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A new *Capsicum baccatum* accession shows tolerance to wild-type and resistance-breaking isolates of *Tomato spotted wilt virus*

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Running head: Resistance and tolerance of a new pepper accession to TSWV

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Abstract

Tomato spotted wilt virus (TSWV) causes economically important losses in many crops, worldwide. In pepper (*Capsicum annuum*), the best method for disease control has been breeding resistant cultivars by introgression of gene *Tsw* from *C. chinense*. However, this resistance has two drawbacks: I) it is not efficient if plants are infected at early growth stages and under prolonged high temperatures, and II) it is rapidly overcome by TSWV evolution. In this work, we selected and evaluated a new accession from *C. baccattum*, named PIM26-1, by using a novel approach consisting in measuring how three parameters related to virus infection changed over time, in comparison to a susceptible pepper variety (Negral) and a resistant (with *Tsw*) accession (PI-159236): 1) The level of resistance to virus accumulation was estimated as an opposite to absolute fitness, $W=e^r$, being r the viral multiplication rate calculated by quantitative RT-PCR; 2); the level of resistance to virus infection was estimated as the Kaplan-Meier survival time for no infection by using DAS-ELISA to identify TSWV-infected plants; 3) the level of tolerance was estimated as the Kaplan-Meier survival time for no appearance of severe symptoms. Our results showed that the levels of both resistance parameters against TSWV wild type (WT) and *Tsw*-resistance breaking (TBR) isolates were higher in PIM26-1 than in the susceptible pepper variety Negral and similar to the resistant variety PI-159236 against the TBR isolate. However, PIM26-1 showed a very high tolerance (none of the plants developed severe symptoms) to the WT and TBR isolates in contrast to Negral for WT and TBR or PI-159236 for TBR (most TSWV-inoculated plants developed severe symptoms). All this indicate that the new accession PIM26-1 is a good candidate for breeding programs to avoid damages caused by TSWV TBR isolates in pepper.

Introduction

Tomato spotted wilt virus (TSWV), the type member of the genus *Tospovirus* of the family *Bunyaviridae*, is one of the most widespread and economically important plant virus affecting many crops such as tomato, pepper, potato, tobacco, peanut, lettuce, bean and ornamental species (Pappu et al. 2009, Turina et al. 2012). TSWV has a wide host range including more than 1000 species and is transmitted in a persistent manner by several thrips species (*Thysanoptera*: *Thripidae*), with *Frankliniella occidentalis* (Pergande) being its main vector (Debreczeni et al. 2014, Whitfield et al. 2005).

TSWV virions are quasi-spherical composed of an outer membrane envelope derived from the host, with two embedded viral-coded glycoproteins (G_N and G_C). Inside there are several copies of the RNA dependent RNA polymerase (RdRp) and nucleoproteines which encapsidate the genome consisting of three negative-sense or ambisense RNA segments: Segment L (~9 kb) encodes a putative RNA-dependent RNA polymerase; segment M (~5 kb) encodes the cell-to-cell movement protein, NSm, and the precursor of surface glycoproteins, G_N/G_C, involved in TSWV transmission by thrips; and segment S (~3 kb) encodes a silencing suppressor, NSs, and the nucleocapsid, N (Plyusnin et al. 2012).

In pepper (*Capsicum annuum*), symptoms caused by TSWV infection vary depending on host genotype and include: stunting of the whole plant, chlorosis and necrosis of the new growth, apical downward leaf curling, mosaic or necrotic lesions on leaves, stems and fruits. The disease can cause the death of the plant or drastically reduce the proportion of marketable fruits (Boiteux 1995, Moury and Verdin 2012, Soler et al. 1998).

Introgression of genes conferring resistance or tolerance against viruses in commercial cultivars from wild relatives by plant breeding is considered the most efficient and simplest strategy for viral disease control, despite of being a long and costly process (Lecoq et al. 2004). Resistance is considered a host characteristic hindering virus infection and/or multiplication, whereas tolerance is considered a host characteristic allowing systemic viral infection while developing milder symptoms than those of more sensitive hosts.

In spite of great efforts and investments in pepper breeding programs, in over seven decades only the gene *Tsw*, identified in several *Capsicum chinense* accessions and mapped to the chromosome 10, was found to confer resistance against a wide spectrum of TSWV isolates (Jahn et al. 2000).

Plants carrying the gene *Tsw* inoculated with TSWV show a hypersensitive response (HR) consisting of a rapid plant cell death in and around the virus entry points to halt cell-to-cell viral movement and avoid systemic infection (Soler et al. 1999). This is manifested as discrete necrotic lesions followed by abscission of the inoculated leaves (Boiteux 1995).

However, *Tsw* fails to confer resistance in plants inoculated at early stages of development and subjected to prolonged high temperatures (>30ºC)(Moury et al. 1998, Soler et al. 1998, Soler et al. 1999). Another problem is due to the high evolutionary and adaptative capacity of TSWV (López et al. 2011, Tentchev et al. 2011, Tsompana et al. 2005) that allowed the emergence of resistance breaking isolates in many areas where resistant cultivars have been grown (Boiteux and Nagata 1993, Hobbs et al. 1994, Margaria et al. 2004, Roggero et al. 2002, Thomas□Carroll and Jones 2003).

