Occurrence and Geographical Distribution of the 'Torrado' Disease in Spain

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Abstract

In surveys to determine the occurrence and distribution of the 'torrado' disease (Tomato torrado virus, ToTV) in the main Spanish tomato growing areas from 2001 to 2008, a total of 584 samples from symptomatic and asymptomatic plants were collected from 92 greenhouses. The tests showed that 451 plants from 85 greenhouses of different areas were infected with ToTV. The majority of the positive samples showed typical symptoms of the disease. However, plants showing different symptoms of necrosis and even asymptomatic plants were infected with the virus. Co-infection of ToTV with *Pepino mosaic virus* (PepMV) occurred in a large number of samples (60.5%), and

several samples were infected with other tomato-infecting viruses, including *Cucumber mosaic virus* (CMV), *Potato virus Y* (PVY), *Tomato spotted wilt virus* (TSWV), *Tomato mosaic virus* (ToMV), *Parietaria mottle virus* (PMoV), *Tomato chlorosis virus* (ToCV) and *Tomato yellow leaf curl virus* (TYLCV). Tomato apex necrosis virus (ToANV) was not detected in any of those samples with similar symptoms to those described for that virus. Additional tests revealed that i) ToTV whitefly transmission is highly efficient and variety-dependent in tomato plants, ii) *Datura stramonium* is another solanaceous species susceptible to this virus, and iii) the tissue-printing hybridization is a reliable technique which could facilitate the routine diagnosis and large-scale analysis of ToTV.

Introduction

Tomato (*Solanum lycopersicum* L.) is an important vegetable crop, the world production of which in 2007 was 126.1 MMT (million metric tons). Spain is the eighth largest tomato producer in the world (3.62 MMT), and the second in the European Union after Italy (FAO, 2008). Nearly 85% of Spanish tomato production is located in four southern regions of the country: Andalucía, Extremadura, the Murcia Region and the Canary Islands; tomatoes are mainly produced in greenhouses, except in Extremadura where production is in open fields (M.A.R.M., Spanish Ministry of Environment and of Rural and Marine Affairs, 2007). Tomato crops are susceptible to a wide range of diseases, of which virus diseases are difficult to control and can result in substantial crop losses, being one of the main limiting factors in intensive protected tomato production (Jones et al., 1991).

In 2001, several greenhouse-grown tomato crops in the Murcia Region showed either an initial yellowing at the base of the leaflet that later developed into necrotic

spots or an extensive necrotic area progressing from the base to the tip. Fruits had necrotic areas that often developed into cracks. These plants generally had overall reduced growth which seriously affected productivity. The disease was named 'torrao' or 'torrado' by the producers, which refers to the general burnt-like appearance of the affected plants (Jordá et al., 2003; Alfaro-Fernández et al., 2006).

In 2007, a new virus named Tomato torrado virus (ToTV) was identified and reported as the causal agent of the 'torrado' disease in tomato samples from the Murcia Region (Spain). This virus was characterized as a Picorna-like virus and is a possible 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). ToANV and ToMarV have been identified in tomato crops in Mexico which showed different necrotic symptoms on leaves and fruits (Turina et al., 2007; Verbeek et al., 2007b). ToTV has isometric particles with a diameter of 28 nm, and its genome is composed of two single-stranded positive-sense RNA molecules which contain three coat protein subunits. This structure resembles those of members of the genera *Sequivirus*, *Waikavirus, Sadwavirus* and *Cheravirus*; however, phylogenetic analyses revealed important differences between ToTV and the viruses of these genera (Verbeek et al., 2007a).

Recently, this virus has been detected in tomato in Poland (Pospieszny et al., 2007, 2009), Canary Islands (Spain; Alfaro-Fernández et al., 2007a), Australia (IPPC, 2008), Panama (Herrera-Vásquez et al., 2009) and Hungary (Alfaro-Fernández et al., 2009a), as well as on weed hosts in Spain (Alfaro-Fernández et al., 2008), and it has been included in the EPPO alert list (EPPO, 2009). Three ToTV Polish isolates were biological and molecularly characterized showing them to be closely related and to the isolate type from Spain (Pospieszny et al., 2009).

The 'torrado' disease has always been associated with large whitefly populations and infestations in the affected greenhouses since the first observation of its symptoms (Jordá et al., 2003). ToTV has been reported to be efficiently transmitted by *Trialeurodes vaporariorum* (Westwood) (Pospieszny et al., 2007, 2009) and *Bemisia tabaci* (Gennadius) (Amari et al., 2008).

Tomato is seriously affected by several viral diseases in which a mixed viral infection may result in synergisms and more severe disease symptoms (García-Cano et al., 2006). In preliminary assays, ToTV was frequently found in tomato samples together with *Pepino mosaic virus* (PepMV), which is widely distributed in the tomato production areas of the country (Alfaro-Fernández et al., 2007b; Verbeek et al., 2007a; Alfaro-Fernández et al., 2009b). However, no data of a mixed infection of ToTV with the other main viruses affecting tomato crops are available.

Despite this disease being present in greenhouse-grown tomatoes in Spain every growing season, there is very limited information about its incidence in Spain. Therefore several surveys have been made in the main tomato production areas in Spain since the outbreak of the disease in 2001. We report here the occurrence and geographical distribution of the 'torrado' disease in Spanish tomato crops. We also assessed the percentage of mixed infections of ToTV with PepMV and whitefly transmission. Finally, we present the useful techniques of tissue-printing and dot-blot hybridization for the detection of ToTV in field samples.

Material and Methods

Surveys and sample collection

Surveys were conducted in greenhouses of commercial tomato crops in 11 different regions of the most important tomato-growing areas in Spain (Fig. 1). Tomato leaves

were collected from 2001 to 2008. A total of 584 plants at different stages of development were surveyed from 92 greenhouses (Fig. 1, Table 1). All the greenhouses contained both symptomatic and asymptomatic plants.

The symptoms in all the collected plants were recorded and marked as symptomatic plants (s) for those plants showing the symptoms currently associated with the 'torrado' disease, plants showing some symptoms of necrosis which could not be clearly associated with the 'torrado' disease (n), and plants without necrotic symptoms (w/s), even though some of them presented another viral symptom (bubbling, mosaic, yellowing etc.). Samples were maintained at -80ºC or as dried material at room temperature until processed. All the leaves were tested individually by the direct antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), molecular hybridization by dot-blot and reverse transcription-polymerase chain reaction (RT-PCR) against different viruses, as explained below.

