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Short communication

Occurrence and diagnosis of *Tomato chlorosis virus* in Portugal

D. Louro[1], G.P. Accotto[2] and A.M. Vaira[2,*]
[1]*Direcção-Geral de Protecção das Culturas – Quinta do Marquês 2784-505 Oeiras, Portugal;* [2]*Istituto di Fitovirologia Applicata – CNR – Strada Delle Cacce 73 10135 Torino, Italy;* [*]*Author for correspondence (Fax: +39 011 343809; E-mail: a.vaira@ifa.to.cnr.it)*

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Abstract

Tomato chlorosis virus (ToCV), a new whitefly-transmitted and phloem-limited *Crinivirus* infecting tomatoes in Europe, is reported for the first time in Portugal. Tomato plants with symptoms of interveinal chlorosis, collected during autumn 1998 and summer and autumn 1999 in Algarve, southern Portugal, were positive in RT-PCR assays using ToCV-specific primers. The amplified 439 bp fragment was sequenced and showed 99% homology with the ToCV sequence in the GenBank database. A digoxigenin–DNA probe was produced and tested in dot-blot with total RNAs extracted from tomato samples. Both the RT-PCR and dot-blot hybridisation procedures enabled rapid and reliable detection of ToCV from field samples.

Tomato chlorosis virus (ToCV) is a whitefly-transmitted, phloem-limited, bipartite closterovirus, now placed in the genus *Crinivirus* (Fauquet and Mayo, 1999). In tomato, ToCV causes interveinal yellowing on leaves that often develop red or necrotic flecks, and brittle and rolling lower leaves (Wisler et al., 1998a). It was noted in Florida in 1989 (Simone et al., 1996), and has been reported elsewhere in the USA and in Taiwan (Wisler et al., 1999). Wisler et al. (1998b) have shown that ToCV is distinct from *Tomato infectious chlorosis virus* (TICV; Duffus et al., 1996), a *Crinivirus* causing similar symptoms in tomato. The two viruses are serologically unrelated, their nucleic acids do not cross-hybridise and TICV is transmitted only by *Trialeurodes vaporariorum* (Westwood), while ToCV is transmitted by *T. vaporariorum*, *T. abutilonea* (Haldeman) and *Bemisia tabaci* (Gennadius) biotypes A and B (Wisler et al., 1998a).

In Portugal, the whitefly *B. tabaci* was first recorded in 1992, in horticultural crops (J. Guimarães, pers. comm.) and since 1995, it has been an important pest in Algarve, southern Portugal. In this region, *B. tabaci* and *T. vaporariorum* occur together, both in the open and in greenhouses, although the former species is more prevalent, except in winter.

During autumn 1998, an unusual yellow leaf disorder of tomato was observed in a greenhouse of the Experimental Station at Patacão, Algarve. Intermediate and lower leaves of plants showed chlorotic mottle and interveinal yellowing, with rolling and brittleness. Later these symptoms developed in the young leaves, together with bronzing and necrotic flecks on older leaves. Some plants also showed symptoms of yellow leaf curling. High populations of *B. tabaci* were present. All attempts to detect viruses, other than *Tomato yellow leaf curl virus-Israel*, (TYLCV-Is; Accotto et al., 2000), a whitefly-transmitted *Begomovirus*, by standard methods, were unsuccessful.

Similar symptoms in tomato were seen during 1999, in commercial Algarve greenhouses. Samples were collected from mature crops in June and young crops in October. A few samples, from central and northern Portugal, showing yellowing, were also examined (see Table 1, samples 6–8). Symptomatic fully expanded leaves from 2 to 4 plants of the same cultivar collected in each greenhouse were pooled. About 0.1 g of leaf tissue was used to prepare total RNA using either RNeasy Plant Mini kit (Qiagen) or RNAWiz reagent (Ambion). The total RNA was diluted to about 0.1–0.5 μg/μl and 1 μl was used for RT-PCR.

