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Screening of pepino (*Solanum muricatum*) and wild relatives against four major tomato diseases threatening its expansion in the Mediterranean region

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SUMMARY

The pepino (*Solanum muricatum*) is an Andean vegetable crop closely related to tomato. In the last decades it has been introduced in the Mediterranean region and other parts of the world as a potential new crop. However, several tomato major pathogens may threaten the expansion of pepino cultivation. We identified *Fusarium oxysporum f. sp. lycopersici* (FOL), *Verticillium dahliae* (VE), pepino mosaic virus (PepMV), and tomato mosaic virus (ToMV) as four of the most likely pathogens to cause damage to pepino crops in Mediterranean climates. In order to evaluate the response of the pepino genepool against these pathogens, as well as to identify sources of tolerance, we inoculated six accessions of cultivated pepino, nine accessions of seven pepino wild relatives, and one interspecific hybrid with FOL, VE, PepMV and ToMV and followed its symptomatology for 30 d (FOL and VE) or 60 d (PepMV and ToMV). ELISA tests were also performed for PepMV and ToMV. Susceptible tomato materials were used as controls. The pepino genepool displayed fewer symptoms than susceptible tomato controls after inoculation with FOL, with most accessions being tolerant or resistant. Regarding VE, a wide variation of values for the symptoms index (SI) was observed, with three cultivated pepino accessions displaying tolerance. For PepMV a wide variation for SI was also observed, with one accession of *S. caripense* being resistant, and several accessions of pepino and other wild relatives displaying different degrees of tolerance. PepMV absorbance values obtained by ELISA tests followed a pattern similar to that of SI. For ToMV no resistances were found, although two wild accessions and the interspecific hybrid displayed low values for the SI and were considered as moderately tolerant. ELISA tests against ToMV revealed that the virus replicated well in all materials. None of the accessions evaluated displayed resistance or high levels tolerance to the four pathogens, but some of them were complementary for resistance or high levels of tolerance. Although the interspecific hybrid tested was not resistant to any of the pathogens, it was tolerant to FOL and PepMV and moderately tolerant to VE and ToMV. A

multivariate hierarchical clustering revealed similar patterns among accessions in the response to the two fungal diseases (FOL and VE) on one side and to the two viral ones (PepMV and ToMV) on the other. The information generated in this study has allowed identifying materials within the pepino genepool for the development of multi-resistant pepino cultivars to major diseases threatening its expansion in the Mediterranean region.

Keywords: DAS-ELISA, *Fusarium oxysporum* f. sp. *lycopersici*, inoculation, PepMV, *Solanum muricatum*, ToMV, *Fusarium oxysporum* f. sp. *lycopersici*; *Verticillium dahliae*.

1. Introduction

The pepino (*Solanum muricatum* Aiton), also known as “pepino dulce" or "sweet cucumber”, is a vegetatively propagated vegetable crop native to the Andean region grown for its fruits (Prohens et al., 1996). Pepino fruits are fleshy, typically of a golden yellow color with purple stripes, and can be consumed as a fresh table fruit in the case of cultivars that have more aromatic and sweet fruits, or as a vegetable in salads, for cultivars with less sweet and more acid fruits (Rodríguez-Burruezo et al., 2011). Although pepino cultivation has been mainly restricted to the Andean region, in the last decades there has been a growing interest in several countries from the Mediterranean region, as well as in China, Japan, New Zealand, or the USA, in introducing pepino as a new vegetable crop (Rodríguez-Burruezo et al., 2011; Herraiz et al., 2015a; Gurung et al., 2016; Kim et al., 2017). However, the introduction of pepino in other countries outside its region of origin is threatened due to susceptibility to pests and diseases of tomato (Nuez & Ruiz, 1996), which is phylogenetically closely related to pepino (Herraiz et al., 2015a, 2016a; Särkinen et al., 2013).

