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Ultrastructural aspects of tomato leaves infected by Tomato torrado virus (ToTV) and co-infected by other viruses

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Optical and electron microscopy studies were carried out to investigate the cytopathology induced in tomato leaves infected by Tomato torrado virus (ToTV), a new picorna-like virus associated with the 'Torrado' disease. Infected leaves, showing typical Torrado disease symptoms were surveyed in commercial greenhouses in the main tomato production areas of Spain. The effect of the co-infection of ToTV with other viruses which commonly infect tomato crops was also studied. Ultra-thin sections of ToTV-infected tomato leaves did not show a strong cellular alteration. However, crystalline arrays of isometric virus-like particles (VLPs) of 20–30 nm in the inclusion bodies were observed in phloem parenchyma cells of the infected tissues. Tissues co-infected by ToTV and either *Tomato chlorosis virus* (ToCV) or *Pepino mosaic virus* (PepMV) presented more severe cellular alterations. The most deleterious consequences for tomato cells were found in triple infections of ToTV, PepMV and *Tomato spotted wilt virus* (TSWV), where characteristic cell wall overgrowth was distinguishable, together with a large amount of necrotic cells.

Keywords: cytopathology, electron microscopy, mixed infection, necrosis, synergism, Torrado disease

Introduction

Since 2001, the new tomato disease referred to as 'Torrao' or 'Torrado' has been affecting several tomato (*Solanum lycopersicum*) crops in Murcia (SE Spain) and the Canary Islands, the most important tomato production areas of Spain (Alfaro-Fernández *et al*., 2006, 2007). The affected plants show an initial plesionecrosis in the basal zone of leaves, close to the central nerve, that later develops into necrotic spots, which are sometimes abscised and/or produce 'shot holes' in the leaflet (hence the Spanish name 'cribado' meaning 'sieve'). Sometimes affected plants show extensive holonecrosis which progresses from the base to the apical area of the leaf. Longitudinal necrotic lesions also appear on stems. Patterns of necrotic lines are usually observed on forming fruits which often crack with fruit growth, rendering them unmarketable. Affected plants have a burnt-like appearance which seriously affects production.

The association of this disease with a virus was confirmed by Verbeek *et al*. (2007a) who characterized it as a picorna-like virus and proposed the name Tomato torrado virus (ToTV), a member type of a new genus, Torradovirus (Verbeek *et al*., 2007a), which also includes two other recently characterized tomato-infecting viruses, Tomato apex necrosis virus (ToANV) and Tomato marchitez virus (ToMarV) (Turina *et al*., 2007; Verbeek *et al*., 2007b). ToTV was also detected on common weeds present in Spanish tomato crops (Alfaro-Fernández *et al*. 2008), and it was transmitted to other solanaceous crops such as pepper and eggplant under controlled conditions (Amari *et al*., 2008). Two whitefly species, *Trialeurodes vaporariorum* (Pospieszny *et al*., 2007) and *Bemisia tabaci* (Amari *et al*., 2008) have been shown to be efficient vectors of ToTV.

Tomato crops are seriously affected by several viral diseases in which mixed viral infection may result in synergisms, causing more severe disease symptoms than those produced in single infections (García-Cano *et al*., 2006). In different surveys conducted since the first outbreak of the disease, ToTV has been seen to affect tomato in single infections, although a high percentage of samples contained double infections with *Pepino mosaic virus* (PepMV), which is widely distributed in tomato production areas of the country (Alfaro-Fernández *et al*., 2007). In addition, other viruses have been detected in some of the samples surveyed, and might induce symptoms in tomato plants by interfering or contributing to the 'Torrado' disease symptoms. Furthermore, necrosis symp-

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toms are also produced by other viral agents when infecting tomato crops, which are not easily distinguishable from Torrado disease symptoms (Córdoba-Sellés *et al.*, 2007).

The aim of this study was to evaluate the microscopic effect of ToTV in plants affected by the Torrado disease. Comparative studies are presented, using optical and electron microscopy, of the cytopathology induced by the viral entity ToTV in single infections, and the ultrastructural effect of the mixed infection of this virus with other related viruses commonly found in Spanish tomato crops.

Materials and methods

Plant material

Leaf samples from tomato plants showing typical symptoms of Torrado disease were collected in commercial greenhouses from areas of Murcia (Mazarrón and Águilas), Almería and the Canary Islands (Las Palmas), where the incidence of disease has been widely reported. A total of 32 samples were taken from plants approximately six weeks after transplanting. These were surveyed, labelled and transported to the laboratory under cold conditions. Leaf samples were collected from the terminal leaflet of the first fully developed leaf from the plant apex of virus-infected tomato plants and used for the comparative and synergism studies. The characteristics of the collected samples are summarized in Table 1. Tomato plants cv. Boludo, grown in a growth chamber under controlled conditions (18–25°C), 16 h daylight and 60% relative humidity, were used as healthy controls. To compare mixed infections of ToTV with the most commonly detected viruses PepMV and *Tomato chlorosis virus* (ToCV) in plants affected by the Torrado disease (see below), single infections of these two viruses

Table 1 Serological and molecular analyses performed on tomato leaf samples infected with one or more viruses