The incomplete effectiveness of the gene *Tsw* in pepper, and the great ability of the virus to generate new virulent isolates have imposed the need to seek and evaluate new sources of resistance or tolerance to TSWV. Although, most breeding programs are aimed to find and implement absolute resistance (no viral infection), considering degrees of resistance (reduction of virus infectivity and/or multiplication) and/or tolerance (reduction of symptom severity) may be useful to rescue valuable phenotypes. This requires developing new analytical tools to asses the level of resistance and tolerance.

In this work, a new accession of *C. baccatum*, PIM26-1, was evaluated for resistance and tolerance to TSWV by measuring how different parameters related to the viral infection changed over time. This accession did not suppose an improvement in terms of resistance with respect to the accessions or cultivars with the gene *Tsw*. However, PIM26-1 was very tolerant not only to TSWV wild type (WT) but also to *Tsw*-resistance-breaking (TBR) isolates, which induce strong symptoms and damage in cultivars carrying the gene *Tsw*.

Material and methods

Plants and viruses

Three pepper accessions were selected from the germplasm collection from Institute for Conservation and Improvement of Valencian Agrodiversity (COMAV) in Valencia, Spain: PIM26-1 from *C. baccatum* L. (the new accession), PI-159236 from *C. chinense* (containing

gene *Tsw*), which was used as a resistant control, and Negral from *C. annuum*, which was used as a susceptible and sensitive control.

Four TSWV isolates were recovered from a collection of biologically characterized TSWV isolates obtained from pepper fields in Eastern Spain (Debreczeni et al. 2014), which corresponded to two biotypes: Da1NL2, of biotype WT, Pilar 1, Alm1 and PC916, of biotype TRB.

Mechanical inoculation of TSWV was performed by grinding 2 g of TSWV-infected tomato leaf tissue in 20 ml of sodium phosphate buffer 0.1 M (pH 7), containing 0.2% of sodium diethyldithiocarbamate trihydrate (DIECA) and 0.2% of carborundum (600 mesh). This preparation was rubbed with cotton-bud sticks to pepper plants with the sixth leaf fully expanded. Some plants were inoculated only with phosphate buffer and carborundum (mock-inoculation) or not inoculated to be used as negative controls and identify possible pathological effects caused by their cultivation in the growth room.

Plants were maintained in a growth room with controlled environmental conditions of 25°C/18°C day/night temperature, 60%/95% day/night relative humidity, and 60-85 μmol s^{-1} m^{-2} of irradiance from Sylvania Grolux fluorescent tubes, and a 14 h-10 h light/dark photoperiod.

Evaluation of parameters related to viral infection

Viral titer in plants was estimated by reverse transcription and quantitative polymerase chain reaction (RT-qPCR) of total RNAs with primers 1M_F (5'-CCAACATGCCATCTGAAAAGC-3') and 1M_R (5'-CAAATGCAGCTGACAGCAGTTT-3') and the TaqMan®MGB probe P_U (5'-6FAM-TCTGAACTGGTCTATTCC-3'). Total RNAs from 0.1 g of fresh leaf tissue from TSWV-infected and non-infected plants were purified by using a phenol-chloroform protocol eluted in 20 μl of RNase-free water, treated with RNase-free DNase(Turbo DNA-free, Ambion, Applied Biosystems, Austin, TX, USA), measured in duplicate with the UV-Vis spectrophotometer Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA) and adjusted to 10 ng/μl to normalize the different extractions (Debreczeni et al. 2011).

RT-qPCR was performed in a LightCycler®480 (Roche Molecular Diagnostics, Indianapolis, IN, USA) using 25 μL of a reaction mix containing 12.5 μL LightCycler®480 Probe Master Mix (ROCHE), 4.38 μL of RNase-free water, 15 U RT Multiscribe

ReverseTranscriptase (Life Technologies, Rockville, MD, USA), 2 U of RNase inhibitor (Applied Biosystems, Foster City, CA, USA), 5 µM of each primer, 0.25 µM TaqMan®MGB probe and 5 µL of total RNA. Cycling conditions consisted of reverse transcription at 48ºC for 30 min, incubation at 95ºC for 10 min and 45 cycles of 95ºC for 15 s and 60ºC for 1 min. For absolute quantification (number of viral RNA molecules per ng of total RNA) a standard curve with serial dilutions of TSWV transcripts was used (Debreczeni et al. 2011).

TSWV-infected plants were identified by double-antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) with polyclonal antibodies (Loewe Biochemica GmbH, Sauerlach, Germany) by following the standard protocol (Clark and Adams 1977) with some modifications (Soler et al. 1999). Absorbance after serological reactions was measured at 405 nm with a microplate reader (model 550, Biorad, Hercules, California, USA). A sample was considered positive (infected) when the absorbance was higher than the mean absorbance of the blank controls (obtained from mock- or non-infected plants) plus three times the standard .deviation.

Symptoms were visually evaluated and plants were classified into: asymptomatic, mild and severe based on the degree of stunting, and leaf yellowing and distortion with respect to mock-inoculated or non-infected plants.

Biological assays

In preliminary assays, the three pepper accessions were inoculated with the four TSWV isolates (10 plants per accession and isolate). At 15 days post inoculation (dpi), infectivity (proportion of infected plants) was determined by DAS-ELISA and symptoms (proportion of plants with severe symptoms) were evaluated by visual observation.