In the Mediterranean region, pepino is mostly grown as a greenhouse crop, following agricultural practices similar to those of tomato (Prohens et al., 1999; Rodríguez-Burruezo et al., 2011). Under these protected cultivation conditions, we have identified two fungal and two viral pathogens that affect tomato (Lahoz et al., 2015), namely *Fusarium oxysporum* f. sp. *lycopersici* (FOL), *Verticillium dahliae* (VE), pepino mosaic virus (PepMV), and tomato mosaic virus (ToMV), that potentially could cause significant damage to pepino crops (Ge et al., 2012; Jones et al., 1980; Nuez & Ruiz, 1996; Pérez-Benlloch et al., 2001). Although late blight (*Phytophthora infestans*) is a serious disease of pepino in its region of origin (Adler et al., 2002), in the Mediterranean area is infrequent in tomato (Lahoz et al., 2015), probably because most of its cultivation, like that of pepino (Rodríguez-Burruezo et al., 2011), is under controlled greenhouse conditions that do not favour its spread.

Fusarium wilt caused by *Fusarium oxysporum* is one of the most devastating fungal diseases of tomato and pepino (Nuez & Ruiz, 1996; Mandal et al., 2009). It is soil-borne and affects both greenhouse and open field cultivation in temperate vegetable production areas through irrigation water and contaminated farm equipment (Maurya et al., 2019). In tomato, FOL directly penetrates roots and colonizes vascular tissue (Srinivas et al., 2019), causing yellowing of the leaves and wilting of the plants, which can lead to a complete loss of production (Nirmaladevi et al., 2016). Under wet conditions, white, pink or orange fungal growth can be seen on the surface of the affected stems (Ajilogba & Babalola, 2013).

Verticillium wilt is caused by VE, a fungal pathogen that affects a wide range of solanaceous hosts (Inderbitzin & Subbarao, 2014; Klosterman et al., 2009), responsible for serious economic losses both in the greenhouse and in open field cultivations (Gayoso et al., 2010). This pathogenic fungus infects roots and then invades the xylem (Hu et al., 2019), causing in tomato and eggplant symptoms of vascular discoloration, wilting and yellow-bronze leaf spots, with reduction of growth, yield and fruit quality, and eventually plant death (Karagiannidis et al., 2002). The pathogen spreads especially by irrigation and infested seeds and locally from field to field through crop management practices (Baroudy et al., 2018; Carroll et al., 2018).

PepMV, a potexvirus that was first isolated from infected pepino plants in 1980 (Jones et al., 1980), causes important losses worldwide in tomato production, especially in Europe and North America (Souiri et al., 2017). The symptoms in pepino include yellow mosaic in young leaves (Jones et al., 1980), while in tomato are very diverse, and may occur in the form of fruit discoloration, chlorosis and yellow angular leaf spots, severe leaf mosaics and occasionally leaf or stem necrosis (Hanssen & Thomma, 2010; Hasiów-Jaroszewska & Komorowska, 2013; Sempere et al., 2016; Soler et al., 2011). PepMV is transmitted mechanically with high efficiency, mainly during cultural pruning and fruit harvesting

practices through contaminated tools and clothing (Hasiów-Jaroszewska et al., 2010). In addition, low rates of transmission have been reported by bumblebees, seeds, vegetative propagation and the soil-borne fungus *Olpidium virulentus* (Alfaro-Fernández et al., 2010; Córdoba-Sellés et al., 2007; Schwarz et al., 2010; Shipp et al., 2008; Van der Vlugt & Stijger, 2009).

ToMV, a member of the genus *Tobamovirus* (Adams et al., 2009), has a wide host range including members of the Solanaceae family such as tomato and pepino, undermining their yield and fruit quality (Ge et al., 2012; Leiva-Brondo et al., 2006; Pérez-Benlloch et al., 2001; Ullah et al., 2019). Symptoms of infected plants, both in pepino and tomato, include local lesions, systemic mosaics on leaves, mottling, malformation, necrosis, and fern-leaf symptoms (Bae et al., 2019; Chitra et al., 1999; Leiva-Brondo et al., 2006; Park & Cha, 2002; Pérez-Benlloch et al., 2001). ToMV is efficiently transmitted through mechanical inoculation, grafting, and infested seed (Ghodoum Parizipour & Keshavarz-Tohid, 2020; Soler et al., 2010).