In 2008, four greenhouses in different locations of the Murcia Region were selected. The symptoms of a total of 1527 plants were recorded in randomly selected rows along each greenhouse, which represented 10% of the total growing plants, in order to evaluate the incidence of the virus inside these greenhouses. In these greenhouses, 47 symptomatic or asymptomatic samples were randomly collected to analyze the presence of other viruses (PepMV, ToTV, ToMV, TSWV, CMV and PVY) individually by RT-PCR or DAS-ELISA, as explained below.

Serological assays

The leaf samples surveyed were tested by DAS-ELISA against different viruses depending on the symptoms observed. Samples collected during the earlier growing seasons (2001-2005), when the necrotic symptoms associated with the 'torrado' disease were not identified to any known virus, were serologically tested with antisera against

different viruses which generally produce necrosis in tomato plants as: CMV, *Groundnut ringspot virus* (GRSV), *Potato virus X* (PVX), *Tobacco necrosis virus* (TNV), *Tomato bushy stunt virus* (TBSV) and *Tomato chlorotic spot virus* (TCSV) (Loewe Biochemica. Sauerlach, Germany); *Alfalfa mosaic virus* (AMV), *Tobacco mosaic virus* (TMV), *Tobacco streak virus* (TSV), *Tomato black ring virus* (TBRV) and *Tomato ring spot virus* (ToRSV) (Bio-rad Phyto-Diagnostics. Marnes-La Coquette, France); *Pelargonium zonate spot virus* (PZSV) and *Pepper mottle virus* (PepMoV) (DSMZ Deutsche Sammlung von mikroorganismen und Zellkulturen, Braunschweig, Germany). Tests for other viruses commonly infecting tomato crops in Spain were also made depending on the symptoms observed in the sampled plants in all the surveys conducted (2001-2008), such as PVY, ToMV, TSWV (Loewe Biochemica, Sauerlach, Germany) and PepMV, (DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen. Braunschweig, Germany). Tissue samples (0.15 g) were homogenized in sample extraction buffer (1:20 w/v). DAS-ELISA was carried out in paired wells using 100 µl of the extracts obtained following the manufacturer's instructions for each specific antiserum supplied. Healthy and virus-infected tomato leaves were included in each ELISA analysis as negative and positive controls, respectively. ELISA reactions were measured spectrophotometrically at 405 nm using a Titertek Multiscan immunoplate reader (Flow Laboratories, Finland). Samples were considered positive if the mean of the absorbance value of the duplicated wells was more than twice that of the corresponding healthy controls.

RNA extraction and molecular hybridization analyses

Total RNA was extracted from 0.1 g of fresh leaf tissue from infected plants using the silica capture protocol (MacKenzie et al., 1997). Total DNA was directly extracted from

those samples selected according to the symptoms observed using the E.Z.N.A® Plant DNA Miniprep Kit (OMEGA Biotech, Doraville, USA) following the manufacturer's instructions. The extracted nucleic acids were stored at -80ºC until used.

Non isotopic dot-blot hybridization was used to detect ToTV in all the surveyed samples. One μl of total RNA was firstly denatured with formaldehyde and then directly applied onto a nylon membrane. An analysis of the total nucleic acids was performed by non-isotopic dot-blot hybridization as previously described by Sánchez-Navarro et al. (1998) using a dig-RNA probe complementary to a fragment of the polyprotein of ORF2-RNA2 of ToTV. Some samples that showed possible symptoms of PMoV were also analyzed by dot-blot hybridization, as described above, using a dig-RNA probe complementary to a fragment of the coat protein of PMoV provided by Dr Pallás.

A total of 103 samples, collected from three different fields, were tested by both dot-blot molecular hybridization and tissue-prints of petioles which were cut transversely and pressed directly onto the nylon membrane. Usually two prints were prepared from each sample. After air-drying, the genetic material was cross-linked by UV. The hybridization and detection procedures were conducted as described above.

RT-PCR amplification and sequencing

RT-PCR was used to verify the presence of ToTV following the procedure described by van der Heuvel et al. (2006). In order to identify the specific isolate of PepMV in the infected samples, a multiplex RT-PCR assay was performed as described by Alfaro-Fernández et al. (2009b). Briefly, this method identifies which of the five currently described genotypes of PepMV is present in the infected sample: European (EU), Peruvian (PE), Chilean 2 (CH2), US2 and/or CH1/US1 (or four strains: US1/CH1, EU, PE and CH2 strains, this last grouping also US2 genotype; Ling, 2007). Based on the

symptoms of the surveyed plants, some other viruses were tested by RT-PCR, such as ToCV, commonly found in Spanish tomato crops, and *Tomato infectious chlorosis virus* (TICV), as previously described (Louro et al., 2000; Vaira et al., 2002, respectively). TYLCV was tested by PCR in several suspected samples as described by Martínez-Culebras et al. (2001). The ten samples collected during the first growing season (survey conducted in 2001) were tested by nested-PCR as described by Lee et al. (1993) to confirm the presence or absence of phytoplasmas. RT-PCR and PCR analyses were performed using the SuperScript III One Step RT-PCR system with the Platinum Taq DNA polymerase kit (Invitrogen Life Technologies, Barcelona, Spain) and NETZYME® DNA polymerase (NEED S.L., Valencia, Spain), respectively.

For the detection of the recently described ToANV, a pair of specific primers were designed using the published sequence of the RNA2 available in the GenBank database (Accession number EF063642): ToANV-D (5´ GTGCAACTGAGCTTACTGGAG 3´) and ToANV-R (5´ CCACCGAATCCAGATGA ACAG 3´), targeting to a fragment of 611 bp of the RNA2 of ToANV. The RT-PCR conditions were an initial incubation at 50ºC for 30 min followed by 2 min at 94ºC and 40 cycles of 94ºC for 15s, 58 ºC for 30s and 68ºC for 45s. A final incubation at 68ºC for 10 min was introduced to finish the incomplete PCR fragments. These samples which presented similar symptoms to those described for ToANV (Turina et al., 2007) were tested against this new virus. A positive control, kindly provided by Dr Turina, was included in the assay.

