Running title: Mixed infection between isolates of TSWV

A severe symptom phenotype in pepper cultivars carrying the *Tsw* resistance gene is caused by a mixed infection between resistance-breaking and non-resistance-breaking isolates of *Tomato spotted wilt virus*

José Aramburu[1], Luis Galipienso[2], Salvador Soler[3], Luis Rubio[2] and Carmelo López[3]

[1]Institut de Recerca i Tecnología Agroalimentaries (IRTA), Ctra. de Cabrils Km 2, 08348 Cabrils, Barcelona, Spain
[2]Instituto Valenciano de Investigaciones Agrarias (IVIA), 46113 Moncada, Valencia, Spain
[3]Instituto de Conservación y Mejora de la Agrodiversidad Valenciana, Universitat Politècnica de Valencia (COMAV-UPV), Camino de Vera s/n, 46022 Valencia, Spain

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Corresponding author: C. López
E-mail: clopez@upvnet.upv.es
Tel.: +34 963877267
Fax: +34 963877429

Abstract

Pepper (*Capsicum annuum*) plants with the *Tsw* resistance gene showing unusual severe symptoms consisting in local lesions, chlorosis, stunting and systemic necrosis on the apical leaves were detected in a commercial field in north-eastern Spain in 2009. Double antibody sandwich enzyme-linked immunosorbent assays (DAS-ELISA) revealed the presence of *Tomato spotted wilt virus* (TSWV) in all diseased plants. After mechanical inoculation of *Nicotiana glutinosa* with infected field samples, TSWV biological clones were isolated from individual local lesions. These biological clones produced two different types of symptoms after inoculation on *Tsw* resistant pepper plants: (i) the typical symptoms caused by resistance-breaking (RB) isolates characterized by chlorosis and stunting, and (ii) the severe symptoms as observed in field plants. Similar symptoms in pepper plants carrying the *Tsw* resistance gene were reproduced under controlled conditions, after simultaneous inoculation of RB and non-resistance-breaking (NRB) isolates. The NRB isolate was detected in a low proportion in the apical uninoculated leaves, whereas NRB isolates could not infect resistant pepper when inoculated alone. Co-infection between NRB and RB isolates induced disease synergism with systemic necrosis on the apical leaves. To our knowledge, this is the first case in which a synergic interaction between isolates of the same virus has been described, which has the ability to overcome a natural genetic resistance. This finding could have epidemiological implications for management of TSWV.

Introduction

Tomato spotted wilt virus (TSWV), the type-member of the *Tospovirus* genus, family *Bunyaviridae*, is one of the most harmful viral pathogens. It ranks second on the list of the most important plant viruses worldwide (Scholthof *et al.* 2011). TSWV has a wide host range including more than 1000 plant species among weed species, ornamental and horticultural crops (Parrella *et al.* 2003; Hanssen *et al.* 2010), such as pepper (*Capsicum annuum*) and tomato (*Solanum lycopersicum*) (Persley *et al.* 2006; Pappu *et al.* 2009). The virus is naturally transmitted by several species of thrips (*Thysanoptera: Thripidae)* in a persistent and propagative manner with *Frankliniella occidentalis* being the most effective vector (Prins and Goldbach 1998). The genome of TSWV consists of three negative-sense or ambisense RNA segments: large (L, 8.9 kb), medium (M, 4.9 kb) and small (S, 2.9 kb). Segment L encodes a putative RNA-dependent RNA polymerase (de Haan *et al.* 1991); segment M encodes the cell-to-cell movement protein, NSm (Li *et al.* 2009), and the precursor of the surface glycoproteins, G_N/G_C, involved in TSWV transmission by thrips (Sin *et al.* 2005; Naidu *et al.* 2008); and segment S encodes the silencing suppressor, NSs (Takeda *et al.* 2002) and the nucleocapsid, N (de Haan *et al.* 1990; Pappu *et al.* 2009).