In tomato, decades of breeding programs, have allowed the identification of QTLs, sources of genetic resistance and major genes, either in the cultivated species or in wild relatives, to *Fusarium*, *Verticillium* and ToMV. These achievements have allowed the development of modern varieties with effective protection against these diseases (Lee et al., 2015). Resistant rootstocks are also commonly used in tomato for resistance to FOL and VE (King et al., 2010). However, so far, no effective resistance against PepMV has been incorporated in tomato (Pechinger et al., 2019), although some sources of resistance have been identified in the wild tomato *S. lycopersicoides* (Soler et al., 2011) and in tomato accessions 11R.412000 and 11R.446400 (US patent US9637757B2). In pepino, several accessions resistant to ToMV have been described, although its genetic control has not been determined so far (Leiva-Brondo et al., 2006; Pérez-Benlloch et al., 2001).

The evaluation of the response of pepino to these four diseases and the search for sources of resistance or tolerance is of great relevance for the development of new cultivars of pepino in the Mediterranean region. In particular, the identification of accessions with multiple resistances or tolerances would facilitate the improvement of breeding programmes to develop new pepino cultivars with multiple resistances and/or tolerances to these major diseases. For the purpose of this paper, we considered a plant as resistant if it did not display symptoms, and as a tolerant if it had mild symptoms without a significant effect on development (Atibalentja et al., 1997; Reis et al., 2004). In this work, we evaluate the response of a collection of cultivated and wild related pepino accessions to FOL, VE, PepMV, and ToMV with the aim of evaluating the threat represented by them for pepino in Mediterranean regions and identifying new sources of variation for breeding to these diseases.

2. Materials and methods

2.1. Plant materials and growing conditions

Six clonal accessions of the cultivated pepino (*Solanum muricatum*), including local Andean varieties and modern cultivars from different locations, were selected for their genetic and phenotypic diversity and for their breeding interest (Herraiz et al., 2015a; 2016b; Prohens et al., 2002; Rodríguez-Burruezo et al., 2004a) (Table 1). In addition, nine clones from seven species of pepino wild relatives (*Solanum* section *Basarthrum*) from Central and South America, plus one interspecific hybrid between cultivated pepino and a wild relative (*S. muricatum* x *S. caripense*), were chosen to represent the wild genepool diversity of pepino (Blanca et al., 2007). Finally, two accessions of tomato (*S. lycopersicum*) were included in the study as susceptible controls for the biotic stresses assessed (Table 1).

All materials came from the Germplasm Bank of the Universitat Politècnica de València and from the collection of the authors. Pepino clones were vegetatively propagated *in vitro* from one individual mother plant per clone (Çavuşoğlu, 2013), whereas wild relatives were germinated from seeds following the protocol of Ranil et al. (2015) and one individual per accession was clonally propagated *in vitro* using the same protocol than for pepino. After acclimatization in a climatic chamber with 16 h light (25 °C) / 8 h dark (18 °C) and relative humidity of 65% to 95% (day and night) regime, the clones were transplanted in the same climatic chamber to 8 × 8 × 6 cm polyethylene pots filled with Neuhaus N3 substrate (Klasmann-Dellmann GmbH, Geeste, Germany). Simultaneously, tomato accessions were germinated and seedlings were maintained in a climatic chamber until transplanting to pots. For each pathogen inoculation experiment, the plants were distributed according to a completely randomized design, with each plant constituting a replicate. The number of plants tested per accession for inoculation with each of the four pathogens varied between 3 to 12 depending on plantlets availability (Table 1). In addition, one plant per accession was kept as a mock-inoculated control. These controls were kept separated in the same climatic chamber to avoid infection with the evaluated pathogens. The pathogens were evaluated one at a time to avoid cross-contaminations.

2.2. Pathogen preparation, inoculation and disease symptoms assessment

Inoculation with *Fusarium oxysporum* f. sp. *lycopersici* (FOL) and *Verticillium dahliae* (VE) was performed, respectively, with FOL race 2 and VE race 0 isolates provided by Variety and Seed Study and Control Group (GEVES, Beaucouzé, France) and were cultured on Potato Dextrose Agar medium (PDA; Scharlab, Barcelona, Spain) at 24º C for 10 d for FOL and 26 ºC for 25 d for VE. Spores of FOL and VE were collected from the PDA culture by flooding the medium surface with 10 mL of sterile distilled water followed by gently scraping with a