All the amplified PCR products were analyzed on 1.2% agarose/TAE gels stained with ethidium bromide. To confirm the correct ToTV amplification, five amplified PCR products corresponding to a fragment of the ORF2 of the RNA2 (van der Heuvel et al., 2006) were purified with the High Pure PCR Product Purification Kit

(Roche Diagnostics, Mannheim, Germany) and directly sequenced. These five positive samples were collected in three different areas of Spain over a five-year period of surveys and marked with a three-letter code to indicate their geographic origin (MUR= Murcia, TEN= Tenerife, GNC= Gran Canaria), followed by the collection year: MUR-03, MUR-05, TEN-07, MUR-08 and GCN-08. These samples were also analyzed by RT-PCR and sequenced with the specific primers to the subunit Vp23 of coat protein (CP) gene described by Pospieszny et al. (2007). Nucleotide sequences were compared with known sequences deposited in the NCBI database using the Blastn program, and an identity/similarity matrix of the amino acid-analysed sequences was calculated using the Matrix Global Alignment Tool software, version 2.02 (http://bitincka.com/ledion/matgat). Phylogenetic analyses were performed with MEGA (Molecular Evolutionary Genetics Analysis), version 3.1 (Kumar et al., 2004). The robustness of the inferred evolutionary relationships was assessed by 1,000 bootstrap pseudoreplicates.

Mechanical back-inoculation and whitefly transmission

Symptomatic tissues from plants collected in the surveys, which were only positive for ToTV, were used to mechanically inoculate tomato plants cvs Marmande and Boludo. Mechanical transmission was carried out with a sap inoculation of 14 ToTV isolates by grinding the leaves in 0.01 M phosphate buffer, pH 7.4 (1:4 w/v). Extracted sap was rubbed onto 56 healthy tomato plants (28 plants of each cultivar), pre-dusted with Carborundum (600 mesh), at the four-leaf-stage of development. Four plants were inoculated with each ToTV isolate. Half inoculated plants were placed in growth chambers at 26ºC/22ºC (day/night) with a 12 h photoperiod and 60% relative humidity, while the others were placed in a greenhouse. Plants were inspected for virus symptoms

periodically for 8 weeks. All the plants were analyzed by RT-PCR using specific primers to ToTV, as described before.

A viruliferous colony of adults of *T. vaporariorum* was collected in the Murcia Region from a greenhouse infected with ToTV and it was released on 66 healthy tomato plants of cvs Marmande (21 plants), Cedrico (12 plants), Boludo (11 plants), Marglobe (10 plants), and 1123 (12 plants) at the six-leaf-stage of development. We also analysed 6 plants of *Datura stramonium* L., and 4 plants of each species of *N. glutinosa* L., *N. occidentalis* Wheeler and *N. rustica* L. All the plants were placed in muslin-covered shelves inside a growth chamber at 27ºC/24ºC (day/night) with a 12 h photoperiod and 60% relative humidity. Plant symptoms were monitored every two days and sampled 15 and 45 days after whiteflies release. These leave samples were analyzed to ToTV by RT-PCR.

Results

Symptom observation

Plants infected with ToTV showed an initial yellowing in defined areas at the base of the leaflet that developed into necrotic spots (Fig. 2a), which sometimes abscised, leaving little holes in the leaflet. This symptom is referred to as 'cribado' (Fig. 2b). Therefore, other plants presented extensive necrotic areas which progressed from the base to tip (Fig. 2c). On the stems, some plants showed necrotic streaking (Fig. 2d and 2e) and fruits appeared distorted with necrotic lines (Fig. 2f). Generally, the affected plants had a burn-like appearance, hence the name of this disease, 'torrado', meaning burnt or roasted (Fig. 2g). Nevertheless, some plants observed in the surveys which also show symptoms of necrosis were positive for other tomato-infecting viruses, such as PVY (Fig. 3a and 3b), TSWV (Fig. 3c and 3d) or PMoV (Fig. 3e), but were negative to

ToTV. When comparing Figs. 2 and 3, clear differences were observed between the symptoms of necrosis developed by ToTV-infection and the plants infected with other tomato viruses.

Surveys

In the surveys conducted from 2001-2008, 584 samples of tomato from 92 greenhouses in different parts of the country were collected (Fig. 1). The results of these surveys are summarized in Table 1. The surveyed plants had a range of necrotic symptoms which were recorded and classified in three different groups: 105 samples were recorded as plants with necrotic symptoms which could not be clearly associated with 'torrado' disease, 373 showed typical symptoms of the disease, while 106 samples had been collected in the diseased fields but showed no symptoms of necrosis (marked with 'n', 's' and 'w/s' in Table 1, respectively). Analysis revealed that 53.3% (56 out of 105), 94.4% (352 out of 373) and 40.6% (43 out of 106) of the 'n', 's' and 'w/s' samples were positive for ToTV, respectively. Of the samples collected, 451 plants from 85 different greenhouses were infected with ToTV. This virus was detected in Mallorca, the Murcia Region, Gran Canaria, Tenerife, Almería, Alicante and Barcelona in the different survey years. PepMV was usually detected in the surveyed samples, as the 357 positive samples to the virus revealed (61.1%) in which the EU genotype was the most prevalent until 2004 (positive EU samples/positive CH2 samples =2). The incidence of the CH2 genotype later increased significantly (positive EU samples/positive CH2 samples = 0.71; Table 1). In all, the EU, CH2 and CH1/US1 genotypes were detected in 214, 272 and 36 of the 357 positive samples of PepMV, respectively. The CH1/US1 genotype was only detected in the Canary Islands (Tenerife and Gran Canaria) mainly in mixed infections with the EU genotype. Besides, 45% of the PepMV positive samples corresponded to a mixed infection between the EU genotype and CH2 (35.2%) or

CH1/US1 (9.8%). A double infection between ToTV and PepMV was detected in 273 samples of the 451 infected ToTV plants (60.5%), where the most prevalent PepMV genotype was CH2 (44.3%), followed by the mixed infection of the EU and CH2 genotypes of PepMV (32.6%), the EU and mixed infection of EU and CH1 (13.2% and 9.5%, respectively) and, finally, CH1/US1 (0.4%). No PE genotype was detected in the analysed samples (Table 1).

Several samples showing necrotic symptoms (marked with 'n') were negative for ToTV, but positive for other viruses such as CMV (5 plants), PMoV (5 plants) PVX (1 plant), PVY (4 plants), TSWV (12 plants), or TYLCV (1 plant). Nevertheless, some samples infected with ToTV were also positive for TSWV (30 plants), ToMV (7 plants) and ToCV (26 plants). Interestingly, PMoV was only detected in 5 samples collected from other areas of Spain, such as Vizcaya, Tarragona and Valencia. None of the 40 samples tested for ToANV resulted positive reactions, although they showed similar symptoms to those described for this virus by Turina et al. (2007).