Two ToCV-specific oligonucleotide primers were designed from the GenBank sequence (acc. no. AF024630), corresponding to the coding sequence of the heat shock protein (HSP70) homolog. The primers, not degenerate and aiming to be specific for ToCV, were the following: ToCV-172(+) (5′-GCTTCCGAAACTCCGTCTTG-3′) and ToCV-610(−) (5′-TGTCGAAAGTACCGCCACC-3′). The expected PCR product was a 439 bp fragment from the 5′ region of the ORF (nucleotides 172–610 of the AF024630 sequence); the phosphate 1 motif was not included in the amplified sequence while the upstream primer was designed on the sequence of the phosphate 2 motif. RT-PCR was performed using the One Step RT-PCR System (Life Technologies): total RNAs were first heat-denatured at 65 °C for 5 min and quickly chilled on ice; the reaction mixture was then added to the PCR tubes. The first step of reverse transcription was done at 50 °C for 30 min, then, after a brief denaturation step at 94 °C, 35 cycles (15 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C) were performed, ending with a terminal extension step of 5 min at 72 °C, in a Perkin Elmer 2400 Thermal Cycler.

The specificity of the assay is illustrated in Figure 1. The expected fragment was amplified from some samples but not from healthy controls. Total RNA extracted from leaves of *Nicotiana benthamiana* infected by TICV (gift of V. Lisa) gave no amplification. When TICV-specific primers (A.M. Vaira and G.P. Accotto, unpublished) were tested on the same samples, no

Table 1. Occurrence of ToCV and TYLCV-Is in tomato field samples showing leaf yellowing symptoms collected in different parts of Portugal

Tomato samples	Serial No.	Date	Cultivar	Place/Region	ToCV		TYLCV-Is
					PCR	Dot-blot	PCR[c]
T7–8[a]	1	Jun, ’99	Brilhante	Laranjeiro, Algarve	+	+	−
T23–24[a]	2	Jun, ’99	Sinatra	Maragota, Algarve	+	+	−
T28–31[a]	3	Jun, ’99	nd[b]	Moncarapacho, Algarve	+	+	−
T32–35[a]	4	Jun, ’99	nd	Moncarapacho, Algarve	+	+	−
T40–42[a]	5	Jun, ’99	nd	Porto Carro, Algarve	−	−	−
T50	6	Aug, ’98	Gama	Vairão, Douro Litoral	−	−	−
T55	7	Jun, ’99	nd	Vila Franca, Ribatejo	−	−	−
T56	8	Jun, ’99	Alpado	Vairão, Douro Litoral	−	−	−
T57	9	Nov, ’98	Daniela	Patacão, Algarve	+	+	−
T8[a]	10	Oct, ’99	Manthus	Paço Branco, Algarve	+	+	+
T9	11	Oct, ’99	Manthus	Paço Branco, Algarve	+	+	+
T10	12	Oct, ’99	Manthus	Paço Branco, Algarve	nt[d]	+	+
T12[a]	13	Oct, ’99	Manthus	Paço Branco, Algarve	+	+	+

[a]Pooled samples. [b]Not determined. [c]With primers TY1(+) and TY2(−), according to Accotto et al., 2000. [d]Not tested.

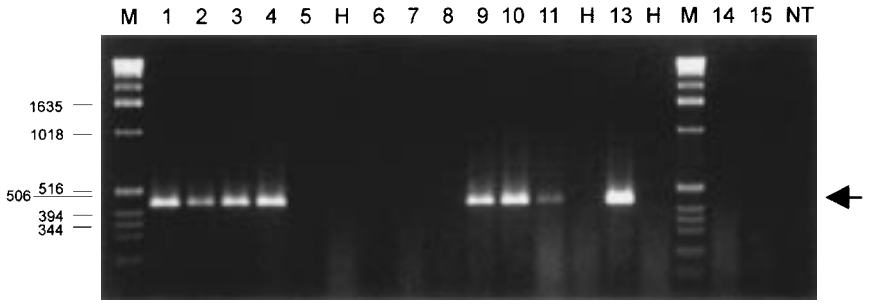

Figure 1. Detection of ToCV by RT-PCR with primers ToCV-172(+) and ToCV-610(−). Ethidium bromide stained 1.7% agarose gel in TBE; H = healthy tomato sample; 14 = TICV-infected *N. benthamiana* sample; 15 = healthy *N. benthamiana* sample; others, field tomato samples (see Table 1). NT = no template; M = Gibco BRL 1 kb DNA ladder, sizes in bp at left; the arrow indicates the amplified fragment.

