Diagnostics
Diagnostic

Cucumber vein yellowing virus (*Ipomovirus*)

Specific scope

This standard describes a diagnostic protocol for *Cucumber vein yellowing virus (Ipomovirus)*.

Specific approval and amendment

Approved in 2007-09.

Introduction

Cucumber vein yellowing virus (*Ipomovirus*) (CVYV) causes severe damages in cucumbers and other cucurbits. The virus is transmitted mechanically and by the whitefly *Bemisia tabaci*, in a semipersistent manner (Mansour & Al-Musa, 1993). It has been reported from Iran, Sudan and from a number of countries in the Mediterranean Basin such as Cyprus, Israel, Jordan, Portugal, Spain and Turkey (Cohen & Nitzany, 1960; Harpaz & Cohen, 1965; Al-Musa *et al*., 1985; Yilmaz *et al*., 1989; Andalucia Department of Agriculture and Fisheries, 2001; Cuadrado *et al*., 2001; Louro *et al*., 2004; Papayiannis *et al*., 2005; OEPP/EPPO, 2005; Bananej *et al*., 2006).

CVYV naturally infects cultivated cucurbit crops such as cucumber (*Cucumis sativus*) (Cohen & Nitzany, 1960), melon (*Cucumis melo*) (Yilmaz *et al*., 1989), watermelon (*Citrullus lanatus*) (Janssen & Cuadrado, 2001) and zucchini (*Cucurbita pepo*) (Rubio *et al*., 2003). Wild cucurbits are also reported as hosts in Jordan (Mansour & Al-Musa, 1993) and in Spain (Janssen *et al*., 2002). Natural infections of weed species belonging to families other than *Cucurbitaceae* have been described from Spain (Janssen *et al*., 2002). Experimental cucurbit hosts such as *Cucurbita moschata*, *Cucurbita foetidissima*, *Citrullus colocynthis*, have been studied using mechanical inoculation and *B. tabaci*-inoculation (Cohen & Nitzany, 1960; Al-Musa *et al*., 1985; Yilmaz *et al*., 1989; Mansour & Al-Musa, 1993). The virus can infect both crops in open fields or under plastic tunnels. The economic impact of the virus is higher in crops cultivated under protection because its vector is more difficult to control in this situation.

Further information can be found in the EPPO data sheet on *Cucumber vein yellowing virus* (*Ipomovirus*) (OEPP/EPPO, 2005).

Identity

Name: *Cucumber vein yellowing virus* (*Ipomovirus*)
Synonyms (including former names): Bottle gourd mosaic virus (Cohen & Nitzany, 1960; Harpaz & Cohen, 1965)
Acronym: CVYV
Taxonomic position: viruses: *Potyviridae: Ipomovirus*.
Phytosanitary categorization: EPPO A2 list no. 316.

Detection

Symptoms

CVYV-infected cucumber plants display vein clearing on the youngest leaves (Cohen & Nitzany, 1960) (Fig. 1). Occasionally, yellow/green mosaic is observed on fruits (Cuadrado *et al*., 2001). Symptoms in melon have been described as vein yellowing, vein clearing and stunting (Yilmaz *et al*., 1989) (Fig. 2). Sudden death was observed on melon in Spain (Janssen & Cuadrado, 2001). In watermelon, a mild leaf chlorosis is sometimes observed (Fig. 3), but most often symptoms are inconspicuous or not expressed (Andalucia Department of Agriculture and Fisheries, 2001; Louro *et al*., 2004). Occasional splitting of fruits has been observed (Janssen & Cuadrado, 2001; Louro *et al*., 2004). In zucchini, symptoms range from chlorotic mottling (Fig. 4) to vein yellowing, or are absent (Andalucia Department of Agriculture and Fisheries, 2001).

Ultramicroscopic observation

Ultramicroscopic observations on cells from infected plants revealed pinwheel-shaped cytoplasmic inclusions characteristic of infection by members of the *Potyviridae* (Lecoq *et al*., 2000).

Fig. 1 Symptoms of CVYV on young leaves of cucumber.

Fig. 2 Symptoms of CVYV on melon leaves.

Fig. 3 Symptoms of CVYV on young leaves of watermelon.