loop. Spore solutions were then filtered through a sterile gauze in order to remove the hyphae and concentrations were measured using a Neubauer Chamber (Celeromics Technologies, Valencia, Spain). At the stage of four true-leaves, which corresponds to the phenological stage 104 of the specific pepino BBCH (Biologische Bundesanstalt, Bundessortenamt, CHemische Industrie) scale (Herraiz et al., 2015b), seedlings were carefully uprooted from the germination trays and the root system was washed with tap water. For inoculation with FOL or VE, 2 cm of the apical part of the root system were excised with a pair of scissors, and dipped for 3 h (FOL) or 12 h (VE), respectively, in a 150 mL conidial solution of 1.0×10^7 spores/mL. Disease severity in each inoculated plant was evaluated at 7, 15, 21 and 30 DAI (Days After Inoculation) according to a symptoms index (SI) based on a numerical scale from 0 to 4, where 0 = absence of symptoms; 0.5 = mild symptoms; 1 = moderate symptoms; 2 = severe symptoms; 3 = very severe symptoms; 4 = dead plant (Atibalentja et al., 1997; Reis et al., 2004).

Inoculation with pepino mosaic virus (PepMV) and tomato mosaic virus (ToMV) was performed, respectively, with PepMV isolate PV-0750 provided by Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), which was obtained from tomato-infected plants collected in the region of Almeria (Spain), and ToMV race 0 provided by GEVES. Inoculum of PepMV and ToMV were prepared using a 1:10 (w:v) proportion of infected tomato leaves and inoculation buffer. The inoculation buffer was prepared using the procedure described by Figàs et al. (2017). The solution was homogenised by macerating 1 g of virus-infected leaves of tomato with 10 mL of inoculation buffer using a mortar and a pestle. Carborundum 1% (w:v) (VWR International S.A.S, Pennsylvania, USA) and 1% (w:v) of activated charcoal (Scharlab, Barcelona, Spain) were added to the solution. PepMV and ToMV were mechanically inoculated rubbing the leaves with a cotton-bud stick, previously dipped in the inoculum when plantlets had four true leaves,

stage 104 of the specific pepino BBCH scale (Herraiz et al., 2015b). All true leaves were inoculated. Mock-inoculation on the non-inoculated control plant was performed using only inoculation buffer and carborundum. Disease severity was visually scored for each individual plant at 15, 30, 45, and 60 days after inoculation (DAI) following a severity scale for the symptoms index (SI): 0 = absence of symptoms; 0.5 = mild symptoms consisting mild mosaic, plant recovered in the apical leaves; 1 = moderate symptoms characterized by intensification of first symptoms, and mottling on leaves; 2 = severe yellow mosaic and mottling on leaves; 3 = very severe mottling and necrotic lesions on stems 4 = plant death. Double Antibody Sandwich - Enzyme-Linked Immunosorbent Assay (DAS-ELISA) was performed on young new leaves of each plant to evaluate the presence and level of virus accumulation. PepMV and ToMV antibodies and their enzyme conjugate were supplied by Loewe Biochemica (Sauerlach, Germany). The absorbance of the serologic reaction was measured at 405 nm with a Bio-Rad iMark 550 microplate reader (Bio-Rad Laboratories, Hercules, California, USA). A sample was considered infected (positive) when the absorbance was higher than the average absorbance of the mock-inoculated controls plus three times their standard deviation, representing a final threshold value of 0.174 for PepMV and 0.123 for ToMV. Samples were considered to be non-infected (negative) when the absorbance value was below these thresholds.

Disease severity was used to discriminate the accessions in four reaction classes depending on the mean maximum symptoms index (MMSI), which was obtained by averaging the maximum value for the symptoms index (SI) of each plant at any of the dates in which symptoms were evaluated. Plants with a MMSI = 0 were considered to be resistant (R); those with a $0 < MMSI \leq 0.5$ were considered to be tolerant (T); those with $0.5 < MMSI \leq 1.0$ were considered to be moderately tolerant (MT); while those with $MMSI > 1.0$ were considered to be susceptible (S).