In the final assay, leaf extracts of TSWV isolates Da1NL2 (WT) and Alm1 (TRB) were quantified by RT-qPCR, equalized to a concentration of 3×10^6 copies of viral RNA copies per ng of total RNA and mechanically inoculated to the three pepper accessions: Negral (susceptible), PI-159236 (with the resistance gene *Tsw*) and PIM26-1 (new accession) by using 200 µl of inoculum per plant. In total 180 plants were inoculated (30 plants per accession and isolate), 6 plants were mock-inoculated (2 plants per accession) and 6 plants were non-inoculated (2 plants per accession). At 7, 14, 21 and 28 days post inoculation (dpi), every plant was evaluated for symptoms and samples from the youngest leaves (not inoculated) were collected for each plant (768 samples) and analyzed by DAS-ELISA to

detect TSWV infection (Table 1). Another part of the samples were used for estimation of virus titer by RT-qPCR from pools of five plants per accession and isolate to obtain six biological replicates (300 pooled samples). Per each sample two RT-qPCR replicates were performed.

Statistical analysis

Resistance and tolerance were evaluated as the host response to virus infection over time (in four periods: 7, 14, 21 and 28 dpi), depending upon two factors: viral biotype (WT and TBR) and plant genotype (Negral, PI-159236 and PIM26-1).

Resistance was estimated from two variables: viral accumulation. The first variable was viral accumulation. Since the host exerts a pressure against TSWV accumulation, absolute fitness (*W*) as an inverse measure of the resistance level of each pepper genotype was used. In evolutionary biology, *W* measures the total number of surviving offspring of an individual or genotype in a given environment (Moya et al. 2004, Orr 2009). In the present study, we were not interested in comparing different viral genotypes in an environment but the performance of each viral genotype in different environments (pepper accessions) (Peña et al. 2014). *W* is calculated as $W = e^r$, where *r*, the Malthusian growth rate, is a normalized measure of the rate of virus accumulation, which was estimated as the slope of the lineal regression of the log-transformed values of the viral titer measured by RT-qPCR (log[number of viral RNA molecules +1]) over time (7, 14, 21 and 28 dpi). Data was obtained from six groups of five pooled plants (six replicates) per each viral biotype, plant genotype and time. *W* was analyzed by using a Generalized Linear Model (Molenberghs and Verbeke 2005), assuming that *W* follows a Gamma distribution and applying a long-link function (Hillung et al. 2013). Differences among treatment means were evaluated using mean standard errors, and a Bonferroni correction (Bonferroni 1936) was applied to protect against type I error.

The second variable to measure resistance was the variation over time (7, 14, 21 and 28 dpi) of the survival to viral infection (proportion of non-infected plants determined by DAS-ELISA) whose distribution was estimated with Kaplan-Meier survival curves (Kaplan and Meier 1958). Log-rank test (Peto and Peto 1972) with the Bonferroni correction (Bonferroni 1936) was used to compare survival distributions. The median survival time *Imd* (in which half of the inoculated plants were not infected) and the mean survival time *Im* (in which a single plant is expected to remain no infected) were used as measures of the

resistance level to viral infection. Data were collected from 30 plants (replicates) per each viral biotype, plant genotype and time.

Tolerance was estimated as the opposite to symptom development over time. Kaplan-Meier survival analysis was used to evaluate the development of severe symptoms. The survival median time *Smd* (in which half of the plants did not present severe symptoms) and the mean survival time *Sm* (in which a single plant is expected to remain without severe symptoms) were used as a measure of the tolerance level. Data were collected from 30 plants (replicates) per each viral biotype, plant genotype and time.

All analyses were performed with *R Statistical* Software (http://www.r-project.org/) by using the packages: survival, stats and multcomp.

Results

In a preliminary assay, four TSWV isolates: PC-916 (biotype TBR), Pilar1 (TBR), Da1NL2 (WT) and Ramiro1 (TBR), whose titers were unknown, were inoculated in the pepper accessions: PIM26-1 (new accession), Negral (susceptible control) and PI-159236 (containing the resistance gene *Tsw*). At 15 dpi, all plants of Negral inoculated with the four isolates resulted infected, with most of them displaying severe symptoms (60, 80, 100 and 100% for isolates PC-916, Pilar1, Da1NL2 and Ramiro1, respectively). As expected none of PI-159236 plants become infected with Da1NL2 as expected whereas most of them were infected with the TBR isolates (90, 100 and 100% for PC-916, Pilar1 and Ramiro1, respectively), with a variable number of plants showing severe symptoms (10, 90 and 40% for PC-916, Pilar1 and Ramiro1, respectively). The number of infected PIM26-1 plants was variable (10, 90, 20 and 80% for PC-916, Pilar1, Da1NL2 and Ramiro1, respectively) but none of these plants showed severe symptoms.

For a precise evaluation of resistance and tolerance, an assay was performed by inoculating equimolar quantities of TSWV isolates Da1NL2 (WT) and Ramiro1 (TBR) in three pepper accessions (PIM26-1, PI-159236 and Negral), and measuring overtime the viral titer by RT-qPCR, infectivity (proportion of infected plants) by ELISA and symptoms (proportion of plants with mild and severe symptoms).

1) Resistance measured as opposition to virus multiplication. TSWV multiplication was estimated for the WT (Da1NL2) and TBR (Ramiro1) isolates (Table 1 and Fig. 1). Except for the WT isolate, which did not infect the cultivar PI-159236 (with the resistance

gene *Tsw*), both TSWV isolates showed an accumulation pattern consisting of an exponential increase of viral titer reaching a maximum peak (mean titer= 2.8×10^6 viral molecules) at 21 dpi, followed by a decrease (mean titer 2.8×10^4 viral molecules) at 28 dpi.