			Serological analysis					Molecular analysis						Type of
Sample	Origin	Variety	PepMV	TSWV	PVY	CMV	ToMV	PepMV[a]	ToTV	ToCV	TICV	PMoV	TYLCV	infection[b]
6269	Las Palmas	Unknown	+	+	–	+	–	+ EU	+	+	–	–	–	M
6295	Murcia	Boludo	+	–	–	–	–	+ EU	+	+	–	–	–	T
6296	Murcia	Boludo	+	–	–	–	–	+ EU	+	+	–	–	–	T
6339	Almeria	Unknown	–	–	–	–	–	–	+	+	–	–	+	T
6343	Almeria	Unknown	–	+	–	–	–	+ CH2	+	+	–	–	–	M
7436	Murcia	unknown	–	–	–	–	+	–	+	+	–	–	–	T
7444	Mazarrón	unknown	–	–	–	–	–	–	+	–	–	–	–	S
7445	Mazarrón	unknown	+	–	–	–	–	+ EU	+	–	–	–	–	D
7446	Mazarrón	unknown	–	+	–	–	–	+ CH2	+	–	–	–	–	T
7449	Mazarrón	unknown	–	+	–	–	–	–	+	–	–	–	–	D
7450	Mazarrón	unknown	+	+	–	–	–	+ CH2	+	–	–	–	–	T
7454	Mazarrón	unknown	+	+	–	–	+	+ CH2	+	–	–	–	–	M
7456	Mazarrón	unknown	+	+	–	–	+	+ CH2	+	–	–	–	–	M
7530	Aguilas	Raferter	–	–	–	–	–	–	+	+	–	–	–	D
7533	Aguilas	Raferter	–	–	–	–	–	–	+	–	–	–	–	S
7534	Aguilas	Raferter	–	–	–	–	–	–	+	+	–	–	–	D
7544	Aguilas	Raferter	–	–	–	–	–	–	+	+	–	–	–	D
7545	Aguilas	Raferter	–	–	–	–	–	–	+	+	–	–	–	D
7547	Aguilas	Unknown	–	–	–	–	–	–	+	+	–	–	–	D
7821-1	Mazarrón	Unknown	–	–	–	–	–	–	+	–	–	–	–	S
7821-2	Mazarrón	Unknown	–	–	–	–	+	+ CH2	+	+	–	–	–	M
7822	Mazarrón	Unknown	–	–	–	–	–	–	+	+	–	–	–	D
7825	Aguilas	Boludo	–	–	–	–	–	–	+	+	–	–	–	D
7826	Aguilas	Boludo	–	–	–	–	–	–	+	–	–	–	–	S
7827	Aguilas	Boludo	–	–	–	–	+	+ CH2	+	+	–	–	–	M
7828	Aguilas	Boludo	–	–	–	–	–	–	+	+	–	–	–	D
7829	Mazarrón	Corly	+	–	–	–	–	+ CH2	+	–	–	–	–	D
7830	Mazarrón	Corly	+	–	–	–	–	+ CH2	+	+	–	–	–	T
7831	Mazarrón	Corly	+	–	–	–	–	+ CH2	+	+	–	–	–	T
487/08	Aguilas	Boludo	–	–	–	–	–	–	+	–	–	–	–	S
491/08	Aguilas	Boludo	–	–	–	–	–	–	+	+	–	–	–	D
498/08	Aguilas	Boludo	–	–	–	–	–	–	+	+	–	–	+	T
Total positive results			10	7	0	1	5	14	32	20	0	0	2	5(S) 12(D)
Percentage of positives			31·25	21·88	0	3·13	15·63	43·75	100	62·50	0	0	6·25	9(T) 6(M)

[a]Genotype of PepMV detected in the multiplex RT-PCR assay (Alfaro-Fernández *et al.*, 2009): EU = European genotype; CH2 = Chilean 2 genotype.

[b]S = single infection, only ToTV was detected; D = double infection, ToTV and one other virus was positive; T = Triple infection, ToTV and two other viruses were positive; M = Multiple infection, more than three viruses were positive, including ToTV.

were also studied in mechanical-inoculated and whitefly-infected tomato plants maintained under controlled conditions as described before. Mechanical inoculation of two isolates of PepMV, characterized as genotypes Chilean 2 (CH2) and European (EU), was carried out with sap inoculation by grinding the leaves in 0·01 M phosphate buffer, pH 7·4 (1:4 w/v). The extracted sap was rubbed onto healthy tomato plants at the 4-leaf-stage of development, pre-dusted with carborundum (600 mesh). For ToCV transmission, a viruliferous colony of adults of *Trialeurodes vaporariorum* was released on healthy tomato plants at the 4-leaf-stage. After a 48 h inoculation period, plants were treated against whiteflies and kept in a whitefly-free growing chamber. One month after inoculation, PepMV and ToCV inoculated plants were analysed to verify the infection as described below, and sampled for further observation.

Serological and molecular diagnostics

Serological analyses were performed with the collected leaf samples by double antibody-sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using polyclonal antisera against *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY), *Tomato mosaic virus (ToMV), Tomato spotted wilt virus* (TSWV) (Loewe Biochemica), and *Pepino mosaic virus* (PepMV) (DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen), as recommended by the manufacturer. Healthy tomato leaves of cv. Boludo were included as a negative control. Positive controls consisted of virus-infected tomato leaf samples. Absorbance values (A405 nm) were measured in a Titertek Multiskan immunoplate reader. Samples were considered positive when the mean absorbance of duplicate wells was more than twice the mean absorbance of the corresponding healthy controls.

For the molecular analysis, total RNA extraction was performed from 0·1 g of fresh leaf tissue from infected plants using the silica capture protocol (MacKenzie *et al*., 1997). Total DNA was extracted using the E.Z.N.A® Plant DNA Miniprep Kit (OMEGA Biotech) following the manufacturer's instructions.