In order to obtain a more detailed information of the distribution of the infected plants in greenhouses, we conducted an exhaustive survey of four greenhouses in the Murcia Region in 2008. A total of 1,527 plants were monitored for the presence or absence of symptoms (Table 2). These greenhouses revealed a variable rate of symptomatology ranging from 37% to 8.4% of plants showing the typical 'torrado' symptoms. Interestingly, 60% of symptomatic plants were located close to doors, windows or main corridors of greenhouses. The testing of 47 samples collected in the four greenhouses revealed that 25, 2 and 1 sample(s) showing 's', 'w/s' and 'n' symptomatology, respectively, were positive for ToTV. Almost all the collected samples presented mixed infections of ToTV and PepMV (25 out of the 28 samples found to be positive to ToTV, Table 2), while all the samples marked with 'n' (13

samples) were positive for TSWV, although 2 samples presented typical ToTV symptoms and were mix infected with ToTV and TSWV.

Due to the high incidence of ToTV in tomato crops, we tested the heterogeneity of the virus population by determining the nucleotide and amino acid sequences of two regions of different isolates from various regions/greenhouses. The analyzed sequences of the fragments of polyprotein RNA2 (partial MP and Vp23 CP subunit) of five different isolates (MUR-03, MUR-05, TEN-07, MUR-08 and GNC-08) revealed a high nucleotide identity (99-97%) in comparison to ToTV sequences deposited in the GenBank database: ToTV-PAN1 (Accession numbers EU934037 and EU357151 for the partial MP and Vp23 CP subunit, respectively), Isolate Wal´03 (Accession number: EU563947), PRI-ToTV0301 (Accession number DQ3888880) and ToTV-CE (Accession number EU476181). However, the percentage of nucleotide identity among ToTV isolates and the two new viruses included in the tentative genus Torradovirus (ToANV and ToMarV) was below 73%. The phylogenetic analysis also confirmed these results (Fig. 4). ToTV studied isolates were grouped in the same cluster and separated from ToMarV and ToANV. Within the ToTV group, the isolate GNC-08 (collected during 2008 in Gran Canaria) was slightly different to the rest in both genome fragments. The percentage of identity of the predicted amino acid sequence for ToTV studied isolates ranged between 98.3-100% and 97.5-100% for the partial MP and Vp23 CP subunit, respectively. This value was slightly lower for the CP subunit, indicating a more variable region as described for other viruses (Aparicio and Pallás, 2002; Fiore et al., 2008; Table 3). By contrast, the percentage of identity of those isolates with the PRI-ToMarV0601 isolate was less than 75.2% (Table 3).

Comparison between tissue-printing and dot-blot hybridization

The high incidence of ToTV and resulting economical losses in tomato crops are strong arguments for using a powerful detection technique that is capable of testing a large number of field samples in a very short time. Non-radioactive molecular hybridization has proved to be a reliable methodology with a good detection limit, even with the direct application of tissue on the membrane (tissue printing). A previous test of the different parts of the ToTV-infected tomato plant revealed that the petioles of apical leaves are a good tissue to perform the tissue-printing analysis. The analysis of 103 tomato plants by dot-blot and tissue printing molecular hybridization using a riboprobe complementary to a fragment of the ORF2-RNA2, revealed that both hybridization techniques were coincident. Fig. 5 shows the results of the tissue-printing (5a) and dot-blot (5b) hybridization of samples collected from the three different fields included in the surveys. The specificity of both the hybridization techniques was confirmed by the lack of cross-reaction with the healthy controls and with tomato plants infected with viruses other than ToTV.

Mechanical back-inoculation and whitefly transmission

Fifty-six tomato plants of cvs Boludo and Marmande were mechanically inoculated with 14 different ToTV isolates. None of the inoculated plants were positive for ToTV 45 days after inoculation. However, whitefly ToTV transmission was observed using a viruliferous colony of *T. vaporariorum* collected from a ToTV-infected greenhouse and different cultivars of tomato, *N. rustica*, *N. occidentalis, N. glutinosa* and *D. stramonium* species. The percentage of infected tomato plants varied according to the cultivar, and ranged from 67.0% (8 of 12 plants of cv. Cedrico), 54.0% (6 of 11 plants of cv. Boludo), 8.3% (1 of 12 plants of cv. 1123) or 0% for cvs Marmande (21 plants) and Marglobe (10 plants). We observed typical necrotic spots associated with ToTV-

infection on the base of the leaflet in only 5 and 6 infected tomato plants of cvs Cedrico and Boludo, respectively. The remaining solanaceous species used for whitefly transmission, 33.3% (2 of 6 plants) of *D. stramonium* and 50.0% (2 of 4 plants) of *N. rustica*, *N. occidentalis* and *N. glutinosa* were ToTV-infected. All infected *Nicotiana* species were symptomless; however, infected plants of *D. stramonium* had symptoms which consisted in interveinal yellowing that developed into necrosis, although similar symptoms were also observed in non-infected plants which could be associated with whitefly feeding.

Discussion

Since 2001, the aggressive 'torrado'disease has been reported to affect tomato crops in two of the most important tomato production areas of Spain (the Murcia Region and the Canary Islands; Jordá et al., 2003; Espino et al., 2007), although similar 'torrado' symptoms were previously observed in 1996 and 1997 in the Canary Islands (Gran Canaria and Tenerife; Espino et al., 2007). This symptomatology has been associated with the presence of ToTV (Verbeek et al., 2007a). In the present study, we conducted an extensive surveys from 2001 to 2008 in different important tomato production areas of Spain to determine the occurrence and distribution of the 'torrado' disease and its new associated viruses. This surveys reveal that ToTV-infected plants are present in tomato crops in Mallorca, the Murcia Region, Gran Canaria, Tenerife, Almería, Alicante and Barcelona. These seven regions cover 40% of the total Spanish tomato production (M.A.R.M., 2007). The incidence of ToTV varied between 58.3% (2001) and 92.2% (2005) of the plants collected from 92 greenhouses in the different tomato production areas, with an average of 77.0% over all the survey years. However, the incidence of the disease recorded in four greenhouses in the Murcia Region in 2008 varied, and could

represent a percentage of affected plants of up to 37.0% of all the plants growing in one greenhouse (Table 2). Furthermore, an incidence of 4.68% (in Tenerife) and 2.99% (in Gran Canaria) of the total cultivated area was reported in other surveys in the Canary Islands in 2007 (Espino et al., 2007).