Virus accumulation in the susceptible cultivar Negral occurred faster than in the other two pepper genotypes (PIM26-1 and PI-159236). Thus, viral titer in Negral was 7.5×10^3 and 8.9×10^4 for isolates WT and TRB, respectively; at 7 dpi whereas no accumulation was detected for the other two pepper accession at that time (Fig. 1). TSWV reached a peak at 21 dpi in the three pepper accessions: Negral (1.7×10^6 and 4.4×10^4 for WT and TRB isolates, respectively), PI-159236 (5.8×10^6 for isolate TRB) and PIM26-1 (5.8×10^5 and 1.7×10^6 for WT and TRB isolates, respectively).

Absolute fitness was used as an opposed measure of the resistance level. Statistical analysis showed different resistance levels against isolate WT for the three pepper varieties (Table 2). As expected, the lowest *W* value was for PI-159236 containing the gen *Tsw* that confers absolute resistance to TSWV WT isolates (none of the plants were infected and therefore the accumulation remained zero over time). PIM26-1 had a lower *W* value (and therefore a higher resistance level) than the susceptible control Negral. With respect to the TRB isolate, both PI-159236 and PIM26-1 showed similar levels of resistance which were significantly higher than that of Negral.

2) Resistance measured as survival to virus infection. Fig. 2 shows Kaplan-Meier survival curves (proportion of plants non-infected by TSWV). Survival (probability of no infection) of the WT isolate in PI-159236 remained 100% since no plant became infected. This was expected since PI-159236 contains the gene *Tsw* conferring resistance against WT isolates. In the other two pepper genotypes survival decreased over time but being faster in the susceptible Negral (less than 0.5% of the plants remained non-infected at 21 dpi) than in the new accession, PIM26-1 (about 80% were not-infected at 21 dpi).

For the TBR isolate the three pepper genotypes became infected (Fig. 2). The most susceptible was Negral (all plants infected at 14 dpi) and survival decreased faster in PI-159236 (30% survival at 21 dpi) than in PIM26-1 (50% survival at 21 dpi).The median (*Imd*) and mean (*Im*) survival time for each TSWV biotype and pepper accession were used measures of the resistance level to viral infection (Table 3). As expected, both *Imd and Im* of the WT isolate in PI-159236 could not be calculated (higher than the time used in this assay) since this pepper genotype presents absolute resistance and none of the plants became

infected. The new accession, PIM26-1 showed much higher level of resistance (*Im*= 26.1) than that of Negral (*Im*= 14.2). Regarding the TRB isolate, the survival times for PI-159236 and PIM26-1 were not significantly different with *Imd* and *Im* values much higher than those for Negral.

3) Tolerance. The probability of plants showing no severe symptoms was evaluated for each time. As expected, all PI-159236 plants inoculated with the WT isolate remained asymptomatic since they were not infected due to their absolute resistance. Some Negral plants inoculated with the WT isolate showed severe symptoms at 7 dpi and the probability of showing severe symptoms increased over time (about 80% at 21 dpi and 100% at 28 dpi). In contrast, the new accession PIM26-1 inoculated with the WT isolate underwent a very slow increase of the number of plants with mild symptoms reaching less than 20% at 28 dpi (Table 1)and none of the plants developed severe symptoms (Fig. 3).

For the TRB isolate, Negral showed a similar response to the WT isolate with more than 80% of plants with severe symptoms after 21 dpi. PI-159236 showed a higher level of tolerance than Negral, with a lower number of plants with symptoms (about 30% mild and about 50% severe at 21 dpi) but almost all plants of both (Negral and PI-159236) had severe symptoms at 28 dpi. In contrast, PIM26-1 never developed severe symptoms, although 70% of these plants had mild symptoms at 28 dpi (Table 1 and Fig. 3).

Regarding the WT isolate, Negral showed a very low level of tolerance whereas PI-159236 and PIM26-1 showed absolute tolerance since none of the plants developed severe symptoms (Table 4). In this case, PIM26-1 showed a true tolerance since the virus infected and multiplied in the host, in contrast to PI-159236 which was never infected, therefore in this case is not tolerance but resistance. Regarding the TBR isolate, PI-15923 showed a little higher, yet statistically significant, tolerance than Negral whereas PIM26-1 had absolute tolerance (Table 4).

Discussion

Introgression of gene *Tsw* into pepper cultivars by plant breeding has been the best method to control TSWV disease in pepper. This gene confers a complete resistance against TSWV infection, although it is not efficient in some conditions (Moury et al. 1998, Soler et al. 1998, Soler et al. 1999) and not durable since TSWV can overcome this resistance after a few years of exposition (Tentchev et al. 2011).

Most breeders only consider absolute or complete resistance when none of the plants becomes infected. In the few cases that the resistance and tolerance levels were estimated (Galipienso et al. 2013, Rubio et al. 2003), they were usually analyzed by taking measures in a single time time. These can be considered as snapshots of the host response and only provides incomplete and inaccurate information given the dynamic nature of biological processes. Evaluation viral infection and symptoms over time is important since the damage severity is highly correlated with the plant growth stage when the virus and/or symptoms become evident.