For the detection of ToTV and *Parietaria mottle virus* (PMoV), RNA extractions were tested using non isotopic dot-blot hybridization, performed as described previously by Sanchez-Navarro *et al*. (1998). Two digoxygenin-labelled RNA probes complementary to a fragment of the polyprotein of the RNA2 for ToTV, based on published primers (van der Heuvel *et al*., 2006), and a fragment of the coat protein of PMoV (kindly provided by V. Pallás, IBMCP-UPV, 46022 Valencia, Spain) were used for the analysis.

To confirm the results obtained by the ToTV analysis, samples were also analyzed by one-step reverse-transcription polymerase chain reaction (RT-PCR) with the SuperScript III Platinum Taq kit (Invitrogen Life Technologies) and with specific primers targeted to the subunit Vp23 of the RNA2 coat protein of the virus (Pospieszny *et al*., 2007). RT-PCR assays were also performed to verify the presence of other viruses, with specific primers for both *Tomato chlorosis virus* (ToCV, Louro *et al*., 2000) and *Tomato infectious chlorosis virus* (TICV, Vaira *et al*., 2002), and by the multiplex RT-PCR procedure for the simultaneous detection of the five PepMV genotypes, as described by Alfaro-Fernández *et al*. (2009). PCR was performed to confirm possible *Tomato yellow leaf curl virus* (TYLCV, Martínez-Culebras *et al*., 2001) infection. The amplified PCR products were separated by electrophoresis on 1·2% agarose in 1 × TAE buffer (40 mM Tris-acetate and 1 mM EDTA at pH 8·0), and were stained with ethidium bromide. Fragment sizes were determined by comparison with a 100 bp DNA standard marker (GeneRuler™ DNA Ladder Plus, MBI Fermentas).

Optical and electron microscopy analysis

Pieces (1 × 1 cm) from the plesionecrotic areas of one leaf per plant were obtained and cut into smaller pieces measuring 0·1 × 1 cm, and were maintained in fixative (glutaraldehyde 2·5% in 0·1 M buffer phosphate pH 7·2) while cutting, to avoid additional stress to cells. They were immersed in the same fixative for 16–24 h. Pieces were washed three times (1 h) in buffer and kept at 4°C until the electron microscopy (EM) process. The material was fixed for 2 h in 2% osmium tetroxide and washed again in buffer. Then the tissue was dehydrated in an ethanol series, from 30 to 100%, and uranyl acetate was added in the 70% phase. Finally, they were embedded in Araldite resin. Semi-thin and ultra-thin sections for optical and electron microscopy studies, respectively, were obtained, placed onto slides and stained with Richardson's blue, or placed on carbon-coated copper grids (200 mesh) and contrasted with uranyl acetate (10 min) and lead citrate (Reynolds' solution, 2 min). Semi-thin sections were examined with an optical microscope while ultra-thin sections were observed with a TEM 910 Zeiss microscope.

Results and discussion

Results of the serological and molecular analyses

The results of the serological and molecular analyses revealed that ToTV was present in single infections, although mixed infections with other tomato viruses was common in commercial greenhouses (Table 1). In addition to the infection of plants with ToTV, 21·8% of the analyzed samples were infected with TSWV, 3·1% with CMV, 15·6% with ToMV, 62·5% with ToCV, 6·3% with TYLCV and 43·8% with PepMV. The multiplex RT-PCR presented greater advantages in the PepMV diagnosis compared to ELISA, as this procedure was able to identify the corresponding PepMV genotype present in the sample, and also to detect a higher number of positives (14 positives) than the serological method (10 positives). The higher sensitivity of this molecular method compared to ELISA is in accordance with previously

reported assays for PepMV (Alfaro-Fernández *et al.*, 2009) and other viruses (Sánchez-Navarro *et al.*, 1998). Of the 14 PepMV-positive samples, four belonged to the European genotype (EU), while 10 corresponded to the Chilean 2 genotype (CH2).

Single infection with ToTV was detected in five samples, and 12 samples presented a double infection of ToTV with ToCV, PepMV or TSWV. Nine of the samples presented a triple infection of ToTV and PepMV/ToCV, ToMV/ToCV, PepMV/TSWV or ToCV/TYLCV. Multiple infections were detected in six of the 32 samples, in which four (ToTV and PepMV/ToCV/TSWV, PepMV/ToCV/ToMV or PepMV/ToMV/TSWV) or five (ToTV and PepMV/ToCV/TSWV/CMV) viruses were positively tested. Tomato crops are usually affected by mixed infections of several viral diseases which might result in synergism and, in some cases, a breakdown in the resistance of the cultivar against one of the viruses involved (García-Cano *et al.*, 2006). TSWV and TYLCV induced more severe macroscopic symptoms, although further studies are required to quantify the macroscopic effect of such mixed infections.

The large number of samples infected with ToTV and ToCV (20 out of 32) was predictable since both are efficiently transmitted by *B. tabaci* and *T. vaporariorum* (Wisler *et al.*, 1998; Pospieszny *et al.*, 2007; Amari *et al.*, 2008), and large populations of whiteflies often co-infect tomato fields worldwide. In addition, since the first outbreak of ToCV in Spain which resulted in more than 30% of plants exhibiting symptoms in individual fields (Navas-Castillo *et al.*, 2000), this virus has commonly infected tomato crops during different growing seasons (EPPO, 2005).

PepMV was detected in almost half of the samples studied (14 out of 32). This virus is endemic in Spain where it was first reported in 2000 (Jordá *et al.*, 2001), and has been frequently detected in co-infection with ToTV in field samples (Alfaro-Fernández *et al.*, 2007).