In tomato and pepper, the best strategy to control the disease has been to use the natural host resistance found in wild *Solanum* and *Capsicum* species (Stevens *et al.* 1992; Boiteux and de Avila 1994). *Sw-5* from *Solanum peruvianum* and *Tsw* from *Capsicum chinense* are the most effective resistance genes in tomato and pepper, respectively, and they are now deployed in commercial cultivars worldwide (Pappu *et al.* 2009). *Tsw* has been found to confer resistance to a wide spectrum of TSWV isolates (Moury *et al.* 1997), although a partial inefficiency by high temperatures after inoculation with wild type isolates on resistant peppers at an early stage of plant development has been

described **(**Moury *et al.* 1998; Turina *et al.* 2012). However, the main setback has been the emergence of TSWV isolates able to overcome this resistance. Several resistance-breaking (RB) isolates of TSWV have been reported from pepper cultivars containing the *Tsw* gene in Louisiana, USA (Black *et al.* 1996), Italy (Roggero *et al.* 2002), Australia (Thomas-Carroll and Jones 2003) and Spain (Margaria *et al.* 2004).

The genetic determinant responsible for *Tsw* resistance breakdown in pepper was mapped in the S RNA segment by analysis of reassortants between RB and non-resistance-breaking (NRB) TSWV isolates (Jahn *et al.* 2000; Margaria *et al.* 2007). Contradictory evidences involving NSs or N proteins in breaking the *Tsw* gene resistance in pepper have been published (Margaria *et al.* 2007; Lovato *et al.* 2008; Tentchev *et al.* 2011), although recent studies have demonstrated that the NSs protein is the avirulence determinant (de Ronde *et al.* 2013 and 2014).

Inoculation of NRB TSWV isolates in resistant pepper triggers a hypersensitive response (HR) inducing localized necrosis at the site of virus infection which prevents systemic infection, whereas RB TSWV isolates produce systemic symptoms consisting in chlorosis and stunting. However, unusual biological behavior of the RB isolates have been observed as for example, the existence of RB isolates inducing severe necrotic lesions on uninoculated upper leaves in *C. chinense* plants (Margaria *et al.* 2007), or evidences of partial reversion from RB to NRB isolates after a few cycles of subculture in susceptible peppers (Tomas-Carroll and Jones 2003). Some of these unusual pathologic alterations could be attributed to different causes: (i) reassortment by exchange of entire genome segments between different TSWV isolates (Qiu and Moyer 1999; Tentchev *et al.* 2011); (ii) existence of a new TSWV pathotype generated by punctual mutations in NSs or another gene and the selective pressure exerted by the resistance genes on TSWV isolates with different fitness (Aramburu *et al.* 2010; López

et al. 2011); (iii) the effect of temperature changes on the HR expression provided by *Tsw* gene (Moury *et al.* 1998; Soler *et al.* 1998); and (iv) synergistic or antagonistic interactions caused by mixed infections (García-Cano *et al.* 2006; Murphy and Bowen 2006; Syller, 2012).

In this article, we analyzed the possible causes of the systemic necrosis found in field samples of resistant peppers infected by TSWV. We reproduced these symptoms by simultaneous inoculation of RB and NRB isolates of TSWV showing that they were produced by synergistic interactions between both types of TSWV isolates.

Materials and methods

Samples collection and TSWV detection

Samples of pepper plants carrying the *Tsw* gene showing systemic necrosis apparently caused by TSWV infection were collected during 2009 growing season from a commercial crop in north-eastern Spain. Selected field samples were tested for TSWV infection by enzyme-linked immunosorbent assay (ELISA) (Clark and Adams 1977), using a specific polyclonal antiserum to TSWV, at recommended dilutions (Loewe Biochemica Gmbh, Sauerlach, Germany), as previously described (Aramburu *et al.* 2010). Samples were also tested by ELISA for the presence of *Cucumber mosaic virus* (CMV), *Parietaria mottle virus* (PMoV) and *Potato virus* Y (PVY), using specific antisera (Aramburu *et al.* 2010) to discard possible infection mixtures with the most frequent viruses found in pepper crops of this area.

Biological cloning

Two field samples positive to TSWV were mechanically inoculated on *Nicotiana glutinosa* plants to obtain homogeneous intra-isolate population of genetic variants.

Several biological clones were obtained after three serial passages of a single local lesion. Mechanical inoculation was performed on plant seedlings at the 2-4 leaf stage by rubbing infected leaves extracts diluted (1:20, w:v) in 0.05 M phosphate buffer, pH 7.2, containing 0.2% 2-mercaptoethanol, 1% polyvinyl pyrrolidone, molecular weight 10.000 and activated charcoal. These TSWV clones were subsequently amplified in *Nicotiana benthamiana* plants and systemically infected leaves were stored at -80ºC until use. TSWV isolates were then mechanically inoculated to pepper cv 'Segura' heterozygous for the *Tsw* resistance gene and pepper cv 'Delfos' without *Tsw* gene (both provided by FITO S.A., Barcelona, Spain) to evaluate the ability to overcome the resistance. In addition to ELISA tests, TSWV infection was monitored by symptoms observation every day since 4 to 21 days post-inoculation (dpi).