2.3. Data analyses

For each combination of accession and disease, the mean and standard error (SE) of the MMSI was calculated. For the two viral diseases, the mean and SE of mean maximum absorbance (MMA) and viral accumulation index, were also calculated. MMA values were obtained by averaging the maximum value for the absorbance of each plant at any of the dates in which symptoms were evaluated, while viral accumulation index values were obtained from the quotient between the viral accumulation of each accession and that of the control, which had a standardized value of 1.00. Pearson linear correlations for MMSI for the four diseases and MMA for PepMV and ToMV were calculated. A hierarchical clustering multivariate analysis using all data from the four pathogens was performed using the package "gplot" as an enhanced version or its basic function stats in R (Warnes et al., 2016). Genotypes were divided into different clusters using Ward's hierarchical clustering method (Kamble, 2010), and the patterns of their disease traits were shown in colors in the heatmap.

3. Results

3.1. Symptoms evolution

3.1.1. Fusarium oxysporum f. sp. *lycopersici* (FOL)

Mild symptoms of chlorosis were observed at 7 DAI on the inoculated leaves of the tomato susceptible control plants, with SI values of 0.11 for MLL and of 0.65 for VLC (Supplementary data S1). Symptoms became more severe at 15 DAI with values of the SI of 0.94 for MLL and 1.75 for VLC, and increased progressively (Figure 1). At 30 DAI, all inoculated susceptible control plants developed severe disease symptoms, reaching values for

SI of 3.10 for MLL and 3.80 for VLC (Figure 2C and Table 2), indicating a high infectivity of the inoculum used and the effectiveness of mechanical inoculation.

Regarding the cultivated *S. muricatum* materials, at 7 DAI, mild symptoms were observed in all accessions, except in Mur2, which did not display any symptoms (Figure 1 and Supplementary Data S1). However, from 15 DAI until the end of the experiment at 30 DAI no more symptoms were observed in any of the *S. muricatum* accessions (Figure 1). Thus, except Mur6, which had an MMSI of 1.15, the pepino clones could be considered as resistant (in the case of Mur2) or tolerant (in the case of the other five accessions) to FOL race 2 (Table 2).

Six out of the ten clones of pepino wild relatives showed mild symptoms of FOL infection at 7 DAI, with SI ranging from 0.05 of Trac to 0.75 of Base (Supplementary data S1). However, just two of them (Cane with SI of 0.33 and Cati with 0.5), plus Car3 (0.57) and Frax (0.17), which were asymptomatic at 7 DAI, exhibited symptoms at 15 DAI. From 21 DAI until the end of the experiment, only Cane continued exhibiting disease symptoms (Figure 2B). Two out of three *S. caripense* accessions, Car1 and Car2, did not develop any symptoms throughout the experiment (Table 2 and Figure 2A). Similarly, the Trac, Cati, Perl, Frax accessions and the hybrid Hb displayed only slight symptoms in responses to FOL infection, with MMSI values ranging from 0.05 to 0.5 (Table 2), and they could be considered as tolerant. Finally, Cari3 and Bas exhibited moderate disease severity, with MMSI values ranging from 0.57 to 0.75, indicating moderate tolerance against FOL, while Cane with MMSI of 1.5 was classified as susceptible (Table 2).

3.1.2. Verticillium dahliae (VE)

Plants of the susceptible tomato controls exhibited moderate symptoms at 7 and 15 DAI, with SI values of 0.78 for MLL and 0.60 for VLC (Supplementary data S1), showing the typical

symptoms of the disease, consisting of leaf chlorosis stem yellowing (Figure 2F). The intensity of symptoms increased with time with SI from 2.00 and 1.50 for MLL and VLC, respectively at 21 DAI, to 3.00 for both tomato accessions at 30 DAI (Figure 1). The MMSI values of 3.00 for controls confirmed that the VE infection was correctly made (Table 2).