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1       GCTTCCGAAA CTCCGTCTTG TTATTTCTAT GATTTGAAAA GATGGGTTGG TGTCACTTCG
61      GTCAATTATG AGGTAGTGAA AGCGAAGATA AACCCAATGT ATAAAACGCG TTTATCTAAT
121     AATAAAGTGT ATATAACTGG TATCAATAAA GGTTTCTCGA CCGAGTTTTC GGTTGAGCAA
181     CTTATATTAC ATTATGTTAA CACTTTAGTT CGATTGTTCT CAAAAACAGA AAACTTAAAA
241     ATAACCGATC TCAATGTGTC TGTTCCGGCT GATTACAAGT CTGGGCAGAG ACTTTTCATG
301     CAGGCAGTTT GTTCCTCTTT GGGTTTCAAT TTACGTCGCA TAGTCAATGA ACCGTCGGCT
361     GCCGCTATTT ACTGCGTTTC TAAATATCCG CAGTATGCTT ATTTCTATAT TTACGATTTT
421     GGTGGCGGTA CTTTCGACA
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Figure 2. Nucleotide sequence of the PCR fragment amplified from the Portuguese ToCV isolate T12 (Acc. No. AF234029). The sequences of the primers used for amplification are in italics.

amplification occurred except with TICV-infected *N. benthamiana* (data not shown).

The ToCV-specific 439 bp fragment amplified from one sample (T12) was purified using the High Pure PCR kit (Roche) and sequenced in both directions using the primers designed for amplification. The sequence obtained (Figure 2) showed 99% identity to that of AF024630, confirming infection by ToCV. Of the four differences found, only one led to a change in the derived amino acid sequence (M instead of T, deriving from the change at position 269).

A digoxigenin-labelled probe was synthesised by PCR using the same primers, with the PCR DIG Probe Synthesis kit (Roche). In dot-blot hybridisations, 3.5 µl of total RNA were spotted on positively-charged nylon membrane (Roche). Pre-hybridisation and hybridisation were done at 50 °C in high SDS buffer (7% SDS, 50% formamide, 5 × SSC, 2% Boehringer blocking reagent, 50 mM sodium phosphate pH7, 0.1% N-laurylsarcosine), followed by high stringency washings and chemiluminescent detection with CDP-Star, according to manufacturer's instructions (Boehringer Mannheim – The DIG System User's Guide for Filter Hybridisation). The ToCV probe hybridised with the same samples that were positive in PCR and no reaction was observed with healthy or TICV-infected samples (Figure 3).

Most samples collected in the Algarve in June 1999 were only infected by ToCV, while most of those collected in the same areas in October were infected with both ToCV and TYLCV-Is. Samples with yellowing symptoms collected in central and northern Portugal were not infected by ToCV (Table 1).

Tomato crops under plastic and in the open have attained major economic importance in many Mediterranean regions, and at the same time, *T. vaporariorum* and *B. tabaci* have become serious pests. Under these circumstances, there is a real danger of ToCV spreading rapidly.

The impact of ToCV, either alone or in mixed infection with TYLCV-Is on tomato production, has yet to be determined in these areas and more information is needed about crop susceptibility and on weed plants that could be virus reservoirs.

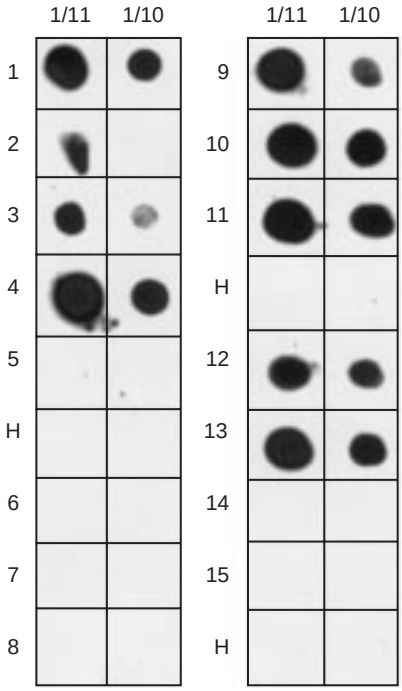

Figure 3. Dot-blot hybridisation of tomato total RNA extracts with ToCV-specific digoxigenin-DNA probe. Undiluted sample on the left, 1/10 dilution on the right. 14 = TICV-infected *N. benthamiana*; 15 = healthy *N. benthamiana*; others, field tomato samples (see Table 1).

Use of a quick and easy method to extract total RNA combined with a simple one-step RT-PCR procedure, and the availability of a stable ToCV-specific DNA probe, both reliable in ToCV diagnosis, could help to understand the dynamics of this new, emerging disease.

This is the first report of ToCV in Portugal, and, together with the concurrent report from Spain (E. Moriones, pers. comm.) it signals the first appearance of this new virus in Europe.

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