Additionally, five TSWV isolates of our collection, previously characterized by their capacity to induce or break the resistance conferred by the *Tsw* gene were used in this work (Table 1). The isolates Da1NL2, Mon1NL2 and GRAU from tomato plants collected in north-east of Spain were grouped as NRB isolates, and the isolates Can2PC2 and Mu2PC2 from resistant pepper collected in Canary Island and in south-east of Spain, respectively, were grouped as RB isolates.

Biological assays to test the effect of temperature

Resistant pepper plants cv. "Segura" were used to test the effect of temperature on *Tsw* resistance expression. The symptoms expression by RB and NRB isolates was conducted in two constant temperature regimes: i) 35ºC, to inactivate the HR and ii) 25ºC to activate the HR. Series of 10 pepper plants were singly inoculated with either NRB (Da1NL2 and Mon1NL2) or RB isolates (Can2PC2 and Mu2PC2). Inoculated plants were maintained during 12 dpi in temperature-controlled growth chambers

(Havell Sylvania SA, Madrid, Spain), with 16-h photoperiod. Four plants were included as control in each serie of plants, two *N. benthamiana* plants used as positive controls of TSWV infection, and two uninoculated pepper plants used to evaluate the effect of temperature on plant growth.

Biological assays to test the interaction of RB and NRB isolates of TSWV

To test the interaction of RB and NRB isolates, pairs of RB + NRB isolates were co-inoculated on series of 10 pepper cv. `Segura' resistant plants. Inoculated plants were kept in a thrips free growth chamber during 12 dpi with 16/8 h light/dark cycle and constant temperature of 25ºC to activate the HR. Inocula of the six possible pairs of RB + NRB isolates were prepared mixing equivalent amounts of each isolate. The amount of each isolate was determined by ELISA and by the number of lesions produced on *N. glutinosa* as previously described (Aramburu *et al.* 2010). Pepper cv. `Segura' and *N. benthamiana* plants singly inoculated with RB and NRB isolates and pepper cv "Delfos" and *N. benthamiana* plants co-inoculated with the same pairs of RB + NRB isolates were used as controls. Plants were monitored by symptoms observation every day since 4 to 12 dpi.

Identification of RB and NRB isolates in co-inoculated resistant peppers plants

Both, molecular and biological analysis at 12 dpi were carried out on resistant pepper plants co-inoculated with Da1NL2 + Mu2PC2 isolates to determine the presence or absence of each isolate. The molecular analysis included: (i) a reverse transcription coupled with polymerase chain reaction (RT-PCR) combined with restriction fragment length polymorphism **(**RFLP) analysis of the M and S segments, and (ii) partial sequence analysis of the RT-PCR products of the M and S segments. RT-PCR products

were amplified using the primer pairs M1F 5´-GTTATAGGATAATTATCTTGTGTC-3´ and M1R 5´-AGAGCAATCAGTGCAAACAAAAACCTTAATCC-3´ and S1F 5´-GATCGAGATGTGCTATAATCAAGC-3´ and S1R 5´-GAACCTGTGCAAAAGATGTGTGAG-3´ for M and S segments, respectively. They were designed from conserved sequences after comparing the full-length sequence of the M and S segments of the two isolates. Differences in the sequence of these RT-PCR products allowed to be selectively digested with *Eco*RI (MBI Fermentas, Madrid, Spain). Ten microlitres of PCR products and 10 U of the restriction enzyme were incubated for 2 h at 37ºC. PCR products or their restriction fragments were separated by electrophoresis in agarose gels, stained with ethidium bromide, and the DNA was visualized under UV lighting.

The biological analysis was performed following the next steps: (i) leaf extracts of resistant pepper plants showing necrosis on apical leaves were inoculated on *N. benthamiana* plants, a non selective host used to favor the multiplication of the infectious mixture; (ii) leaf extracts showing symptoms of systemic infection were inoculated on *N. glutinosa* plants to obtain biological clones; (iii) leaf tissue disks of 30 local lesions obtained 4 dpi were inoculated in individual *N. benthamiana* plants to multiply the different clones; (iv) each biological clone was inoculated to three resistant pepper plants to evaluate the symptoms; and (v) finally, infectious tissues of *N. benthamiana* corresponding to the clones that only induced HR on inoculated pepper leaves were analyzed by molecular techniques, as described above, to identify the TSWV isolate present in the infection.