Fig. 4 Symptoms of CVYV on leaves of zucchini.

Sampling

Preliminary studies using antibodies in western blot experiments revealed the presence of viral coat protein in all organs and tissues, including roots, stems, leaves, petioles, tendrils, flowers, and fruits of inoculated cucumber plants (Martínez-García *et al*., 2004). However, in field conditions the virus showed irregular distribution within cucumber, melon and zucchini plants when studied using hybridisation of tissue prints with non-radioactive cDNA probes (Rubio *et al*., 2003). Consequently, samples from different parts of plant should be taken, to reliably detect CVYV.

Mechanical and whitefly-mediated transmission to test plants

CVYV is readily transmissible by inoculation to cucurbits, but does not cause symptoms (either local or systemic) to

Chenopodium quinoa. The recommended test plant for both mechanical and whitefly-mediated transmission is *Cucumis sativus* (vein clearing followed by vein yellowing in systemically infected leaves). Inoculation of a set of differential hosts for viruses infecting cucurbits, e.g. CMV, WMV and *Papaya ringspot virus* (PRSV), can further identify and discriminate CVYV from other, mechanically transmitted plant viruses. In particular, absence of symptoms on *C. quinoa* or *C. amaranticolor* can differentiate CVYV from 10 other sap-transmissible viruses infecting cucurbits, including CMV, PRSV, and WMV. Infection of CVYV in cucurbit species has also often been found in combination with other *Bemisia tabaci*-transmitted viruses such as *Cucurbit yellow stunting disorder virus* (Janssen *et al*., 2003; Louro *et al*., 2004). In addition, co-infection with *Trialeurodes vaporariorum*-transmitted *Beet pseudo-yellows virus* should not be ruled out, although this combination has not been reported so far. Of these three viruses (CVYV, *Cucurbit yellow stunting disorder virus*, and *Beet pseudo-yellows virus*), only CVYV is mechanically transmitted to cucumber test plants, producing distinctive vein yellowing symptoms, without local lesions.

The efficiency of sap inoculation depends on a buffer that reduces the instability of the virus in plant sap, and on a method of inoculation and incubation of the test plants which is adequate to the development of symptoms. This detection method is simple and sensitive, although of limited diagnostic value when used alone. At least two plants per species should be inoculated. Test-plant inoculations are useful not only for virus detection but also for isolation and propagation of the virus in plant tissues to be used in subsequent identification methods, such as tissue-print hybridization and RT-PCR. Additionally, the virus bioassays are essential for host range studies and the test-infected plants are used for the maintenance of reference virus isolates. Details on mechanical transmission are given in Appendix 1.

An alternative to mechanical transmission is inoculation of cucumber seedlings using viruliferous adults of *Bemisia tabaci*. Details on CVYV transmission by *B. tabaci* are given in Appendix 1.

Identification

Identification of CVYV is possible by reverse transcriptase-polymerase chain reaction (RT-PCR) and nucleic acid hybrization.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

This method detects CVYV isolates from Israel, Spain, Portugal, and Cyprus. Negative and positive control samples must be included in the analysis, together with the test samples. The detailed protocol is given in Appendix 2 and is recommended as it has been thoroughly tested.

Nucleic acid hybridisation

Nucleic acid hybridization is the most suitable method when large numbers of plants need to be tested. The method for nucleic acid hybridization using total RNA or tissue prints is presented in Appendix 3.

The procedures for detection and identification described in this protocol and in the flow diagram in Fig. 5 should be followed. If symptoms of vein yellowing and/or mosaic are seen on cucurbits in association with the presence of *Bemisia tabaci* (or, generically, of whiteflies), and the disease is readily transmissible by mechanical inoculation to cucurbits only, but does **not** cause symptoms (either local or systemic) to *Chenopodium quinoa*, it is highly probable that CVYV is present. The procedures do not change in the absence of symptoms and signs of the presence of whiteflies.

A positive identification requires at least **one** of the following:

- Amplification of a 450-bp fragment in RT-PCR with specific primers
- Nucleic acid hybridization of extracted total RNA or tissue-printing.

In critical cases, confirmation by inoculation to *C. sativus* or using the molecular method not used in the first assay is recommended.