Cytopathology of infection by ToTV alone

The tomato tissue of samples infected with ToTV alone, analyzed by optical (Fig. 1b) or electronic microscopy, did not appear to be over disrupted or have necrotic

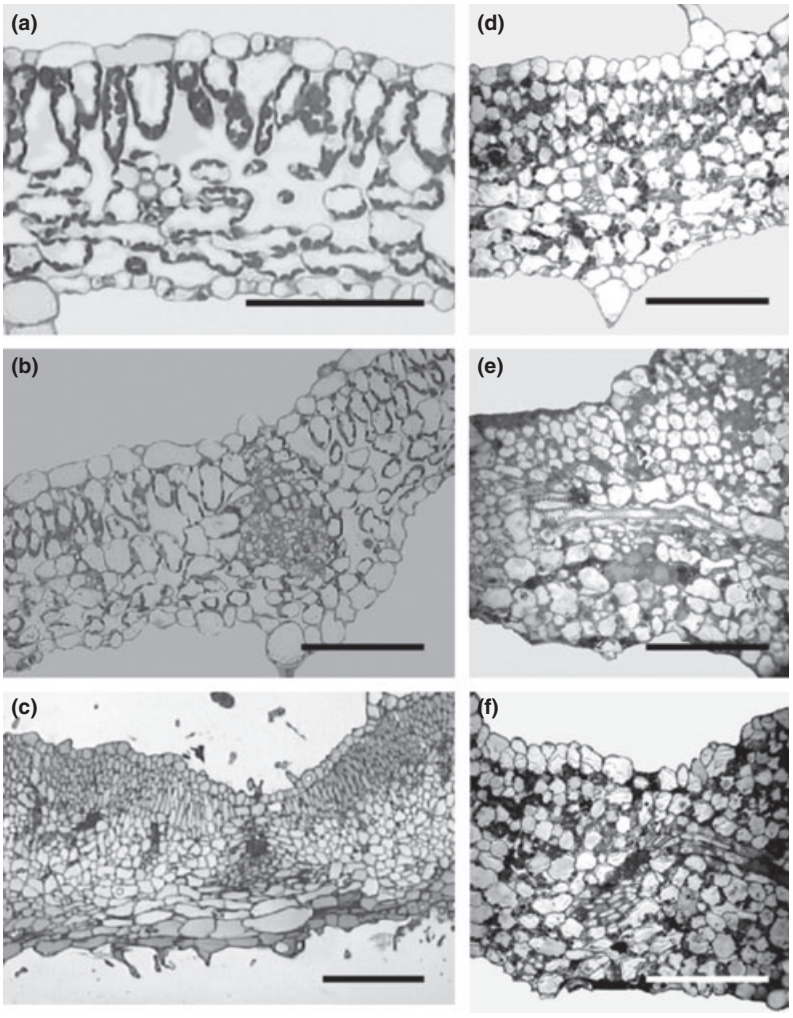

Figure 1 Light micrographs showing semi-thin sections of healthy tomato leaves (a) and leaves infected with Tomato torrado virus (ToTV) (b), *Tomato chlorosis virus* (ToCV) (c), *Pepino mosaic virus* (PepMV) Chilean 2 genotype (d), co-infection with ToTV/PepMV (e) and multiple infection with ToTV/PepMV/*Tomato spotted wilt virus* (TSWV) (f) (bars = 42 μm).

cells. All the semi-thin sections observed under optical microscopy (Fig. 1) were compared with a healthy leaf section (Fig. 1a), although this sample had larger intercellular spaces due to cultivation in a growth chamber, and tissues appeared more swollen. Under EM, crystalline inclusions of virus-like particles (VLPs) of 20–30 nm were only observed in phloem parenchyma cells (Fig. 2a,b). The presence of crystal-like arrays of viral particles in infected tomato leaf tissues is considered a characteristic feature of ToTV (Pospieszny *et al.*, 2009). No crystals were observed outside the vascular tissue. Cells of ToTV-infected tissue displayed cytoplasmic vesiculation (Fig. 2c), and the sieve elements exhibited no abnormal presence of the p-Protein (Fig. 2c). Only a few of the observed samples presented hypertrophy of the mitochondria and strong cytoplasm disorganization, but the mitochondria and chloroplasts usually appeared normal (Fig. 2c). The results of this work reveal that a