Results

Biological characterization of TSWV causing severe symptoms in field resistant peppers

Pepper samples with the *Tsw* gene showing severe and unusual symptoms consisting in necrotic lesions, chlorosis, stunting and apical necrosis which later on evolved to petiole and stem collapse were collected from the field (Figure 1). Two pepper samples that tested positive for TSWV and negative for CMV, PMoV and PVY by ELISA were selected to obtain biological TSWV clones by isolating local lesions in *N. glutinosa* plants. After transmission by mechanical inoculation to pepper plants carrying the *Tsw* gene, these biological TSWV isolates segregated in two different types of symptoms: (i) severe symptoms consisting in necrotic lesions on inoculated leaves at 4-6 dpi, chlorosis, stunting, systemic necrosis with asymmetrical distribution on uninoculated leaves and petiole collapse of some leaves at 8-10 dpi, and stem collapse at 12-14 dpi, that eventually induced plant dead at 16 dpi (Figure 2A), and (ii) the typical symptoms of RB isolates on resistant peppers characterized by chlorosis and stunting of apical leaves (Figure 2B).

Effect of temperature on symptom expression in pepper plants carrying the *Tsw* gene

To assess whether the effect of temperature could be involved in the expression of systemic necrosis, two regimes of constant temperature, 25 and 35ºC, were assayed after inoculation of pepper plants carrying the *Tsw* resistance gene with RB (Can2PC2 and Mu2PC2) or NRB (Mon1NL2, Da1NL2 and GRAU) isolates of our collection. Results were consistent in five independent experiments for each assay condition. Uninoculated pepper plants kept at 35°C did not show any significant change in grown compared to those maintained at 25°C after 12 days. Plants inoculated with NRB

isolates only showed HR lesions on inoculated leaves at 25ºC, whereas plants were systemically infected at 35ºC. Plants inoculated with RB isolates were systemically infected at both 25ºC and 35ºC and without HR lesions in inoculated leaves. In all cases, systemic symptoms consisted in chlorosis and stunting but none showed the apical necrosis observed in field peppers (data not shown).

Synergism between NRB and RB isolates of TSWV in resistant pepper plants

Pepper plants carrying the *Tsw* resistance gene were co-inoculated with pairs of RB + NRB isolates to assess whether mixed infections could be involved in the expression of systemic necrosis. Plants were grown at constant temperature of 25ºC to maintain activated the HR. These plants showed local lesions on inoculated leaves and systemic infection, which in several plants (Table 1) consisted in the typical chlorosis and stunting (as shown in Figure 2B), whereas a clear systemic necrosis was observed in other plants (as shown in Figure 2A). The proportion of infected pepper plants showing systemic necrosis for each RB + NRB combination was: Can2PC2 + Mon1NL2 (0/10), Can2PC2 + Da1NL2 (3/10), Can2PC2 + GRAU (4/6), Mu2PC2 + Mon1NL2 (10/10), Mu2PC2 + Da1NL2 (5/10) and Mu2PC2 + GRAU (1/10). Furthermore, new lots of 10 pepper resistant plants inoculated with leaf extracts from eight different pepper plants showing systemic necrosis reproduced the segregation of the two types of symptoms previously described (data not shown).

Identification of TSWV isolates present in co-inoculated pepper plants

To confirm the presence of RB + NRB isolates on co-inoculated resistant peppers, the Mu2PC2 + Da1NL2 combination was randomly selected and upper leaves showing necrotic symptoms were subjected to RFLP and sequence analysis. For this purpose, the primer pairs M1F/M1R and S1F/S1R were designed to amplify, respectively, the last

692 nt of the M segment and a specific region of 545 nt into the NSs gene (avirulence determinant) of the S segment. Amplified products of M and S segments from samples inoculated with Da1NL2 or Mu2PC2 isolates alone were digested with *Eco*RI. The fragment amplified of the M segment from Da1NL2 inoculated plants resulted into two bands of 93 bp and 599 bp each after digestion with *Eco*RI (Figure 3A, lane 1), whereas no digestion bands were obtained for the fragment amplified from the S segment with this same enzyme (Figure 3B, lane 1). In contrast, the fragment amplified of the M segment from Mu2PC2 inoculated plants was not digested with *Eco*RI (Figure 3A, lane 2), whereas two bands of 127 bp and 418 bp each were obtained after digestion of the fragment amplified from the S segment (Figure 3B, lane 2).