The response of the accessions tested of pepino and wild relatives' genotypes varied considerably. Symptoms of VE on pepino clones appeared 7 DAI after the inoculation, except for Mur3 that exhibited slight symptoms (SI=0.50) at this date (Figure 1 and Supplementary Data S1). At 21 DAI, also Mur5 (0.08), Mur6 (0.25) and Mur1 (0.50) showed light symptoms while those of Mur3 substantially increased (2.00). At the end of the experiment, all clones reached higher symptoms levels, with MMSI values ranging from 0.50 to 3.00 (Figure 1 and Table 2). Based on the records, three of them (Mur2, Mur4 and Mur5) could be considered as tolerant, being Mur2 and Mur4 the most promising since they showed mild symptoms (0.50) only at 30 DAI (Figure 2D and Supplementary data S1). The rest of the accessions exhibited severe symptoms, ranging from 1.58 of Mur6 to 3.00 of Mur3, indicating higher susceptibility to VE race 0 (Figure 2E and Table 2). Regarding pepino wild relatives, they exhibited mild symptoms at 7 and 15 DAI, while only Car1, Car2 and Hb showed no symptoms (Supplementary data S1). At 21 DAI, all the accessions displayed a considerable increase in the severity of the symptoms, reaching MMSI values between 1.07 and 3.00 at the end of the experiment (Table 2 and Figure 1).

None of the wild accessions performed better than the best pepino clones. In fact, the best performance was of Hb (0.55 at 30 DAI), which is a hybrid between "Sweet Long", a pepino clone not included in this study, and Car1 that exhibited one of the worst results (MMSI of 3.00). However, the *S. caripense* accession Car2 displayed the lowest symptoms among the wild relatives, with 1.00 MMSI values, and could be considered as moderately tolerant against

VE race 0 (Table 2). The rest of the wild accessions displayed moderate to severe symptoms and therefore were classified as susceptible (Table 2).

3.1.3. Pepino mosaic virus (PepMV)

The susceptible tomato control MLL showed severe symptoms (2.58) already at 15 DAI and increased progressively during all the experiment (Figure 1 and Supplementary Data S1). At 60 DAI all inoculated susceptible control plants developed severe mosaic in leaves (Figure 2I) with MMSI values of 3.03 (Table 2). The serological analyses of the plants indicated that MLL had high virus titre, with MMA values of 3.08 (Table 3 and Supplementary Data S2). Both symptoms and viral accumulation displayed high levels throughout the experiment (Figures 1 and 3). Therefore, also for this pathogen, the inoculum and inoculation were successful.

The behaviour of the tested cultivated pepino clones varied considerably among the different accessions. While some clones at 15 DAI exhibited no symptoms (Mur6) or very light ones, such as Mur 2 (0.06) and Mur4 (0.25), the rest showed mild (Mur 5 with 0.85) or severe symptoms (Mur3 with 1.50 and Mur1 with 2.00) (Figure 1 and Supplementary Data S1). After increasing at 30 DAI, the symptoms generally reached their maximum at 45 DAI and maintained stable until the end of the experiment (Figure 1). The lowest MMSI was found in Mur6 with a value of 0.50, followed by Mur2 and Mur 4 with a value of 1.00 and finally Mur3 and Mur5 with the highest MMSI (2.00) (Table 2). These symptoms results followed the same patterns of the MMA values (Supplementary Data S2). In this way, Mur6 absorbance levels were the lowest with an MMA value of 0.30, followed by Mur2 (1.43), Mur4 (1.61) and Mur3 (1.99), and finally by Mur1 (2.28) and Mur5 (2.54) (Table 3). Taking account all these data and also the normalized ones, using the susceptible control for the viral accumulation index

(Table 3), we could consider Mur6 as tolerant, and Mur2 and Mur4 as moderately tolerant to PepMV (Figure 2 and Table 2).

Wild relatives exhibited a wide range of performance after infection with PepMV. First symptoms, from light to mild, were registered already at 15 DAI, except for Car1 and Hb (Figure 1) and similar to pepino clones they generally reached their higher values around 45 DAI (Supplementary data S1). Accession Car1 and its hybrid Hb displayed a good response against this pathogen. In this way, Car1 did not exhibit any symptoms during the test and could be considered as a resistant accession, while Hb showed mild symptoms only at 45 and 60 DAI with MMSI of 0.5 and was classified as tolerant (Table 2). Other accessions that presented moderate symptoms and low MMSI values were Perl (0.79), Car2 (0.95), Trac and Base (1.00), being moderately tolerant to PepMV (Table 2). Overall, the progression of absorbance values (Figure 3 and Supplementary Data S2) and MMA values (Table 3) were consistent with those of the progression of symptoms (Figure 1 and Supplementary Data S1) and MMSI values (Table 2).

