 13 000 rev min^{-1} for 10 min. Wash precipitate by addition of 500 µL of 70% ethanol and centrifuge for 5 min at 13 000 rev min^{-1}. Carefully discard ethanol. Desiccate for 15 min to dry the pellet. Resuspend pellet in 100 µL of 1 × TE (Appendix 4). Alternatively the EZNA extraction kit Omega Biotek is recommended.

Appendix 3

PCR tests

PCR method 1

For the TYLCV/TYLCSV PCR test in plants (Accotto *et al*., 2000), prepare the PCR reaction mix in a 1.5 mL microfuge tube. Final concentrations for PCR reaction components (kept on ice) are: 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM (final concentration) primer TY1, 0.4 µM (final concentration) primer TY2, 0.4 U µL^{-1} Taq DNA polymerase for a total of 25 µL. For further reactions, calculate the quantity of each component for the required number of reactions (25 µL per sample aliquot). Make up 1 more than needed for easier pipetting. For PCR after mixing the components, transfer 25 µL of PCR reaction mix, with 1 µL of nucleic acid DNA to a 0.2 mL Eppendorf tube. Place the tubes in the thermal cycler heating block. Run the following programme on the thermal cycler: 1 cycle (4 min at 95°C), 35 cycles (30 s at 95°C, 30 s at 60°C, 30 s at 72°C), 1 cycle (7 min at 72°C). Analyse 5 µL of PCR product on a 1% agarose gel or store tubes at –20°C until analysis is performed.

Interpretation of the PCR test result

If an amplicon of the expected size of 580 bp is present, precipitate with ethanol the remaining 20 µL of PCR product and carry out RFLP to confirm TYLCV identity as described. If no amplicon is present or amplicon of unexpected size, PCR method 2 should be performed to determine whether the sample is negative or other ssDNA virus species present.

RFLP

Resuspend DNA (ethanol-precipitated 20 µL volume of PCR product) and digest with Ava 11 in 10 µL volume. Load all the reaction mix on a 3% NuSieve agarose gel (or 2% agarose). Restriction products of 68 bp, 360 bp, and 150 bp are expected for TYLCSV. Restriction products of 277 bp and 302 bp are expected for TYLCV.

PCR Method 2

For the MPCR/PCR test for geminiviruses in plants (TYLC viruses and ToMoV) (Deng *et al*., 1994), prepare the PCR reaction mix in a 1.5 mL microfuge tube. A typical PCR reaction mixes is: 10 × PCR buffer 5 µL, 1.75 mM $MgCl_2$ 3.5 µL, 0.2 mM dNTPs 1 µL (of 100 µL stock solution), 0.2 µM (final conc) primer 540 2 µL, 0.2 µM (final conc) primer 541 2 µL, 2 U Taq DNA polymerase 0.4 µL, sterile ultra-pure water 34.6 µL, total 48.5 µL. For further reactions, calculate the quantity of each component for the required number of reactions (48.5 µL per sample aliquot). Make up 1 more than needed for easier pipetting. For PCR after mixing the components, transfer 48.5 µL of PCR reaction mix, with 1.5 µL of DNA to a 0.5 mL Eppendorf tube. Place the tubes in the thermal cycler heating block. Run the following programme on the thermal cycler: 1 cycle (2 min at 94°C), 1 cycle (1 min at 55°C), 1 cycle (2 min at 72°C), 32 cycles (45 s at 94°C, 1 min at 55°C, 2 min at 72°C), 1 cycle (45 s at 94°C, 1 min at 55°C, 5 min at 72°C). Analyse PCR product or store tubes at –20°C until analysis is performed.

Analysis of PCR product

The PCR fragments are detected by agarose gel electrophoresis and stained with ethidium bromide. Prepare a 1–2% agarose gel by gently bringing to the boil agarose in 1 × TBE (Appendix 4). Cool the molten agarose to 50–60°C, pour into the mould and insert the comb. Allow the gel to set. Remove the comb; submerge the gel in 1 × TBE. Add 10 µL of loading buffer to tubes containing 50 µL of sample, flick to homogenize the solution. Load the wells carefully (12 µL of sample + buffer). Include appropriate markers and positive control, amplified DNA. Run gel at 100 V/40 mA for 1 h until the gel dye front is within 1 cm of the end of the gel. Remove gel and stain in ethidium bromide solution (0.5 µg mL^{-1}) for 45 min. Rinse the gel in distilled water. Visualize the amplified DNA fragments by UV *trans*-illumination. The PCR product for TYLCV with degenerate primers (Appendix 4) is 540 bp in length. Verify results against DNA marker and positive control. Photograph the gel to provide a permanent record.

Interpretation of the PCR/MPCR test result

Where more than one PCR product is present per lane, the test is termed MPCR. The test is negative if the characterized 540 bp fragment (TYLC or other geminivirus) or the 377 bp fragment (ToMoV) is not detected and the fragments for the respective positive control isolate(s) are detected. The test is positive if the 540 bp fragment (TYLC or other geminivirus) or the 377 bp fragment (ToMoV) are detected and the fragment is identical with the positive control isolate(s).

RFLP utilising PCR products derived from PCR method 2

Sequence analysis is required to confirm a first record of ToMoV. Definitive RFLP patterns are not available for all TYLCV species, so sequencing is recommended to confirm virus identity.

Appendix 4

Materials for detection of TYLC viruses or ToMoV in plants by PCR

Oligonucleotide primer sequences
TY1 (+): 5′-GCC CAT GTA (T/C) C G (A/G) AAG CC-3′
TY2 (–): 5′-GG (A/G) TTA GA (A/G) GCA TG (A/C) GTA C-3′
Deng 541: 5′-TAA TAT TAC CKG WKG VCC SC-3′
Deng 540: 5′-TGG ACY TTR CAW GGB CCT TCA CA-3′
(where K = G or T, R = A or G, S = C or G, W = A or T, Y = C or T, B = C, G or T, and V = A, C or G)

Extraction buffer for DNA extraction method 1: 100 mM Tris-HCl pH 8, 50 mM EDTA, 500 mM NaCl, 1% SDS, 10 mM β-mercaptoethanol or 10 mM citric acid.

Extraction buffer for DNA extraction method 2: 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 L with distilled water. Store solution at ambient temperature. Add PVP and sodium sulphite fresh to aliquot of stock buffer (this will keep for about 2 weeks).
TE Buffer for DNA extraction methods 1 and 2: 10 mM Tris-HCl, 1 mM EDTA, molecular grade water. Adjust to pH 8.0.
10 × Tris borate EDTA buffer (for gel electrophoresis): 108 g Tris HCl, 55 g boric acid, 7.4 EDTA. Make up to 1 L with molecular grade water and adjust to pH 8.2.
Restriction enzymes: Ava11 TYLCV/TYLCSV.
Lateral flow devices (LFDs): Product Code 14-772 (10 tests) – ADGEN; Product code 14-773 (100 tests)– ADGEN.