In this work, the variation over time of the resistance and tolerance levels were evaluated and integrated. Resistance was evaluated by absolute fitness, *W*, from viral accumulation measured by RT-qPCR and the Kaplan-Meier survival time to viral infection, measured by DAS-ELISA. RT-qPCR is very sensitive and gives a very accurate estimate of viral titer (Debreczeni et al. 2011, Mackay et al. 2002) whereas DAS-ELISA is much less sensitive and, in spite that is considered semi-quantitative, provides a more limited information (in this case ELISA detects the virus if the titer has surpassed a certain threshold). In addition, data from RT-qPCR can be log-transformed to follow a normal distribution which translates into a higher statistical power than the count data from DAS-ELISA. However, RT-qPCR is much more expensive and laborious than DAS-ELISA and limits the number of replicates compared to DAS-ELISA. In the present work, six replicates (groups of five plants) were used for RT-qPCR vs 30 replicates (individual plants) for DAS-ELISA, reaching the same conclusion with both techniques.

Our results showed that the new accession, PIM26-1, has a certain resistance level for viral infection and accumulation against both TSWV biotypes: WT and TBR, much higher than the susceptible Negral, and similar to PI-159236 (with the resistance gene *Tsw*) against the TBR isolate. Therefore the new accession does not represent an improvement with respect to resistance because PI-159236 showed absolute resistance against WT isolates. However, the tolerance of PIM26-1 for both TSWV isolates was very high or absolute (none of the plants developed severe symptoms during the assay) in contrast to Negral for both isolates and PI-15236 for the TBR isolate, which ended with most plants developing severe symptoms.

The high tolerance of PIM26-1 was observed for different isolates in previous assays and in field (data not shown) suggesting that can be valid for a wide spectrum of TSWV

isolates. In our previous work, we found that different TSWV isolates had similar fitness for accumulation in plant (*Datura stramonium*, non-resistant pepper and non-resistant tomato) and transmission by the thrips *Frankliniella occidentalis* (Debreczeni et al. 2011, Debreczeni et al. 2014).

The advantage of tolerance versus resistance is its durability. Resistance exerts a selective pressure favoring mutations increasing fitness in resistant cultivars (higher infectivity and/or multiplication) so that the virus would evolve to overcome the resistance under appropriate conditions (Garcia-Arenal and McDonald 2003). The *Tsw*-based resistance is not durable since TSWV resistance-breaking isolates have been detected in many areas after releasing resistant cultivars. Recently, the TSWV avirulence determinant of the *Tsw*-based resistance has been identified in the gene NSs (de Ronde et al. 2013). Nucleotide analyses suggest that mutations in several sites of this gene could trigger resistance breakdown (Margaria et al. 2007, Tentchev et al. 2011) in opposite to the breakdown of the resistance conferred by gene *Sw-5* in tomato which only one mutation in one of two loci are allowed in TSWV NSm gene (López et al. 2011, Peiró et al. 2014). Also, the loss of efficiency at higher temperatures or at early stages of growth could exert a partial selective pressure reducing virus fitness but allowing multiplication which may favor the emergence of resistance-breaking mutants such as it has been observed for RNA interference-mediated resistance (Lafforgue et al. 2011).

In contrast, true tolerance (without decreasing virus infection and multiplication) can be favored during virus evolution as the plant defenses would have a low negative effect on virus fitness and harming the host would decrease the probability of virus transmission to new plants. According to the avirulence hypothesis, parasites should evolve towards avirulence and the parasite fitness would be related to the host fitness. When virulence is related to virus multiplication, the tradeoff hypothesis suggests that virulence will evolve to a level at which virulence and transmission would balance to maximize the spread of the virus (Alizon et al. 2009). The disadvantage of tolerance vs resistance is from the epidemiological point view because tolerant pepper plants carry the virus that can be transmitted to other crops.

In conclusion, this new accession PIM26-1, obtained from *C. baccatum*, can be used to avoid the damages by TSWV infection including those isolates able to infect pepper cultivars with the gene *Tsw*, widely used for disease control. It would be of great interest to obtain cultivars combining this tolerance with the *Tsw* resistance. This requires further

research to identify the source of tolerance and the feasibility to incorporate it in commercial pepper cultivars.

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Figures and tables

Figure 1. Time-course accumulation (number of viral RNA copies per ng of total RNA) of two TSWV isolates: Da1NL2 (Biotype wild type, WT) and Alm1 (biotype *Tsw* resistance-breaking, TRB) in three pepper accessions: Negral (susceptible), PIM26-1 and PI-159236 (with the resistance gene *Tsw*). Mean values of six replicates per isolate and pepper accession are shown.

Figure 2. Kaplan-Meier survival curves showing the probability of no infection over time for two TSWV isolates: Da1NL2 (Biotype wild type, WT) and Alm1 (biotype *Tsw* resistance-breaking, TRB) and three pepper accessions: Negral (susceptible), PIM26-1 and PI-159236 (with the resistance gene *Tsw*). Thirty replicates were used per isolate and pepperaccession.

Figure 3. Kaplan-Meier survival curves showing the probability of no presence of severe symptoms over time for two TSWV isolates: Da1NL2 (Biotype wild type, WT) and Alm1 (biotype *Tsw* resistance-breaking, TRB) and three pepper accessions: Negral (susceptible), PIM26-1 and PI-159236 (with the resistance gene *Tsw*). Thirty replicates were used per isolate and pepper accession.