3.1.4. Tomato mosaic virus (ToMV)

The susceptible tomato control MLL showed the characteristic symptoms of ToMV infection, with light and dark green leaf mosaic, mottling and deformation of leaves (Figure 2L). At 15 DAI moderate symptoms were observed, becoming severe from 30 DAI on until the end of the experiment (Figure 1 and Supplementary Data S1). The absorbance values were high at 15 DAI but decreased slightly at the end of the experiment with a MMA of 1.86 (Figure 3 and Supplementary Data S2). Again, this indicates that the conditions and the inoculation method were adequate.

All pepino clones showed symptoms at 15 DAI, ranging from light (Mur1 and Mur6 with 0.50) to severe (Mur4 with 2.42) (Supplementary Data S1). However, while some accessions were able to avoid the onset of more severe symptoms, others like Mur4 (2.92) and Mur5 (3.15) reached MMSI values higher than the tomato control MML (Figure 1 and Table 2). Nevertheless, all pepino clones reached MMSI values higher than 1.00 and therefore were considered susceptible to ToMV (Table 2). The large differences recorded for symptoms were not observed for the absorbance and the viral accumulation index (Table 3 and Supplementary Data S2). During all the experiment, the difference in the absorbance values among pepino clones was limited and MMA values ranged from 1.08 of Mur5 to 1.44 of Mur6 (Table 3 and Supplementary Data S3). All wild relatives, except Hb which did not display symptoms (Figure 2), showed from mild (Trac with 0.50, Cati with 0.65 and Car1 with 0.75) to moderate symptoms (Car2 with 2.15) at 15 DAI (Supplementary Data S1). Symptoms became more severe after 30 DAI and at the end of the experiments Car1 (2.75), Car2 (2.30), Cane and Frax (3.00) recorded higher MMSI values than the control MLL (Figure 1 and Table 2). Better performances were observed for Car3, Base and Hb (MMSI at 1.00) and they were considered as moderately tolerant (Table 2). However, except Base, these accessions were not the ones that registered lower absorbance values along the experiment (Figure 3 and Supplementary Data S2), confirming that pepino wild relatives exhibit different symptoms severity at similar viral concentrations. Also, some wild relatives Car1, Car2 and Trac displayed similar symptoms severity and absorbance than the control MLL and therefore are considered as susceptible.

3.2. Hierarchical clustering analysis

Unsupervised hierarchical clustering of disease traits and accessions revealed several clusters (Figure 4). Clustering for traits revealed two major clusters. Cluster I is formed by the

MMSI of both fungal diseases (FOL-MMSI and VE-MMSI), while cluster II grouped tightly MMSI and MMA of PepMV. ToMV-MMSI and ToMV-MMA were placed in two separated branches. The heatmap is in agreement with the disease traits correlation values observed (Supplementary Data S3). Genotypes were also grouped into two major clusters (Figure 4). Cluster A included the wild accessions Cati, Frax and Cane, which exhibited high values for MMSI and MMA. Cluster B is the most heterogenous and is subdivided into two sub-clusters. The sub-cluster (a) included Car3 and Hb on one branch and Base and Mur6 in another branch, with these accessions generally displaying a good performance against the two viruses, except for Car3 and the hybrid Hb for ToMV-MMA. On the contrary, these two accessions displayed a good performance against VE (Figure 4). The sub-cluster (b) is divided in turn in three groups, being the first one comprised by Car2, Mur2, Mur4 and Mur5 that shared good response to FOL-MMSI, VE-MMSI and ToMV-MMA (except Car2). The second one, which included Trac, Perl, Mur1 and Mur3 shared good behavior for FOL-MMSI and ToMV-MMSI but severe symptomatology for VE-MMSI. Finally, the third group is formed only by Car1 which showed good response to all the traits, except for VE-MMSI and ToMV-MMSI.