In resistant peppers co-inoculated with the combination of isolates Mu2PC2 + Da1NL2 only the M and S segments corresponding to the Mu2PC2 isolate were detected at 12 dpi by RFLP assays (Figure 3A, lanes 3-7 and Figure 3B, lanes 3-7, respectively). Sequence analysis of these RT-PCR products from five plants confirmed that only Mu2PC2 isolate was present in the upper leaves (data not shown).

On the other hand, leaf extracts from resistant peppers co-inoculated with the combination of isolates Mu2PC2 + Da1NL2 were inoculated on *N .glutinosa* plants to obtain biological clones. Thirty biological clones were analyzed by inoculation on resistant pepper plants which induced diverse symptomatology: two clones induced the typical symptoms of NRB isolates characterized by local lesions on inoculated leaves (HR), 19 clones induced the typical symptoms of RB isolates characterized by chlorosis and stunting and nine clones reproduced the severe systemic necrosis (Figure 4, left, middle and right, respectively). Sequence comparison analysis of the RT-PCR products from *N. benthamiana* plants infected with the two clones that only induced HR showed

a 100% homology with the M and S segments of the Da1NL2 NRB isolate (data not shown).

Discussion

Pepper plants carrying the *Tsw* resistance gene showing unusual severe systemic necrosis were collected in a field survey during 2009 growing season in north-eastern Spain. Similar symptoms were reproduced when leaves extracts of these samples were directly inoculated on resistant peppers plants under controlled conditions. After ruling out the presence of other viruses in the field samples, we concluded that these symptoms could be due to the existence of infrequent TSWV RB isolates. These isolates would produce necrotic lesions on the upper uninoculated leaves as consequence of an inefficient HR, as it has been previously described (Moury *et al.* 1997; Margaria *et al.* 2007). However, TSWV biological clones obtained from field infected samples segregated into two types of different symptoms in resistant peppers: (i) the typical symptomatology caused by RB isolates characterized by chlorosis and stunting, and (ii) the severe systemic necrosis described above. This segregation suggested the possible existence of mixed infection with different types of TSWV isolates in the original field samples.

The influence of elevated temperatures in TSWV HR has been widely studied (Black *et al.* 1991; Gil and Luis 1994; Moury *et al.* 1997 and 1998; Soler *et al.* 1998 and 1999), but inconclusive results have been reported. One explanation could be that these experiments were performed by alternating high and low temperatures according to daily cycles (night/day). In fact, tests conducted by Roggero *et al.* (1996) at constant temperature of 33ºC showed that resistant pepper plants developed systemic infection after inoculation with wild-type isolates, while all the inoculated plants grown at lower

301 temperatures of 18 to 24ºC showed necrotic local lesions by HR independently of plant
302 age. For this reason, HR evaluation in our study was performed using regimes of
303 constant temperature at 25ºC or 35ºC, which provided homogeneous results on each
304 repetition. We found that HR was inactivated at 35ºC on resistant peppers and both
305 NRB and RB isolates induced indistinguishable systemic infection, whereas at 25ºC,
306 HR blocked the multiplication of NRB isolates on inoculated leaves. Considering these
307 results, the systemic necrosis observed at constant temperature of 25ºC in some resistant
308 pepper plants, after co-inoculation with mixtures of RB + NRB isolates, should not be a
309 consequence of HR inactivation against the NRB isolate. These symptoms could
310 suggest the presence of the NRB isolate on uninoculated apical leaves. However, the
311 detection of the NRB isolate in several uninoculated apical leaves of resistant peppers
312 co-inoculated with RB + NRB isolates was not possible by RT-PCR-RFLP assays and
313 nucleotide sequence analysis. In both cases, it was only possible to detect M and S
314 segments of the RB isolate. It should be noted that RT-PCR-RFLP assays are useful for
315 determining the prevalence of one isolate in a mixture of isolates, but fails to detect one
316 isolate when its presence in the mixture is at very low title (Aramburu *et al.* 2010).
317 Finally, the presence of the NRB isolate in the uninoculated apical leaves of resistant
318 peppers was demonstrated using biological clones from individual local lesions
319 obtained on *N. glutinosa.* These biological clones were obtained after mechanical
320 inoculation of extracts from apical necrotic leaves, followed by sub-culturing in the non
321 selective host *N. benthamiana* to promote viral replication. Of a total of thirty clones,
322 only two of them induced the typical symptoms of the NRB isolates consisting in HR on
323 inoculated leaves and absence of systemic infection. The nucleotide sequence analysis
324 of M and S segments from these two clones confirmed that both belonged to the NRB
325 isolate.