Reference material

All plant reference materials, i.e. CVYV isolates and isolates of the most important viruses infecting cucurbits are available at the Plant Virus Division of the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. Plasmids carrying cloned CVYV, total nucleic acid preparations of CVYV-infected cucumbers, freeze-dried plant material and quality controlled digoxigenin-labelled RNA or cDNA probes can also be obtained there (www.dsmz.de). GenBank accession codes are AF233429 and AJ301640.

Reporting and Documentation

Guidance on reporting and documentation is given in EPPO Standard PM 7/77 (1) *Documentation and reporting on a diagnosis*.

Acknowledgements

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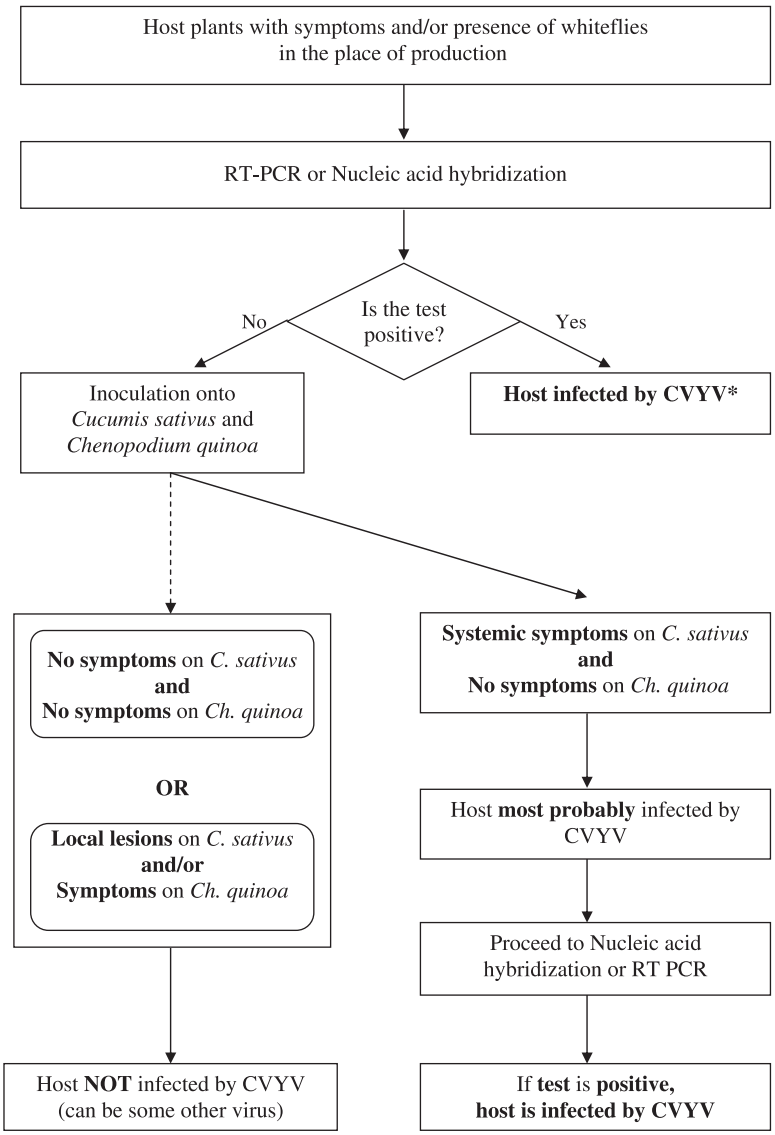

Fig. 5 Flow diagram for the detection and identification of CVYV.

* in critical cases, confirmation by inoculation to *C. sativus* or using the molecular method not used in the first assay is recommended.

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Appendix 1

Mechanical transmission

Seedlings of cucurbit test plants should preferably be inoculated at the cotyledonal stage, i.e. when cotyledons are fully expanded, but before expansion of the first true leaf. Inoculation onto the first true leaf is also acceptably sensitive.