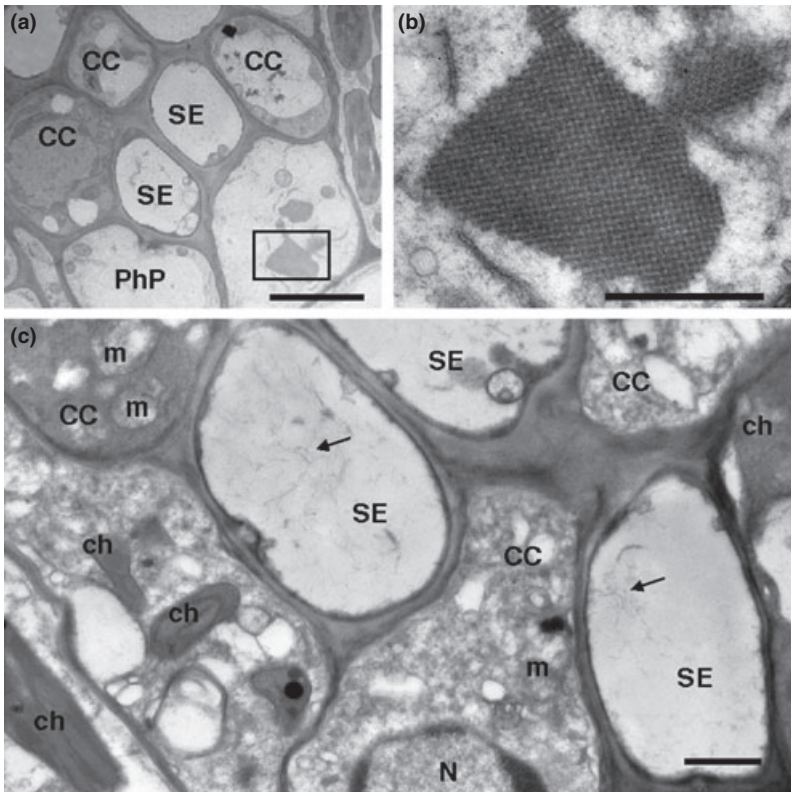

Figure 2 Electron micrographs of tomato leaf cells infected with Tomato torrado virus (ToTV) alone. (**a**) Vascular zone showing a defined crystal of isometric virus-like particles (VLPs) of 20–30 nm in a phloem parenchyma cell (PhP). A small amount of flexuous particles, probably corresponding to the P-protein, are present in the sieve tubes (SE). Bar = 3·63 μm. (b) Detail of a. Bar = 1·45 μm. (c) Vascular area showing three companion cells (CC) with cytoplasm vesiculation and sieve tubes (SE) containing a small amount of P-protein (arrows). Chloroplasts (ch) do not show alterations. Mitochondria (m) appear hypertrophied. No special cell wall overgrowth is present (N = nucleus; bar = 0·9 μm).

Table 2 Effect on cell and organelles in tomato leaf vascular tissues infected by different viruses

	Cytoplasm vesiculation			Cell wall overgrowth			Virus-like particles (VLPs)		Chloroplasts		Mitochondria	
Virus infection	CC[a]	PhP	BS	CC	PhP	BS	Crystals	Aggregates	H	OG	H	P
ToTV[b]	+[c]	+	±	−	−	−	+	−	−	−	±	−
ToCV	+	±	−	+	+	−	−	+[d]	−	±	−	+
PepMV	+	+	+	−	−	−	.	+[d]	+	+	+	+
ToTV + ToCV	+	+	±	++++	++	−	+	+	−	+	+	+
ToTV + PepMV	+	+	+	−	−	−	+	+	+	+	+	+
ToTV + PepMV + ToCV	+	+	+	+++	++	−	+	+	+	+	+	+
ToTV + PepMV + TSWV	+	+	+	++	++	++	−	+	+	+	+	+
ToTV + ToCV + TYLCV	+	±	±	++	++	−	+	+	±	±[e]	+	+

[a]Letters indicate the cells of the vascular tissues observed (CC = companion cell, PhP = phloem parenchyma cell, BS = bundle sheet cell) or alteration observed (H = hypertrophy, OG = osmiophilic globules, P = proliferation/increase in number).
[b]ToTV: *Tomato torrado virus*, ToCV: *Tomato chlorosis virus*, PepMV: *Pepino mosaic virus*, TSWV: *Tomato spotted wilt virus*, TYLCV: *Tomato yellow leaf curl virus*.
[c]+ indicates that the given feature was seen in greater or lesser quantities for the respective viral infection; ± indicates that the observation is not conclusive.
[d]Forming masses of flexuous VLPs.
[e]Chloroplast also showed an abnormal starch accumulation.

single infection of ToTV induces more cellular alteration at the phloem level than in other leaf tissues (Table 2). This vascular tissue is considered to be the translocation route used by this virus in tomato (Sánchez-Pina *et al.*, 2008). Chloroplasts generally showed no observable differences with healthy tissues (Fig. 2c).

Cytopathology of double infection with ToTV + ToCV

Leaf tissues infected with ToCV alone revealed flexuous virus-like particles (VLPs) in the sieve tubes and some hypertrophied chloroplasts showing some osmophilic globules (Fig. 3a), normally associated with viral infection of other viruses (Francki *et al.*, 1985). The typical cytoplasmic vesiculation (Table 2) and the *Beet yellows virus* (BYV)-type membranous inclusion bodies (Fig 3b) induced by *Crinivirus* infection were also observed (Medina *et al.*, 2003). No plasmalemma deposits were shown like those described for *Lettuce infectious yellows virus* (LIYV) (Medina *et al.*, 1998). Under an optical microscope, the leaf tissues infected with ToCV only showed a few necrotic cells of the parenchyma mesophyll (Fig. 1c).

The observation of a double infection of ToTV and ToCV reveals that the cytopathic effect of ToCV was more evident in these samples (Table 2; Fig. 3c–g). Two types of VLPs were distinguishable: large crystalline masses of isometric VLPs, contained in the