The present study shows that NRB isolates can infect resistant peppers in presence of RB isolates inducing severe symptoms consisting in systemic necrosis due to HR. Our assays demonstrate that this type of synergistic interaction would occur frequently and besides, it is facilitated when NRB isolates were inoculated 3 to 6 days after the inoculation with RB isolates (data not shown). The continuous effect of HR in the whole plant against the NRB isolate causes systemic necrosis and sometimes collapse, which indirectly affects the multiplication of the RB isolate as well. The low proportion of the NRB isolates in resistant pepper plants infected with a mixture of RB + NRB isolates could have been the cause of some erroneous conclusions. Margaria and co-workers (2007) reported the existence of some TSWV RB isolates that induced systemic HR on resistant peppers. Subsequently, these observations were explained as a result of a partial recognition of the avirulence determinant of RB isolates (de Ronde *et al.* 2013), as described for *potato virus* X and the *R*x resistance gene. However, in view of our results, the systemic HR could be also explained as consequence of a mixed infection between RB and NRB isolates, despite the isolates were purified after three serial passages of a single local lesion. Thomas-Carroll and Jones (2003) also showed evidences of a partial reversion of TSWV RB isolates to NRB after five serial passages in susceptible peppers. However, it is highly unlikely that mutations able to break the resistance conferred by the *Tsw* gene repetitively can revert to wild-type behavior after a few passages in a susceptible host. This result could be also explained by a random selection of NRB isolates after several passages in a non selective host from a mixture of RB + NRB isolates able to break the *Tsw* resistance due to the synergistic interaction. Resistant pepper plants co-infected with both types of isolates showing severe symtomatology could have epidemiological implications. As consequence of a

continuous HR in whole plant the virus multiplication, the acquisition by thrips feeding or even the insect reproduction could decrease the secondary spread of the virus.

Although synergistic interactions are known to be produced predominantly by unrelated viruses that infect the same host, for example breakdown of resistance to TSWV in tomato (García-Cano *et al.* 2006), they have also been reported for more or less closely related virus species belonging to the same family (Syller, 2012). However, to our knowledge, this is the first description of a synergic interaction occurring between isolates of the same virus species. Synergistic interactions have a facilitative effect on both, or at least one of the viruses, manifested by an increase in virus(es) replication (Syller, 2012). This is true in our case, since NRB isolates are able to infect systemically pepper plants with the help of RB isolates. However, in a second phase HR triggered by the NRB isolates, indirectly hampers the replication of RB isolates due to widespread necrosis, therefore, this interaction become antagonistic for RB isolates.

Currently, the exact mechanism that makes possible this interaction remains unknown. It could be consequence of a reduced HR, equivalent to the amount of elicitor, which in a RB + NRB mixture would be only provided by the NRB isolate. This insufficient HR could trigger programmed cell death, not fast enough to block the multiplication of the virus mixture into the local lesions, which would allow that a few NRB virions continuously escape from the restriction area inducing a systemic HR in the whole plant.

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Figure legends

Figure 1. Field resistant pepper plant showing unusual severe symptoms of TSWV characterized by local lesions, chlorosis and apical necrosis.

Figure 2. Symptoms in resistant pepper plants after inoculation with biological clones of TSWV obtained from field samples. **A,** Image showing local lesions on inoculated leaves, chlorosis, stunting and local necrosis on uninoculated leaves and stem collapse at 21 dpi. **B,** image showing chlorosis and stunting of apical leaves at 10 dpi.