Inoculum is prepared by grinding the infected material, preferably tissues from young symptomatic leaves, in 0.05 M Na/K phosphate buffer, pH 7.0, containing 1 mM Na_2EDTA, 5 mM sodium diethyl dithiocarbamate (DIECA), 5 mM thioglycolic acid, using a chilled mortar and pestle. A spatula of active charcoal is added to the homogenate, which is kept chilled on ice until the end of inoculation. The inoculation is done by gently rubbing the inoculum over the surface of the cotyledon leaves, previously dusted with carborundum powder. After the inoculation, the plants should be rinsed with water and kept in a glasshouse or a growth chamber at 22–26°C.

Symptoms appear after 8–12 days on cucumber test plants kept at an average temperature of 25°C and are usually visible on the second true leaf. At lower temperatures the development of the symptoms is slow, and the symptoms are milder or even absent on some infected plants.

Whitefly transmission to test plants

If possible, allow adults of *B. tabaci* from a CVYV-free colony to feed on suspected field samples for an 8 to 32 h acquisition access period (AAP) in a plastic cage. At the end of the AAP, transfer insects to test plants, clipping the insects (about 15 per plant) in small clip cages (see, for example, Muniz & Nombela, 2001) on the first or second true leaf of test plants. Remove cages with whiteflies after at least 12 h of inoculation access period (IAP), marking the inoculated leaf with a permanent marker. Keep the test plants at 24–26°C. In order to avoid the development of adult whiteflies, remove the inoculated leaf of each plant after about 10 days from the end of IAP. If a CVYV-free colony of *B. tabaci* is not available, insects can be collected on symptomatic plants or leaves and transferred for 24 h of IAP as described above. In this case, more insects per plant should be used, possibly in different clip cages.

Symptoms normally appear within 15 days from the end of IAP.

Appendix 2

Reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA extraction

Leaf samples (about 200 mg) are ground in an iced mortar, in liquid nitrogen. One mL of total nucleic acid isolation reagent (TRI Reagent, Ambion, USA) is added. The preparation is thawed at room temperature and transferred to a 1.5 mL test tube. Manufacturer's instructions (Ambion) are then followed.

The pellet is dried and resuspended in 15 µL of DEPC-treated water.

1 µL of the resuspended RNA is used and diluted 1:70 for the RT-PCR reaction. The total RNA extraction can be stored at –80°C.

TRIzol Reagent (Invitrogen, USA) or other similar monophasic acid phenol-chloroform solutions can also be used in place of TRI Reagent.

Alternatively other kits for RNA extraction are commercially available and are suitable for CVYV detection (e.g. RNeasy, Qiagen, Germany has been used and shown to be effective).

RT-PCR

A one-step RT-PCR system (Invitrogen) is used according tothe manufacturers' instructions, and using the primers described by Cuadrado *et al*. (2001). Using the primer pair 5′-AGCTAGCGCGTATGGGGTGAC-3′ [CVYV(+)] and 5′-GCGCCGCAAGTGCAAATAAAT-3′ [CVYV(–)] yields a 450-bp fragment from the CVYV coat protein gene.

The diluted RNA is denatured at 65°C for 5 min and quickly chilled on ice. 5 µL of the denatured RNA is aliquoted into chilled PCR tubes (0.2 mL) to perform a 25 µL reaction, and 20 µL of the reaction mixture containing 0.25 µM of each primer and 0.5 µL of the RT-Taq mix are added.

The tubes are transferred to a pre-heated thermocycler (50°C) for immediate reverse transcription followed by a denaturation step and PCR amplification (35 cycles). Conditions for RT-PCR are 50°C for 30 min, 94°C for 2 min, 35 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 1 min) and 72°C for 5 min.

Five µL aliquots of each reaction product should be analysed on 2% agarose gel in TBE buffer.

Presence of CVYV in a suspected plant sample is confirmed when fragments of 450 bp are found in the RT-PCR .

Appendix 3

Nucleic acid hybridization

Sample preparation

Best results are obtained with total RNA preparations made either with a commercially available RNA isolation kit or solution (e.g.: RNeasy, Qiagen, Germany; TRI Reagent, Ambion, USA; TRIzol Reagent, Invitrogen, USA) following the manufacturer's instructions or using established protocols. As an example, the following procedure is taken from Celix *et al*. (1996).