485 **Figure 1**

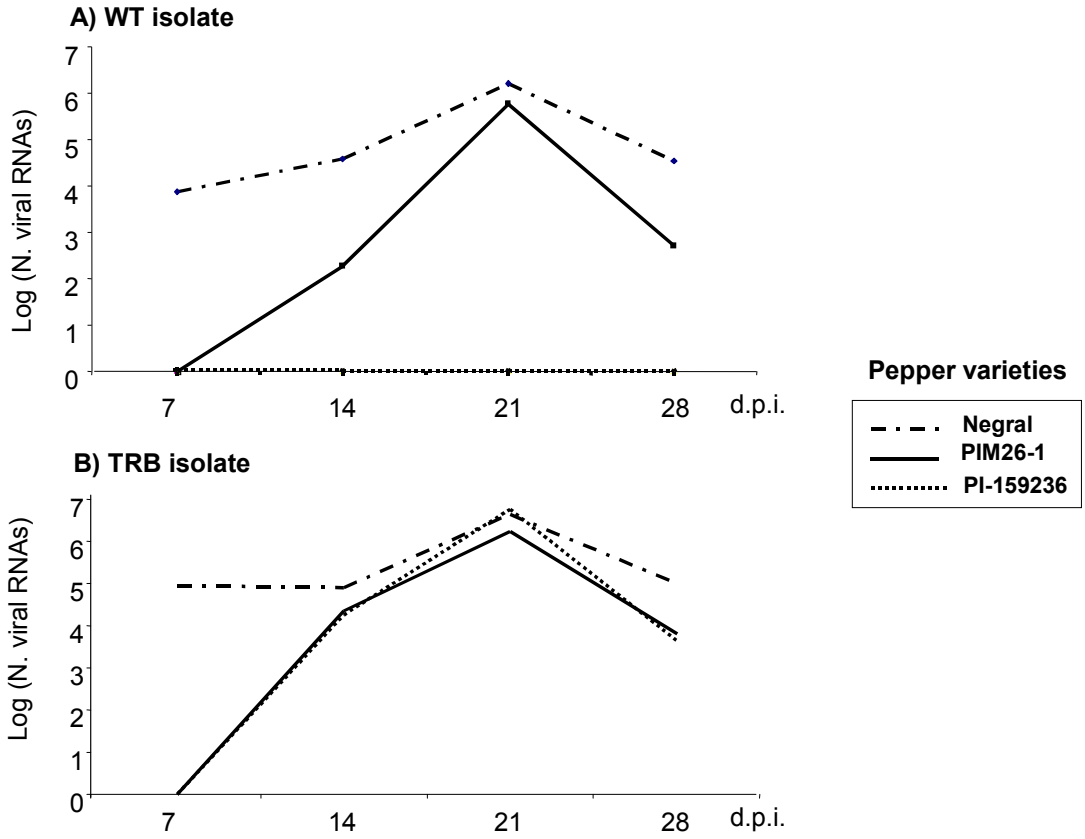

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Figure 2

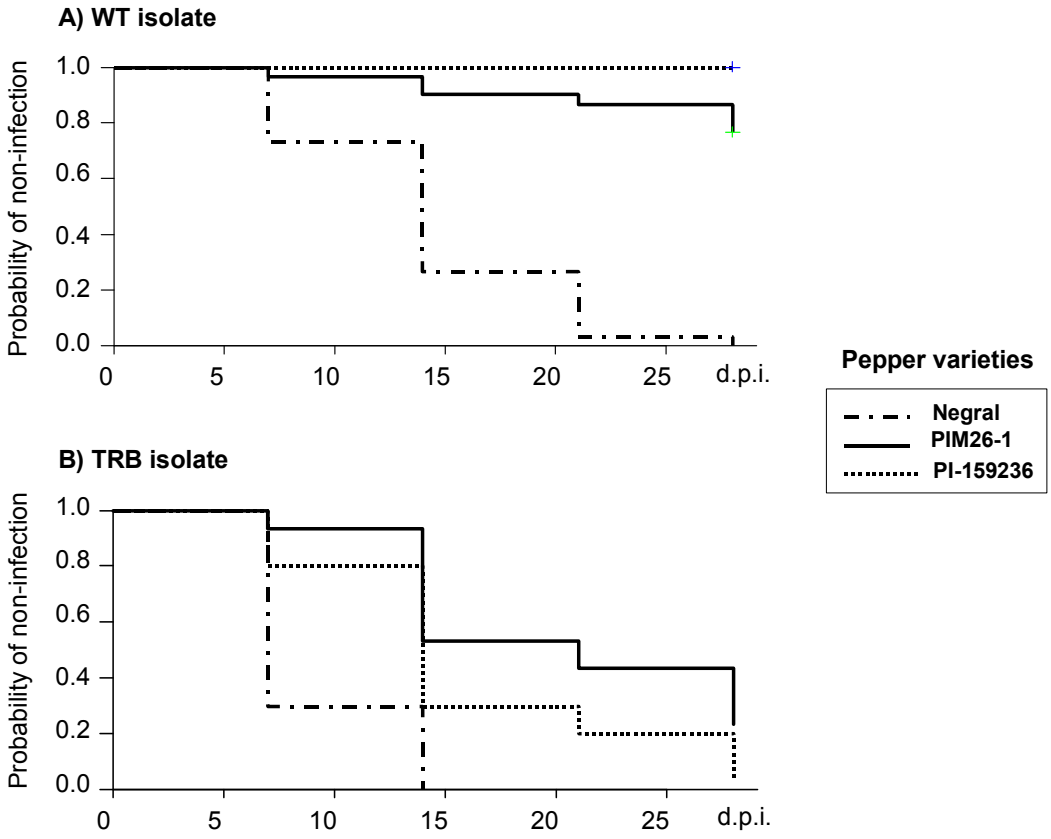
A) WT isolate
Probability of non-infection
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
d.p.i.
B) TRB isolate
Probability of non-infection
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
d.p.i.
Pepper varieties
Negral
PIM26-1
PI-159236