To gain a better understanding of the correlation between the 'torrado' symptoms and the presence of ToTV, we visually classified the surveyed plants into three symptomatology categories: 'torrado' disease with typical necrotic symptoms (s), necrotic symptoms whose appearance differs from that of the 'torrado' disease (n) and plants without necrotic symptoms (w/s). Furthermore, 94.4% of 's' plants were ToTV-infected, and 53.3% and 40.5% were 'n' and 'w/s' plants, respectively, were also ToTV-positive. These data indicated the no complete correlation between symptomatology and ToTV infection, and the presence of latent infections that represent a potential risk for virus spread. Several viruses could cause different necrotic symptoms in tomato plants (Córdoba-Sellés et al., 2007), which make diagnosis of some symptomatic samples difficult. In addition, symptoms and their severity vary with the virus isolate, cultivar, plant stage and environmental conditions in which infection takes place (EPPO, 2004). Our results clearly show that ToTV symptoms are not only related to the 'torrado' symptomatology, but also to other necroses, or even to asymptomatic phenotypes. Thus, some of the symptomatic (s) and necrosis samples (n) were infected with other tomato viruses which commonly induce necrosis, such as TSWV, PVY, PMoV, CMV, ToCV and TYLCV. PMoV, which induced brown patches on tomato fruits and necrotic mosaic on leaves that progressed to apical stem necrosis, is easily confused with CMV, TSWV (Galipienso et al., 2003) or ToTV symptoms (Jordá et al., 2003). Tomato plants infected with PMoV, marked with 'n' in the symptomatology evaluations, were only detected in restricted areas of north and east Spain (Vizcaya, Tarragona and Valencia).

These results agree with previous surveys in which PMoV was detected in tomato crops from north-eastern (Cataluña; Aramburu, 2001; Gallipienso et al., 2003) or east Spain (Comunidad Valenciana; Aparicio et al., 2008).

The high incidence of ToTV in some areas of Spain may present a potential risk for more aggressive or adaptive viral variants. To analyze the heterogeneity of the ToTV population in Spain, we characterized the nucleotide and amino acid sequences of two regions of the RNA 2 (partial MP and subunit Vp23 of CP) of five Spanish isolates from different geographic areas and collected in different time. This confirmed the high genetic similarity of the Spanish ToTV isolates to each other as well as their close relationship to ToTV isolates from other countries as Poland (Budziszewskza et al., 2008; Pospieszny et al., 2009) or Panama.

Given the high percentage of ToTV-infected tomato plants and the lack of an available commercial antibody, we explored alternative detection techniques to the standard serological ELISA method to be used to screen a large amount of samples. Molecular hybridization (MH) using non radioactive riboprobes has proved to be an interesting detection technique for carnation (Sánchez-Navarro et al., 1996), stone fruit (Herranz et al., 2005) and tomato crops (Saldarelli et al., 1996). The analysis of the five ToTV Spanish isolates reveals that the fragment which includes the putative MP in the ORF2-RNA2, is a conserved region that represents a good viral portion to be detected by MH. The use of a riboprobe target to that fragment was probed to specifically detect ToTV using total RNA extracted from tomato tissue. The same result was obtained when the samples were applied directly onto the membrane by tissue-printing, thus avoiding the RNA extraction procedure. Tissue printing hybridization proves to be a reliable technique with satisfactory results as a first detection step to determine the phytosanitary status of tomato plants in fields. This technique also saves time and

simplifies the handling procedure in large-scale testing (Aparicio et al., 2008; Gallipienso et al., 2003). Furthermore, the MH allows the simultaneous detection of different viruses affecting tomato crops by either mixing the corresponding riboprobes in the same hybridization solution (Saldarelli et al., 1996) or using polyprobes (Aparicio et al., 2008). Our results indicate that ToTV is easily detected by MH to allow its inclusion in the previously described simultaneous detection procedures. However, it could prove very interesting to analyze whether the tissue-printing approach allows the multiple detection of the main viruses affecting tomato crops.

Due to the large number of viruses affecting tomato crops, another aspect to consider is the presence of mixed infections. The testing of tomato plants infected with ToTV revealed that a high number of these plants were co-infected with another virus in which PepMV was the most prevalent (46.7%), followed by ToCV and TSWV (5.7% and 6.7%, respectively). However, other than ToTV and PepMV, only suspicious plants were tested for the remaining viruses, which indicates that the percentage could be even higher. PepMV is widespread in tomato crops in Spain. The surveys from 2000 to 2004 revealed that the PepMV EU strain showed a high incidence in just single (80%), and the presence of the PE or US2 PepMV strains at lower frequency relative to EU (2 and 8 out of 64 isolates respectively; Pagán et al., 2006). Since then, new PepMV isolates have been identified and, four major PepMV strains may now be distinguished EU, PE, CH1/US1, and CH2 (US2 genotype belongs to CH2 strain; Ling et al., 2007). The distribution and occurrence of some of these strains in other European countries has also been reported and show that tomatoes infected with PepMV present the EU and CH2 genotypes (Hanssen et al., 2008). A recent analysis performed by our group using a specific multiplex RT-PCR that discriminates among the five PepMV genotypes shows how the EU strain is the most prevalent (94.0% of the infected plants), followed by CH2