Total RNA is extracted from 0.2 g of leaf tissues ground in liquid nitrogen with 2 volumes of 2% sodium dodecyl sulfate (SDS), 100 mM Tris-HCl (pH 8.0) and 10 mM EDTA. After vortexing, samples are extracted with 1 volume of phenol-chloroform, and the aqueous phase is adjusted to 2 M LiCl. After overnight incubation at 4°C, samples are centrifuged at 12 000 g for 15 min, and the pellets dissolved in 100 µL of sterile H_2O.

A tissue print assay format is also possible by firmly pressing cross-sections of freshly cut petioles onto nylon membrane (Rubio *et al*., 2003). Cross sections of several petioles from the same sample plant need to be blotted on the membrane. After air-drying, the membrane is irradiated with UV light (50 mJ) in a cross-linking oven. Although tissue printing is a simple and fast method when many samples are to be tested, interpretation of results is generally easier when total nucleic acid extracts from plants are spotted on membranes. This provides more consistent and clear positive signals and is recommended for multiple, successive hybridization analyses to tests for presence of several viruses.

Nucleic acid probes

In general, it is recommended to use RNA probes for detection of CVYV although dsDNA probes work well (Rubio *et al*., 2003).

RNA probes

Using digoxigenin-11-UTP-labelled RNA probes synthesized by *in vitro* transcription from plasmids carrying respective viral sequences, high sensitivity and specificity in virus detection is reached. Probes against the most significant viruses infecting cucurbits and including CVYV are available at the DSMZ Plant Virus Division, Braunschweig, Germany, http://www.dsmz.de and can be either obtained as plasmid clones or as DIG-labelled RNA probes.

Hybridization with RNA probes should be run according to the instructions, distributed with the diagnostic, that supply sufficient information to perform the hybridization, including temperatures and durations of the various steps. RNA probes, once used in formamide-containing hybridization buffer, can be reused several times without loss of signal.

dsDNA probes

The following procedure is taken from Rubio *et al*. (2003).

To synthesize a CVYV-specific probe by RT-PCR, the following primers have been designed:

VVA1: (5′-GGGTCATGATGTTGTCCCCAGCTTCATTT-GAATC-3′), which encompasses positions 559–580 of CVYV RNA (GenBank sequence AF233429) and includes a *Rca* I restriction site (underlined nucleotides), and VVA2 (5′-CCCCGCTTAGCAAATACAGTAATTACTAAGAACG-3′), which is complementary to positions 1701–1723 of same sequence and includes a *Cel* II restriction site (underlined nucleotides). Total RNAs from CVYV-infected plants were extracted with TRIzol Reagent (Invitrogen) following the manufacturer's instructions. An aliquot of this extract equivalent to 2 mg of fresh tissue is denatured at 95°C for 5 min, chilled on ice and immediately reverse-transcribed by incubation at 42°C for 1 h in a 20 µL reaction mixture containing first strand buffer, 1 mM DTT, 1 mM of each dCTP, dATP, dGTP and dTTP, 0.4 µM of primer VVA2, 4U of RNASEOUT Ribonuclease inhibitor (Invitrogen) and 20U of Superscript II RNAse H-(Invitrogen).

The labelling with digoxigenin is as follows: an aliquot (1/10) of this preparation is PCR amplified in a 20 µL reaction mixture containing PCR buffer, 1.5 mM $MgCl_2$, 1 mM of each of the four dNTPs, 70 µM DIG-UTP (Roche), 0.5 U of *Taq* DNA polymerase (Invitrogen), and 0.2 µM of each primer, VVA1 and VVA2, according to the following conditions 94°C for 2 min, 35 cycles of (94°C for 30 s, 50°C for 30 s, 72°C for 1 min), 72°C for 5 min.

Five µL aliquots of each reaction product are analyzed on 2% agarose gel in 0.5X TBE buffer and the amount of probe produced is estimated.

Pre-hybridize for 2 h in ULTRAhyb hybridization buffer (Ambion) at 55°C, then add about 25 ng of digoxigenin-labelled probe and incubate overnight at 55°C.

The membrane is then washed twice (5 min each) in 2× SSC and 1% SDS at room temperature and twice (15 min each) in 0.1× SSC and 0.1% SDS at 65°C.

Hybridization is revealed using the DIG Luminescent Detection kit (Roche) following the manufacturer's instructions.

To enable distinction between positive and negative reactions healthy plants should be included in the test.