Figure 3. RT-PCR-RFLP pattern of M (**A**) and S (**B**) segments amplified from apical necrotic leaves samples of resistant peppers inoculated with TSWV Mu2PC2, Da1NL2 or a mixture of both isolates. Lane M: 100 bp DNA ladder marker; lanes 1 and 2 *Eco*RI digestion products of Da1NL2 and Mu2PC2 isolates respectively; lanes 3–7 *Eco*RI digestion products of Mu2PC2 + Da1NL2 co-inoculation.

Figure 4. Detail of symptoms in leaves of resistant pepper inoculated with TSWV isolates and maintained at constant temperature of 25ºC. **Left,** local lesions induced by HR in inoculated leaves with a NRB isolate at 6 dpi. **Middle,** chlorosis at 10 dpi on apical uninoculated leaves caused by systemic infection of an RB isolate. **Right,** chlorosis and necrotic lesions on apical uninoculated leaves induced by systemic infection of a mixture of RB + NRB isolates at 10 dpi.

Table 1. Symptoms on uninoculated upper leaves of *N. benthamiana* and pepper plants 12 days post inoculation singly with resistance-breaking (RB) and non-resistance-breaking (NRB) isolates or co-inoculated with different pairs of RB + NRB isolates of *Tomato spotted wilt virus* (TSWV).

TSWV isolates[a]	*N. benthamiana*	Delfos[b]	Segura[c]	
RB	c, s	c, s	c, s	
NRB	c, s	c, s	ll	
Can2PC2+Mon1NL2	c, s	c, s	10/10 ll, c, s	
Can2PC2+Da1NL2	c, s	c, s	7/10 ll, c, s	3/10 ll, c, s, sn
Can2PC2+GRAU	c, s	c, s	2/6 ll, c, s	4/6 ll, c, s, sn
Mu2PC2+Mon1NL2	c, s	c, s		10/10 ll, c, s, sn
Mu2PC2+Da1NL2	c, s	c, s	5/10 ll, c, s	5/10 ll, c, s, sn
Mu2PC2+GRAU	c, s	c, s	9/10 ll, c, s	1/10 ll, c, s, sn

[a] RB, resistance-breaking isolates = Can2PC2 and Mu2PC2 and NRB, non-resistance-breaking isolates = Mon1NL2, Da1NL2 and GRAU.
[b] Pepper hybrid susceptible to TSWV.
[c] Pepper hybrid carrying the *Tsw* gene.
Number of infected plants/number of co-inoculated plants.
Legend: c = chlorosis, s = stunting, ll = local lesions on inoculated leaves, sn = systemic necrosis.