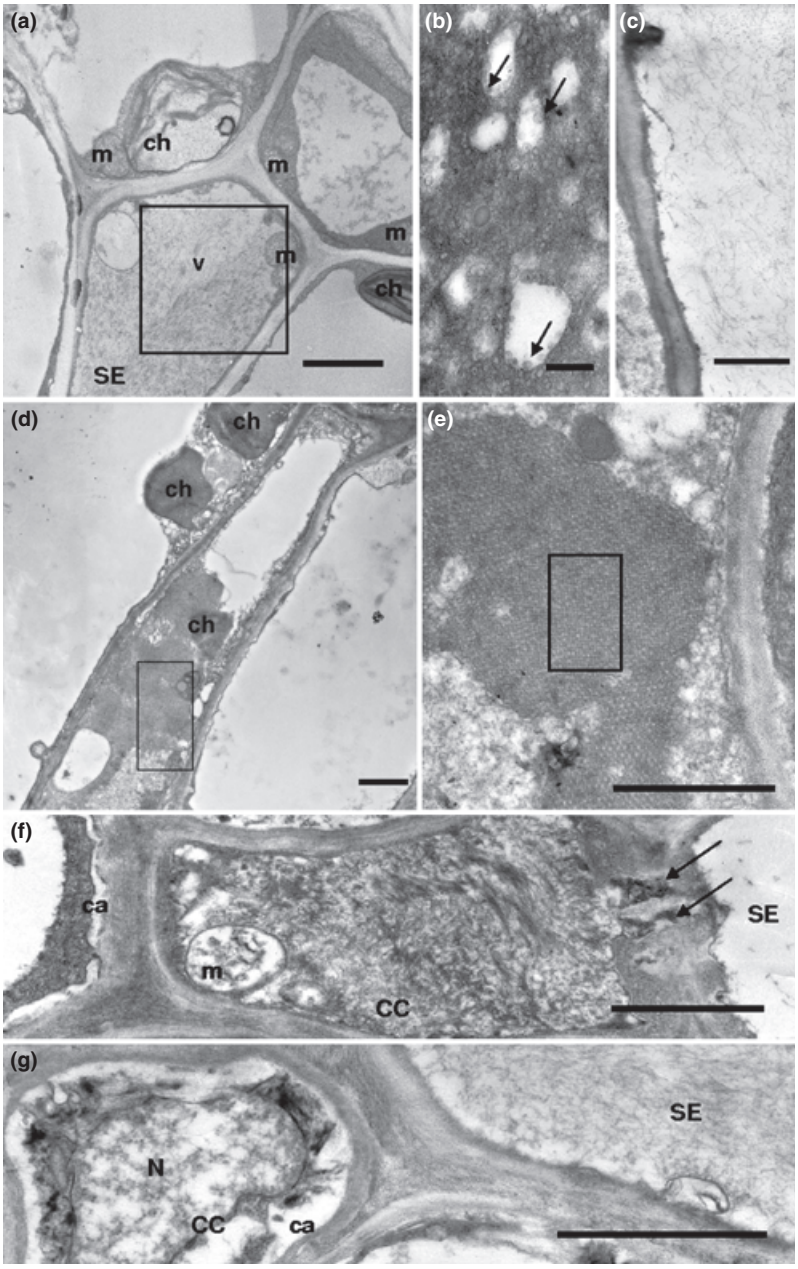

Figure 3 Electron micrographs of tomato leaf cells infected with *Tomato chlorosis virus* (ToCV) alone (a,b) and double-infected with Tomato torrado virus (ToTV) and *Tomato chlorosis virus* (ToCV) (c–g). (a) Vascular area showing hypertrophied chloroplast (ch) and flexuous virus-like particles (VLPs) in a sieve tube in ToCV single-infected tissues. Bar = 1·5 μm. (b) Membranous inclusion body showing vesicles (arrows) in lacunes in ToCV single-infected tissues. Bar = 0·91 μm. (c) Sieve tube showing flexuous VLPs together with fibres of the P-protein in ToTV and ToCV co-infected tissues. Bar = 1·45 μm. (d) Companion cell of ToTV and ToCV co-infected leaf tissues, showing a large crystalline mass of isometric virus-like particles (VLPs). Chloroplasts (ch) show slight alterations. Bar = 2·9 μm. (e) Companion cell of ToTV and ToCV co-infected leaf tissues showing a large crystalline mass of isometric virus-like particles (VLPs). Bar = 1·45 μm. (f) Companion cell (CC) of ToTV and ToCV co-infected leaf tissues crowded by masses of flexuous VLPs and with hypertrophied mitochondria (m). Note the considerable increased size of the plasmodesmata (arrows) connecting with a phloem tube, the general callose (ca) deposition in plasmalemma and the cell wall overgrowth. Bar = 1·45 μm. (g) Vascular detail showing a sieve tube of ToCV and ToTV co-infected leaf tissues full of flexuous VLPs and an enormous callose deposition in a companion cell. Bar = 1·45 μm (N = nucleus).

companion cells of the phloem (Fig. 3d,e) and masses of flexuous VLPs inside the sieve tubes (Fig. 3f,g), together with p-Protein fibers (Fig. 3c). Chloroplasts revealed only slight alterations (Fig. 3d), although some mitochondria appeared to be hypertrophied (Fig. 3f, Table 2). Moreover, stronger cell wall growth and callose deposition were observed in the companion cells (Fig. 3f,g, Table 2), as common alterations characteristic of *Criniviruses* infection (Medina *et al.*, 2003). A noticeable increase in size of the plasmodesmata connected with a phloem tube was shown (Fig. 3f), which is a reported mechanism of cell-to-cell movement of other viruses (Wolf, 1989; McLean *et al.*, 1993). Cytoplasm disruption was stronger and viral accumulation in the phloem was clear in the ToTV and ToCV mixed infected samples, probably because cell wall growth and callose deposition hindered the possible cell-to-cell viral spreading, as reported for plant virus infections in general (Francki *et al.*, 1985).