(61.0%) and CH1/US1 (25.0%) (Alfaro-Fernández et al., 2009b). Our results show the genetic variability of the PepMV to be associated with 'torrado' disease symptoms. Then 61.1% of tomato plants were infected with PepMV (357 plants of 584) in which the EU, CH2 and CH1/US1 genotypes represented 13.2%, 44.3% and 0.4%, respectively. Meanwhile, mixed infections of EU-CH2 and EU-CH1/US1 represented 32.6% and 9.5%, respectively. Regarding the different PepMV genotypes, we observed that CH2 has been detected in samples since 2001, and is mainly associated with the Murcia Region. This observation contrasts with previous studies in which the CH2 strain (referred then as US2 strain) was only found in samples collected in 2004 in the Murcia Region (Pagán et al., 2006). In addition, the incidence of CH2 in the analyzed tomato plants increased from 8.3% (2001) to 73.3% (2008). The fact that the incidence of the EU genotype in single infections decreased from 2001, especially in the Murcia Region, could be interpreted as the CH2 genotype displacing the EU variant. However, more analyses are required to confirm this notion. Unlike PepMV population studies performed by Pagan et al. (2006), PE genotype was not detected in any of the tested samples. Nevertheless, the incidence of this genotype in these studies was quite low (4 out of 64 isolates) and was only detected in the Canary Islands in 2001 and 2003, and in 2 isolates from Murcia collected 2004, studying the TGB region of the PepMV genome (Pagan et al., 2006). All these results revealed that PE genotype was present, but not highly distributed in tomato crops in Spain, although our study only focused on one area of the PepMV genome. Otherwise, no specific PepMV genotype is associated with ToTV infection, which contrasts with previous assays performed in the Murcia Region (Alfaro-Fernández et al., 2007b). Interestingly, the co-infection of ToTV and PepMV is mainly associated with a more aggressive symptomatology (73.3% and 14.3% of the plants infected with both viruses showed symptomatology of 's' and 'n'), indicating a

putative synergism. This phenomenon could be explained by the expression of an RNA silencing protein, as previously described (Yang and Ravelonandro, 2002). In this sense, the TGB1 protein expressed by potexvirus is an RNA silencing inhibitor that shows different silencing levels (Voinnet et al., 2000; Senshu et al., 2009). Indeed, we checked the capacity of the TGB1 protein of PepMV to influence ToTV symptomatology.

Unlike mechanical transmission, ToTV is easily and efficiently transmitted by whiteflies (up to 67.0% in some tomato cultivars). In previous tests, the efficiency of the mechanical and approach-grafting inoculation of tomato plants was low, resulting in a few positive plants (1.8%; Alfaro-Fernández et al., 2006). The higher efficiency of whitefly transmission compared to the mechanical transmission of ToTV was previously reported by Pospieszny et al. (2007; 2009). Furthermore, the results obtained in whitefly transmission assays reveal that 66.7%, 54.5% and 8.3% of tomato plants cvs Cedrico, Boludo and 1123 were infected with ToTV. In addition, we observed that tomato plants cv. Marmande were not infected, indicating that ToTV transmission to *S. lycopersicum* is variety-dependent as previously reported Budziszeska et al. (2008). It should be noted that there are tomato plants with a natural resistance to ToTV which confers them at least an allele of one gene. However, these virus-resistant plants and the method to produce them have been patented (Maris et al., 2007). Some other solanaceous species tested in the assay were ToTV-infected as: *N. glutinosa*, *N. rustica*, *N. occidentalis* and *D. stramonium*. Remarkably, the three *Nicotiana* species had already been reported as virus hosts as well as *D. inoxia* (Verbeek et al., 2007a; Pospieszny et al., 2007; 2009; Amari et al., 2008). However, we report the susceptible response of *D. stramonium* to ToTV infection (33.3%) for the first time.

Therefore several studies have confirmed the vector-assisted transmission of this virus by whiteflies (Pospieszny et al., 2007, 2009; Amari et al., 2008), which means that

the distribution of this virus throughout the production areas has been totally determined by both the climate and the vector, although plenty of epidemiological aspects of ToTV still remain unknown. Moreover, ToTV is an emerging disease which has been reported in several countries (Australia, Hungary, Panama, Poland and Spain) and has been recently included in the EPPO alert list due to the risk of spread within the European countries (EPPO, 2009). The efficiently whiteflies transmission of ToTV in tomato cultivation may mean that it becomes a serious agricultural problem for tomato production in forthcoming years. Whitefly populations have increased worldwide since the 1970s, probably due to the combination of different effects, such as: i) increased use of synthetic organic insecticides with an increased resistance to them, ii) changing climatic conditions, iii) intensified agricultural practices, or iv) international movement of plant materials as part of the nursery and horticultural trade (Wisler et al., 1998). In Spain, a displacement of *T. vaporariorum* by *B. tabaci* in melon crops cultivated in greenhouses of south-east Spain has been reported to change the incidence of a whitefly-transmitted closterovirus (Berdiales et al., 1999; Celix et al., 1996). Otherwise, the two whitefly species are present in tomato, and *T. vaporariorum* is usually the most important species in north and north-east Spain, whereas *B. tabaci* is predominant in south Spain where it causes severe damage. In a mid-transition area, both species co-exist all year long, especially during winter, and they even co-exist within a plant, exhibiting *T. vaporariorum* with a greater preference for younger leaves than *B. tabaci* (Arnó et al., 2006). Although both species have been reported to transmit ToTV (Pospieszny et al., 2007; Amari et al., 2008), the specificity and the parameters of the whitefly transmission remain unknown. Furthermore, ToTV has also been found in weeds to show that, besides the main crop host, the virus and the vector have alternative

hosts for their dissemination. Therefore, weed management demands particular attention to effectively control the spread of ToTV (Alfaro-Fernández et al., 2008).

Our research provides more current data on the occurrence and distribution of ToTV in Spain, which is usually found in mixed infections with PepMV. The impact on crop productivity and the potential spread of this virus to other growing regions with resident whitefly populations emphasizes the need for further research to develop and implement effective control strategies.

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FIGURE CAPTIONS

Fig. 1: Map of Spain showing the location of regions 1 to 11 where greenhouse tomato crops were surveyed during the 2001-2008 growing seasons.

Fig. 2: Symptoms observed in the plants showing the 'torrado' disease. Defined areas at the base of the leaflet of the affected tomato leaves showing yellowing and necrotic spots **(a)**, which sometimes abscised, leaving little holes in the leaflet ('cribado') **(b)**. Extensive necrotic areas progressed from the base to the tip of the leaflet **(c)**. Necrotic streaking observed on the stems **(d,e)**. Fruits appeared distorted with necrotic lines **(f)**. Burnt-like appearance of the plants affected with the 'torrado' disease (**g**).

Fig. 3: Necrotic symptoms observed on the leaves of tomato plants infected with PVY **(a)** and **(b)**, TSWV **(c)** and **(d)** or PMoV **(e)** collected in the different surveys.