Figure 3

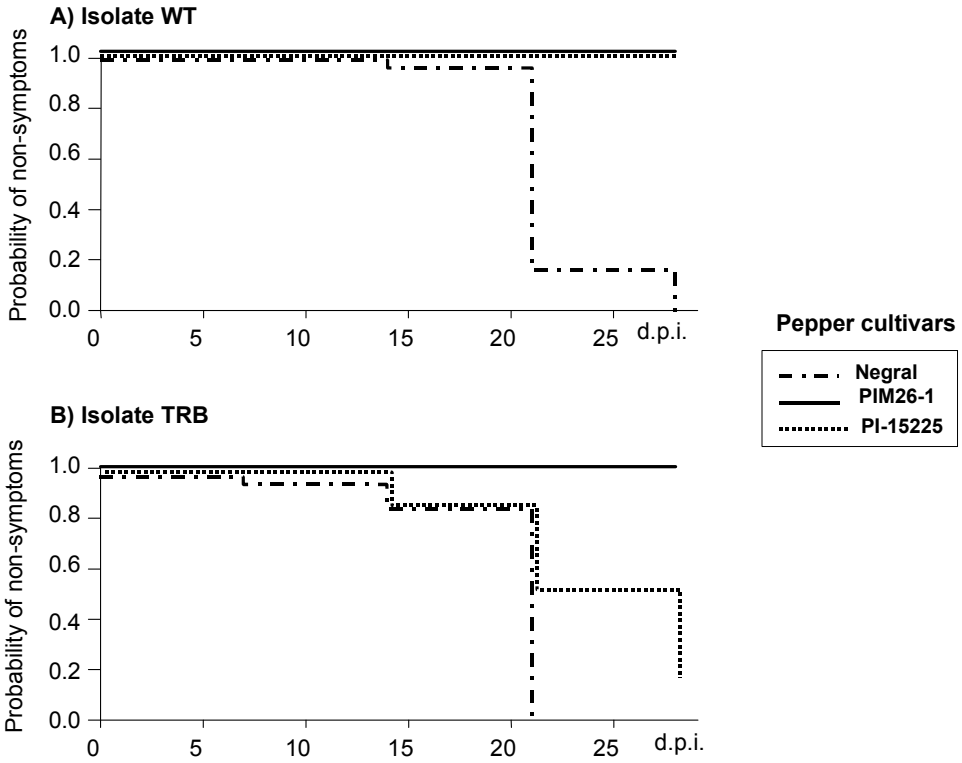
A) Isolate WT
Probability of non-symptoms
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
d.p.i.
B) Isolate TRB
Probability of non-symptoms
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
d.p.i.
Pepper cultivars
Negral
PIM26-1
PI-15225

Table 1. Analysis of three variables over time to evaluate resistance and tolerance of three pepper genotypes to two TSWV biotypes

		Negral[a]			PI-159236[a]			PIM26-1[a]		
		WT[b]	TBR[b]	C[b]	WT[b]	TBR[b]	C[b]	WT[b]	TBR[b]	C[b]
RT-qPCR[c]	**N[d]**	**6**	**6**	**2**	**6**	**6**	**2**	**6**	**6**	**2**
	7 dpi[e]	7.5 ± 4.5^{3}	8.9 ± 2.9^{4}	0	0	0	0	0	0	0
	14 dpi[e]	3.9 ± 0.9^{4}	8.2 ± 0.9^{4}	0	0	1.8 ± 0.6^{4}	0	1.8 ± 1.8^{2}	2.3 ± 1.3^{4}	0
	21 dpi[e]	1.7 ± 0.5^{6}	4.4 ± 0.7^{6}	0	0	5.8 ± 4.2^{6}	0	5.8 ± 2.9^{5}	1.7 ± 0.6^{6}	0
	28 dpi[e]	3.4 ± 1.6^{4}	9.7 ± 2.0^{4}	0	0	4.5 ± 1.8^{3}	0	5.2 ± 5.2^{2}	6.3 ± 5.7^{3}	0
ELISA[c]	**N[d]**	**30**	**30**	**4**	**30**	**30**	**4**	**30**	**30**	**4**
	7 dpi[e]	8	21	0	0	6	0	1	2	0
	14 dpi[e]	22	30	0	0	21	0	3	14	0
	21 dpi[e]	29	30	0	0	24	0	4	17	0
	28 dpi[e]	30	30	0	0	29	0	7	22	0
Symptoms[c]	**N[d]**	**30**	**30**	**4**	**30**	**30**	**4**	**30**	**30**	**4**
	7 dpi[e]	15(0)	23(1)	0	0	3(0)	0	0	0	0
	14 dpi[e]	21(2)	27(4)	0	0	15(4)	0	1(0)	4(0)	0
	21 dpi[e]	30(25)	30(30)	0	0	22(14)	0	3(0)	13(0)	0
	28 dpi[e]	30(30)	30(30)	0	0	30(25)	0	5(0)	21(0)	0

[a]Pepper genotypes: Negral (considered as susceptible), PI-159236 (with the resistance gene *Tsw*) and PIM26-1 (new accession).