Cytopathology of double infection with ToTV + PepMV

The analysis of PepMV single-infected tissue showed that a very small amount of necrotic cells were present (Fig. 1d). Mitochondria were not hypertrophied, although their number increased because of infection (Fig. 4a,b), an effect also seen in *Nicotiana glutinosa* leaves infected with *Tobacco mosaic virus* (TMV), and in virus-infected apple tissues (Šutić & Sinclair, 1991). Overall, some cells presented a slight hypertrophy of chloroplasts visible under EM, showing osmiophilic globules (Fig. 4b). Details of the alteration of the tissues observed in PepMV single-infected samples are provided in Table 2. Large masses of virions (viroplasms) were present in the different cells which induced alterations and a disruption of contents in extensive areas of the epidermal cells (Fig. 4a,b). These aggregates containing arrays of filamentous virus-like particles were previously

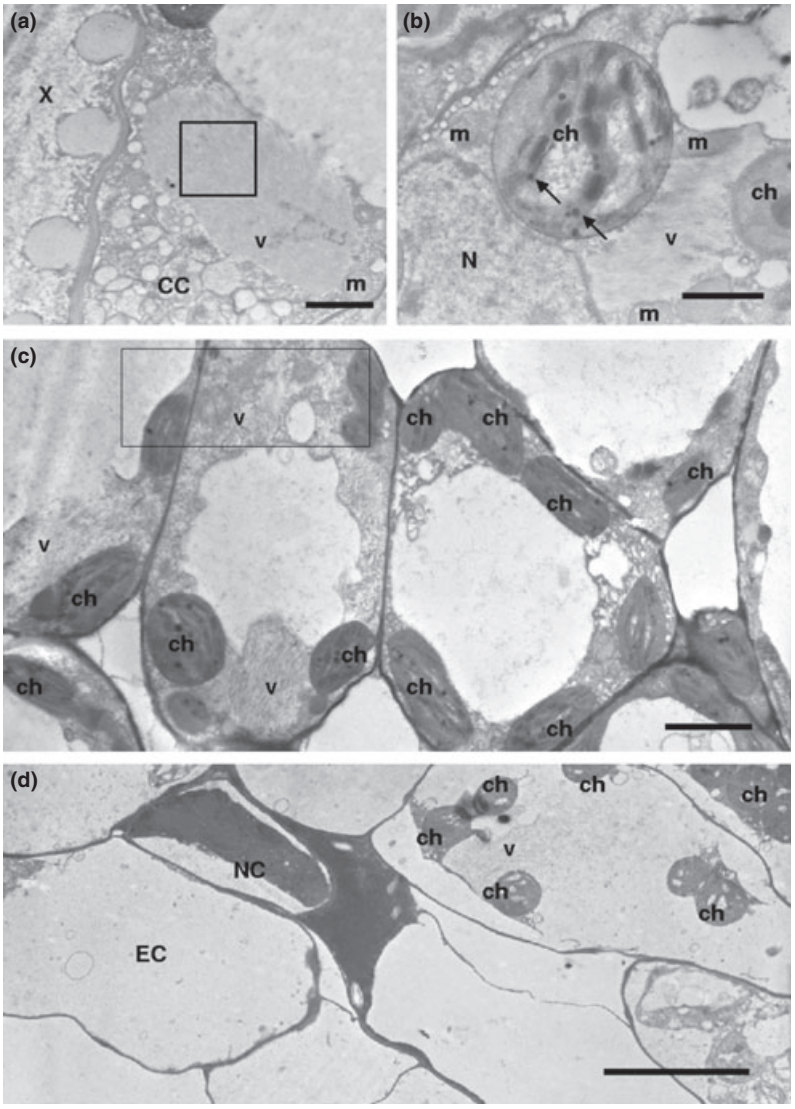

Figure 4 Electron micrograph of tomato leaf cells infected with the CH2 genotype of *Pepino mosaic virus* (PepMV) alone (a, b) and double-infected with PepMV-CH2 and Tomato torrado virus (ToTV) (c, d). (a) Phloem cell of leaf single-infected with PepMV showing strong cytoplasmic vesiculation and a mass of flexuous VLPs (viroplasm, v) close to a xylem element (X). Bar = 1·81 μm. (b) Mesophyll cells of leaf single-infected with PepMV showing masses of flexuous VLPs, hypertrophied chloroplasts (ch) with osmiophilic bodies (arrows) and cytoplasmic vesiculation. Bar = 1·81 μm. (c) Mesophyll cells of leaf double-infected with PepMV and ToTV, close to epidermis (upper part) showing a general infection by PepMV-like particles (v). The flexuous virus-like particles (VLPs) appear scattered inside the vacuoles. Bar = 3·63 μm. (d) Some epidermal cells (EC) and lacunar mesophyll cells of tissues double-infected with PepMV and ToTV appear necrosed and/or disrupted (NC). Bar = 7·3 μm.

observed in ultrathin sections of *N. glutinosa* leaves infected with the first described isolate of PepMV detected on pepino in Peru (Jones *et al.*, 1980). There was no obvious difference in the cytopathic effect between the two genotypes of PepMV (CH2 and EU) studied in single- or multiple-infected tissues.