Figure 1. Aramburu et al., 2015

Figure 2. Aramburu et al., 2015

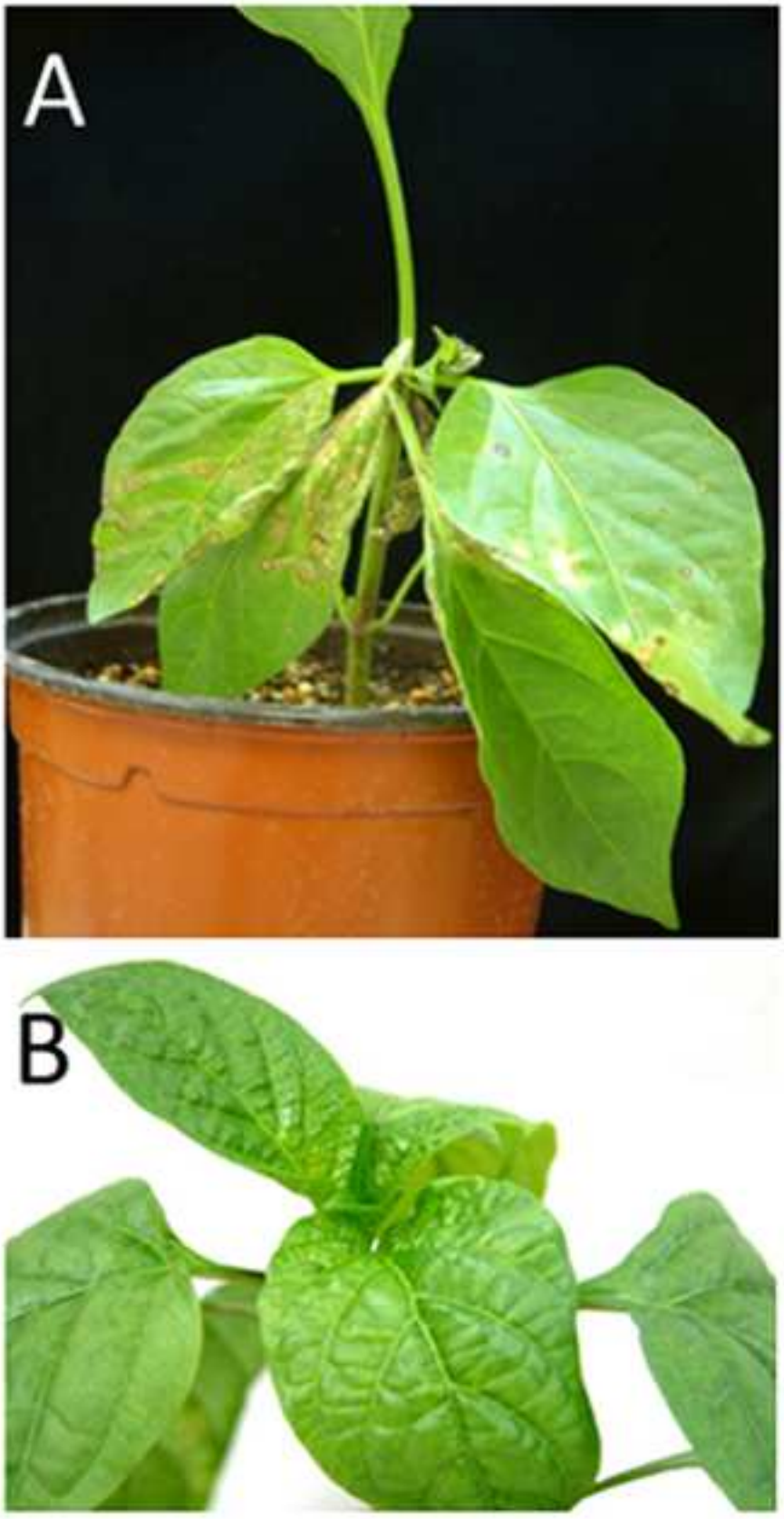

Figure 3. Aramburu et al., 2015

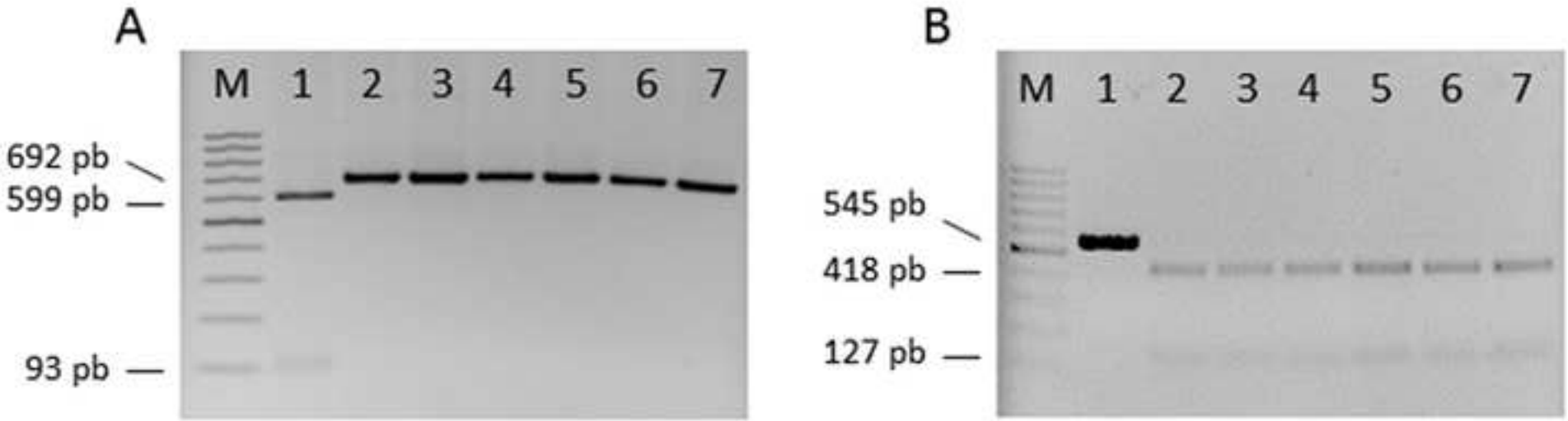

Figure 4. Aramburu et al., 2015