Fig. 4: Phylogenetic analysis based on nucleotide sequences of partial movement protein (a) and subunit Vp23 of coat protein **(b)** of analyzed ToTV isolates (MUR-03, MUR-05, TEN-07, GNC-08 and MUR-08) and sequences published in the GenBank Database of ToTV isolates (PRI-ToTV0301, Wal03, ToTV-Pan1, ToTV-CE) and other related viruses (PRI-TMar0601 and ToANV-VE434). The phylogenetic tree was constructed and visualized with MEGA 3.1 using neighbor-joining algorithm and 1,000 bootstrap values. The number above nodes indicates the percentage of bootstrap

replicates which supported the branching. Internal branches with percentage of bootstrap replicates lesser than 50% were not indicated. The accession numbers of the sequences used in the analysis were as follows: ToTV-CE, Accession number EU476182; ToTV-PAN1, Accession numbers FJ357161 and EU934037; PRI-ToTV0301 Accession number DQ388880; Wal03, Accession number EU563947; PRI-ToMarV06031, Accession number NC010988; and ToANV-VE434, Accession number EF063642.

Fig. 5: Comparative analisis of tissue-printing **(a)** and dot-blot **(b)** molecular hybridization procedures. The tomato plants previously analyzed by dot-blot hybridization (b) were analyzed by tissue printing using petioles which were transversely cut and directly pressed onto the nylon membrane (a). The membranes were crosslinked and hybridized using a specific dig-RNA ToTV probe. The positive and negative controls corresponding to infected or healthy tomato plants are indicated as PC and HT, respectively. Samples infected with viruses other than ToTV are indicated as 1 (TSWV), 2 (ToCV), 3 (TYLCV), 4 (ToMV), 5 (PMoV) and 6 (PVY).

TABLES

Table 1: Results of the surveys performed between 2001 and 2008: location, number of fields and samples surveyed, symptoms observed, and viruses detected.

Year	No. Map[a]	Region	Fields	No. Samples	Symptoms observed[b]			Virus detection						
									PepMV					Double infection ToTV+PepMV
					n[b,c]	s[b,c]	w/s[b,c]	ToTV[d]	Total of positives	EU	CH1/US1	CH2	Mixed infection	
2001	5	Alicante	1	1	1 (0)	0	0	0	1	1	0	0	0	0
	8	Mallorca	1	2	2 (1)	0	0	1 (1)	2	2	0	0	0	1
	6	Murcia	2	9	9 (6)	0	0	6 (2)	1	1	0	1	1	1
2002	6	Murcia	7	20	9 (5)	11 (11)	0	16 (5)	9	8	0	2	1	6
	11	Vizcaya	1	2	0	2 (0)	0	0	0	0	0	0	0	0
2003	10	Gran Canaria	1	2	2 (1)	0	0	1 (1)	1	1	0	0	0	0
	6	Murcia	6	22	9 (4)	12 (12)	1 (1)	17 (6)	12	8	0	10	6	8
	9	Tenerife	4	5	4 (2)	1 (1)	0	3 (2)	3	2	0	1	0	2
	4	Valencia	1	1	1 (0)	0	0	0	0	0	0	0	0	0
2004	7	Almería	1	2	0	2 (2)	0	2 (1)	1	0	0	1	0	1
	3	Castellón	1	1	1 (0)	0	0	0	0	0	0	0	0	0
	10	Gran Canaria	3	9	6 (6)	3 (3)	0	9 (3)	2	2	0	0	0	2
	6	Murcia	5	8	1 (1)	6 (6)	1 (0)	7 (5)	4	4	0	0	0	3
	2	Tarragona	1	2	2 (0)	0	0	0	0	0	0	0	0	0
	9	Tenerife	1	1	1 (0)	0	0	0	1	1	0	0	0	0

Year	No. Map[a]	Region	Fields	No. Samples	Symptoms observed[b]			Virus detection						
					n[b,c]	s[b,c]	w/s[b,c]	ToTV[d]	PepMV					Double infection ToTV+PepMV
									Total of positives	EU	CH1/US1	CH2	Mixed infection	
2005	10	Gran Canaria	1	10	0	10 (10)	0	10 (1)	1	1	0	1	1	1
	6	Murcia	11	90	11 (9)	75 (73)	4 (2)	84 (11)	50	13	0	47	10	48
	4	Valencia	1	2	2 (0)	0	0	0	0	0	0	0	0	0
2006	6	Murcia	10	135	8(7)	89 (84)	38 (18)	109 (10)	98	54	0	94	50	77
	10	Gran Canaria	4	21	7 (3)	9 (8)	5 (4)	15 (4)	2	2	0	0	0	1
	9	Tenerife	1	2	2 (2)	0	0	2 (1)	2	2	0	2	2	2
2007	5	Alicante	2	12	2 (0)	9 (9)	1 (1)	10 (1)	12	8	0	7	3	10
	7	Almeria	1	1	0	1 (1)	0	1 (1)	0	0	0	0	0	0
	10	Gran Canaria	3	22	0(0)	20 (18)	2 (2)	20 (3)	8	6	3	4	4	8
	6	Murcia	4	52	4 (4)	31 (28)	17 (5)	37 (4)	41	11	0	40	10	31
	9	Tenerife	9	75	11 (1)	47 (44)	17 (7)	52 (7)	46	45	33	7	36	34
2008	1	Barcelona	1	2	2 (2)	0	0	2 (1)	2	0	0	2	0	2
	10	Gran Canaria	1	3	1 (0)	1 (1)	1 (1)	2 (1)	3	0	0	3	0	2
	6	Murcia	7	70	7 (2)	44 (41)	19 (2)	45 (7)	55	42	0	50	37	33
	TOTAL		92	584	105 (56)	373 (352)	106 (43)	451 (85)	357	214	36	272	161	273

[a] The number indicates the corresponding region in Fig. 1.
[b] The symptoms were marked as symptomatic plants (s), for those plants which showed the symptoms currently associated with the "torrado disease"; plants which showed some necrotic symptoms that could not be clearly associated with the "torrado disease" (n), and plants without necrotic symptoms (w/s).
[c] The number represents the plants showing the corresponding symptomatology. The numbers in parentheses correspond to the samples which were positive for ToTV.
[d] Number of positive plants. The number of fields from which the positive samples were collected are shown in parentheses.