The combined infection of ToTV and PepMV led to some necrotic cells of the parenchyma mesophyll (Fig. 1e) and induced considerable cytopathic effects for tomato leaf cells. Cytoplasm vesiculation was observed in the vascular tissues (Fig. 4c), as in *Beet necrotic yellow vein virus* infections (BNYVV) (Šutić & Sinclair, 1991). Chloroplasts and mitochondria appeared to be hypertrophied (Fig. 4c), as in Chinese cabbage infected by *Turnip yellow mosaic virus* (TuYMV), which induced chloroplast hypertrophy with a round shape and appeared aggregated toward the cell walls, or in cucumber cells infected with *Cucumber green mottle mosaic virus* (CGMMV) which showed abnormal mitochondria (Šutić & Sinclair, 1991). However, no cell wall overgrowth was determined in the affected samples (Table 2). The tissues affected by this double infection sometimes resembled badly embedded samples, which also occurred in the triple infection of ToTV/PepMV/TSWV. Aggregates of virions were observed in mesophyll cells, and some scattered VLPs in the vacuoles were also present due to the disruption of tonoplasts (Fig. 4c). Occasionally, some necrotic cells were seen in leaf tissues in a larger amount than in other double or multiple infections (Figs 1e, 4d).

Cytopathology of ToTV in triple and multiple infections

Cells with a disrupted cytoplasm and necrotic cells were present in greater numbers in triple-infected samples with ToTV, PepMV-CH2 and TSWV (Fig. 1f). In such samples, mesophyll cells presented large cell wall overgrowth and, as in the double infection of ToTV/ToCV, scattered flexuous VLPs in the vacuoles (Fig. 5a) and necrotic cells were observed (Fig. 5c). Osmophilic bodies were seen in the chloroplasts (Fig. 5b), while granular inclusion bodies were detected in the mesophyll cells (Fig. 5c, d). Other triple infections of ToTV with PepMV/ToCV or ToCV/TYLCV also produced evident cell wall overgrowth of the companion cells and the phloem parenchyma cells. However, this cytological change in the bundle sheath cells was only observed in tomato samples infected with ToTV, PepMV and TSWV (Table 2). The presence of necrotic cells in the plesionecrotic areas under study was less evident in those samples affected with viruses which were confined to the phloem, i.e. ToTV, ToCV or TYLCV, where the alteration was limited to the vascular tissue (data not shown). The reaction induced by the infection of ToTV/ToCV produced a general obstruction of the phloem tubes, as observed before (Fig. 3c), which might prevent PepMV or other viruses spreading (Francki *et al.*, 1985), and consequently, the damage caused by this virus may not occur in these cases.

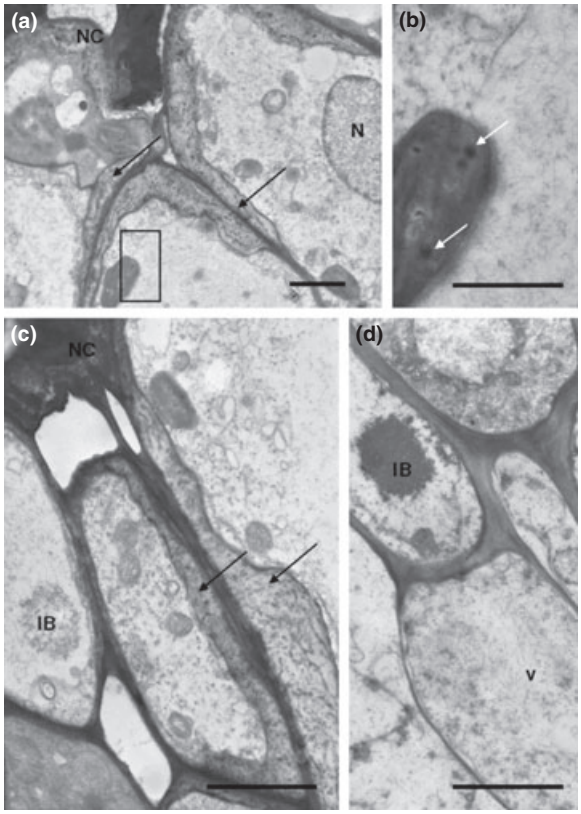

Figure 5 Electron micrograph of tomato leaf tissues triple-infected with Tomato torrado virus (ToTV), *Pepino mosaic virus* (PepMV) and *Tomato spotted wilt virus* (TSWV). (a) Mesophyll cells around a necrotic cell (NC) showing spectacular cell wall overgrowth (arrows) and scattered flexuous virus-like particles (VLPs) in their vacuoles. Bar = 2·9 μm. (b) Detail of a. Chloroplasts showing osmiophilic bodies (white arrows). Bar = 1·45 μm. (c) Palisade mesophyll cells close to a necrosed epidermal cell showing strong cell wall overgrowth and disrupted cytoplasms. One of them shows a granular inclusion body (IB). Bar = 2·3 μm. (d) Cells close to a vascular area showing disruption, flexuous VLPs and dense granular IBs. Bar = 2·3 μm. (N = nucleus).

The results detailed in Table 2 show that hypertrophied chloroplasts were more visible in the samples infected with PepMV in any of the combinations studied. Cell wall overgrowth appeared to be a typical feature of ToCV infection. ToCV did not induce observable symptoms in cells outside the phloem, at least in the plesionecrotic tissues studied. As for the multiple infections with ToTV, cell necrosis was more evident, and embedding for EM was harder due to the cell wall overgrowth of cells caused by infection, which made the penetration of fixatives and resins difficult.

In conclusion synergism was observed at the ultrastructural level between different viruses that co-infected samples affected by Torrado disease. ToTV in single infections led to alterations in cells mainly at the phloem level. However, the co-infection of this virus with other viruses, such as the double-infection of ToTV with PepMV or ToCV, and the multiple infections of ToTV,

TSWV and PepMV, produced more severe reactions in affected plants in either the phloem or other plant tissues.

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