[b]Inocula: TSWV isolates of biotype wild type (WT) and *Tsw*-resistance-breaking (TBR) and mock- or non-inoculated controls (C).

[c]Analysis. **RT-qPCR** to evaluate viral accumulation (mean viral titer for 6 replicates, corresponding to 6 groups of 5 plants or two groups of two plants for controls and standard error), **ELISA** to evaluate the number of TSWV-infected plants, and **Symptoms**, number of plants with symptoms (number of plants with severe symptoms is between parentheses) evaluated by visual inspection. Viral accumulation is presented simplified, ex. is $7.5\times10^{3} \pm 4.5\times10^{3}$.

[d]N= number of replicates. **RT-qPCR**: 6 groups of 5 plants for WT and TBR and 2 groups of 2 plants for C, **ELISA** and **symptom**s: 30 individual plants for WT and TBR and 4 for C (2 mock- and 2 non- inoculated).

[e]Time of taking measurements: 7, 14, 21 and 28 days post-inoculation (dpi). Mean number of TSWV RNA molecules (for 6 replicates), number of TSWV-infected plants and number of plants with symptoms or severe symptoms (between parentheses) are indicated for each time.

Table 2. Absolute fitness (*W*) for evaluation of resistance levels to TSWV

TSWV biotype[a]	Pepper variety[b]	*W* (mean)[c]	GLM test[d]
WT	**Negral**	1.239±0.041	A
	PI-159236	1.000±0.000[e]	B
	PIM26-1	1.086±0.063	C
TBR	**Negral**	1.290 0.019	A
	PI-159236	1.204±0.047	B
	PIM26-1	1.157±0.056	B

[a]Three pepper varieties: Negral (susceptible), PI-159236 (with resistance gene *Tsw*) and PIM26-1 (new accession).

[b]Two TSWV isolates: wild type (WT) and *Tsw* resistance breaking (TBR).

[c]Mean and standard error of absolute fitness ($W = e^r$, being r the Malthusian growth rate) for six replicates (6 groups of 5 plants).

[d]For each virus biotype, different letters indicate significant differences according to a Gamma generalized linear model (overall p-value < 0.05 by using Bonferroni correction).

[e]Null accumulation as the virus never infected the host, indicating absolute resistance.

Table 3. Survival time to viral infection for evaluation of resistance levels to TSWV

TSWV biotype[a]	Pepper variety[b]	*Imd* (median)[c]	*Im* (mean)[d]	Log-rank test[e]	Number of infected plants at 28 dpi
WT	**Negral**	14	14.2±1.0	A	30
	PI-159236	N[f]	N[f]	B	0
	PIM26-1	N[f]	26.1±0.9	C	7
TBR	**Negral**	7	9.1±0.6	A	30
	PI-159236	14	16.1±1.3	B	29
	PIM26-1	21	20.3±1.3	B	23

[a]Two TSWV biotypes: wild type (WT) and *Tsw* resistance breaking (TBR)

[b]Three pepper varieties: Negral (susceptible), PI-159236 (with resistance gene *Tsw*) and PIM26-1 (new accession)

[c]*Imi*, median survival time (that in which 50% of the plants remain non-infected) estimated according to a Kaplan-Meier survival analysis for 30 replicates (plants)

[d]*Im*, mean and standard error of survival time (that in which a single plant is expected to remain no infected) estimated according to a Kaplan-Meier survival analysis for 30 replicates (plants).

[e]For each virus biotype, different letters indicate significant differences according to a long-rank test (overall p-value < 0.05 by using Bonferroni correction).

[f]N means it cannot calculated indicating absolute resistance(no plant became infected)or almost absolute resistance.

Table 4. Survival time to severe symptoms for evaluation of tolerance levels to TSWV

TSWV biotype[a]	Pepper variety[b]	*Smd* (median)[c]	*Sm* (mean)[d]	Log-rank test test[e]	Number of plants with severe symptoms at 28 dpi
WT	**Negral**	21	21.9±0.5	A	30
	PI-159236	N[f]	N[f]	B	0
	PIM26-1	N[f]	N[f]	B	0
TBR	**Negral**	21	19.8±0.6	A	30
	PI-159236	28	23.8±0.9	B	25
	PIM26-1	N[f]	N[f]	C	0

[a]Two TSWV biotypes: wild type (WT) and *Tsw* resistance breaking (TBR)

[b]Three pepper varieties: Negral (susceptible), PI-159236 (with resistance gene *Tsw*) and PIM26-1 (new accession)

[c]*Smd*, median survival time (that in which 50% of the plants remain did not developed severe symptoms) estimated according to a Kaplan-Meier survival analysis for 30 replicates (plants).

[d]*Sm*, mean and standard error of survival time (that in which a single plant is expected to remain without severe symptoms) estimated according to a Kaplan-Meier survival analysis for 30 replicates (plants).

[e]For each virus biotype, different letters indicate significant differences according to a long-rank test (overall p-value < 0.05 by using Bonferroni correction).

[f]N means it cannot calculated indicating absolute tolerance (no plant develop severe symptoms)or almost absolute tolerance.