Table 2: Results of the surveys conducted in 2008 in four greenhouses of the Murcia Region.

| Greenhouse | No. of monitored plants | % plants with symptoms | No. samples collected | Symptoms observed[a] | | | Virus detection | | | | | | | |
				n[b]	s[b]	w/s[b]	ToTV	PepMV Total of positives	EU	CH1/US1	CH2	Mixed infection	Double infection ToTV+PepMV	Other analyses[c]
1	115	37	16	2 (1)	10 (10)	4 (0)	11	16	12	0	14	10	11	TSWV (2)
2	132	37	10	2 (0)	6 (6)	2 (1)	7	7	4	0	6	3	5	TSWV (3)
3	648	8.4	9	1 (0)	8 (8)	0 (0)	8	8	7	0	7	6	7	TSWV (3)
4	632	9	12	8 (0)	1 (1)	3 (1)	2	12	11	0	11	10	2	TSWV (8)
TOTAL	1527	37-8.4	47	13 (1)	25 (25)	9 (2)	28	43	34	0	38	29	25	TSWV (15)

[a] The symptoms were marked as symptomatic plants (s), for those plants which showed the symptoms currently associated with the "torrado disease"; plants which showed some necrotic symptoms that could not be clearly associated with the "torrado disease" (n), and plants without necrotic symptoms (w/s).

[b] Number of samples showing the corresponding symptomatology. The numbers in parentheses correspond to samples which were positive for ToTV.

Table 3: Comparison of amino acid sequences of the partial MP and the Vp23 CP subunit of RNA2 of ToTV isolates studied with other ToTV isolates and ToMarV sequences available in the GenBank database. The identity/similarity matrix was calculated using the Matrix Global Alignment Tool software (http://bitincka.com/ledion/matgat).

	MUR-03		MUR-05		TEN-07		GCN-08		MUR-08		PRI-ToTV0301		ToTV-CE		Wal03		ToTV-PAN1	
	MP	Vp23 CP	MP	Vp23 CP	MP	Vp23 CP	MP	Vp23 CP	MP	Vp23 CP	MP	Vp23 CP	MP	Vp23 CP	MP	Vp23 CP	MP	Vp23 CP
MUR-05[a]	Id. 100.0 Sim. 100.0	Id. 100.0 Sim. 100.0																
TEN-07[a]	Id. 99.3 Sim. 99.3	Id. 99.4 Sim. 100.0	Id. 99.3 Sim. 99.3	Id. 99.4 Sim. 100.0														
GCN-08[a]	Id. 99.3 Sim. 99.3	Id. 99.4 Sim. 99.4	Id. 99.3 Sim. 99.3	Id. 99.4 Sim. 99.4	Id. 98.6 Sim. 98.6	Id. 98.8 Sim. 99.4												
MUR-08[a]	Id. 100.0 Sim. 100.0	Id. 100.0 Sim. 100.0	Id. 100.0 Sim. 100.0	Id. 100.0 Sim. 100.0	Id. 99.3 Sim. 99.3	Id. 99.4 Sim. 100.0	Id. 99.3 Sim. 99.3	Id. 99.4 Sim. 99.4										
PRI-ToTV0301[b]	Id. 100.0 Sim. 100.0	Id. 98.8 Sim. 99.4	Id. 100.0 Sim. 100.0	Id. 98.8 Sim. 99.4	Id. 99.3 Sim. 99.3	Id. 98.2 Sim. 99.4	Id. 99.3 Sim. 99.3	Id. 98.2 Sim. 98.2	Id. 100.0 Sim. 100.0	Id. 98.8 Sim 99.4								
ToTV-CE[b]	Id. 99.3 Sim. 99.3	Id. 98.1 Sim. 98.8	Id. 99.3 Sim. 99.3	Id. 98.1 Sim. 98.8	Id. 98.6 Sim. 98.6	Id. 97.5 Sim. 98.8	Id. 98.6 Sim. 98.6	Id. 96.9 Sim. 97.5	Id. 99.3 Sim. 99.3	Id. 97.5 Sim. 98.8	Id. 96.9 Sim. 98.1	Id. 96.9 Sim. 98.1						
Wal03[b]	Id. 99.3 Sim.100.0	Id. 100.0 Sim. 100.0	Id. 99.3 Sim. 100.0	Id. 100.0 Sim. 100.0	Id. 98.6 Sim. 99.3	Id. 99.4 Sim.100.0	Id. 98.6 Sim. 99.3	Id. 99.4 Sim. 99.4	Id. 99.3 Sim. 100.0	Id. 100.0 Sim. 100.0	Id. 100.0 Sim. 100.0	Id. 98.8 Sim. 99.4	Id. 98.6 Sim. 99.3	Id. 98.1 Sim. 98.8				
ToTV-PAN1[b]	Id. 100.0 Sim. 100.0	Id. 99.4 Sim.100.0	Id. 100.0 Sim. 100.0	Id. 99.4 Sim. 100.0	Id. 99.3 Sim. 99.3	Id. 100.0 Sim. 100.0	Id. 99.3 Sim. 99.3	Id. 98.1 Sim. 98.8	Id. 100.0 Sim. 100.0	Id. 100.0 Sim. 100.0	Id. 98.1 Sim. 99.4	Id. 98.1 Sim. 99.4	Id. 99.3 Sim. 99.3	Id. 97.5 Sim. 98.8	Id. 99.3 Sim. 100.0	Id. 99.4 Sim. 100.0		
PRI-TMarV0601[b]	Id. 75.2 Sim. 88.3	Id. 69.6 Sim. 85.1	Id. 75.2 Sim. 88.3	Id. 69.6 Sim. 85.1	Id. 74.4 Sim. 87.5	Id. 69.6 Sim. 84.5	Id. 75.2 Sim. 89.2	Id. 69.6 Sim. 85.1	Id. 75.2 Sim. 88.3	Id. 69.6 Sim. 84.5	Id. 69.6 Sim. 85.1	Id. 69.6 Sim. 85.1	Id. 74.4 Sim. 87.5	Id. 69.6 Sim. 83.9	Id. 75.2 Sim. 88.3	Id. 69.6 Sim. 85.1	Id. 75.2 Sim. 88.3	Id. 69.6 Sim. 8...

Id., identity; Sim., similarity

[a]These isolates are marked with a three-letter code indicating their geographical origin (MUR= Murcia, TEN= Tenerife, GNC= Gran Canaria), followed by the collection year.
[b]The accession numbers of the sequences used in the analysis: PRI-ToTV0301 (DQ3888880), ToTV-CE (EU476181), Wal03 (EU563947), ToTV-PAN1 (EU934037 and EU357151), PRI-ToMarV (NC010988).