4. Discussion

The success of a new vegetable crop such as pepino in regions where it is being introduced depends largely, among many other factors, on the availability of resistant varieties (Nelson et al., 2018). In the Mediterranean region, tomato and other solanaceous crops are widely cultivated and therefore their pests and diseases can difficult the introduction of pepino in this region (Nuez & Ruiz, 1996; Hanssen and Lapidot, 2012; Lee et al., 2015). Among the most threatening diseases, we have identified four tomato pathogens that, for their efficient mode of transmission and wide distribution (Lahoz et al., 2015; Janssen et al., 2018), are

especially threatening in the case of pepino, which is phylogenetically closely related to tomato (Herraiz et al., 2015, 2016b; Särkinen et al., 2013).

In order to increase the likelihood to find stable and multiple disease resistance sources, in addition to cultivated clones, we have also selected pepino wild relatives, due to their greater diversity and for having been demonstrated their usefulness for improving pepino quality through introgression breeding (Blanca et al., 2007; Herraiz et al., 2015a; Rodríguez-Burruezo et al., 2011). All of the pepino wild relatives selected for this study, with the exception of *S. canense* and *S. fraxinifolium*, are cross-compatible with the cultivated pepino, producing fertile interspecific hybrids (Prohens et al., 2003; Rodríguez-Burruezo et al., 2011), and so, suitable to transfer the desired traits from the wild to the cultivated background. By incorporating wild species in the materials screened, we tried to mimic the approach used in tomato, where the majority of the disease resistance genes incorporated nowadays in the high performing tomato commercial cultivars come from its wild genepool (Kaushal et al., 2020). In this way, resistance genes, *I-2* and *Ve-2*, found respectively in the *S. lycopersicum* × *S. pimpinellifolium* hybrid PI126915 and in *S. lycopersicum* accession Peru Wild, that confers resistance against FOL race 2 and VE (Lee et al., 2015; Stall & Walter 1965) have been introgressed to modern commercial tomato varieties. Regarding *Tm-1*, *Tm-2* and *Tm-2*^{2} genes, that confer resistance against ToMV, was originally identified from *S. habrochaites* (*Tm-1)*PI126445 and in an *S. peruvianum* (*Tm-2* and *Tm-2*^{2}) (Lanfermeijer et al., 2005; Lee et al., 2015).

Surprisingly, in this study, some of the pepino cultivated clones have revealed as sources of variation for the disease resistance of the pathogens screened that were as good as or even better than the wild ones, which suggests that, unlike other traits such as soluble solids content (Prohens et al., 2005; Herraiz et al., 2015a), cultivated materials may be of great interest as sources of resistance in pepino breeding. This may facilitate developing new pepino resistant

cultivars, since using cultivated clones instead of wild relatives would drastically reduce the linkage drag of undesired traits typical of interspecific crosses (Prohens et al., 2017).

The results indicate that in cultivated pepino germplasm resistant or tolerant accessions to *Fusarium* and *Verticillium* can be identified, so it may be possible to select and develop varieties resistant or tolerant to these pathogens by using the diversity present in the cultivated species. Furthermore, given the phylogenetic closeness to tomato (Blanca et al., 2007; Herraiz, Blanca, et al., 2016; Spooner et al., 1993), pepino resistant clones could be tested as potential tomato rootstocks against these pathogens (Singh et al., 2017).

Regarding the viruses screened, one wild accession from *S. caripense* (Car1) has been resistant to PepMV. This is in contrast to tomato, where no complete resistance has been found yet to PepMV (Pechinger et al., 2019). The fact that viable somatic hybrids between tomato and pepino have been obtained (Sakomoto & Taguchi, 1991), may represent a way to transfer the resistance from *S. caripense* accession Car1 to tomato. However, as in tomato, some cultivated accessions and wild materials have shown different degrees of tolerance to PepMV (Soler et al., 2011). In the case of ToMV, no resistance has been found in the evaluated cultivated and wild materials selected for this study, so we suggest resorting to other materials that have previously been identified as resistant (Leiva-Brondo et al., 2006), and which in fact have already been used to develop a ToMV resistant cultivar (Rodríguez-Burruezo et al., 2004b). Our results also show that while symptomatology and virus titer are well correlated in the case of PepMV suggesting, that greater multiplication of the virus is associated to more severe symptoms, for ToMV they are not correlated. These results indicate that for ToMV there may be different mechanisms of tolerance to ToMV infection, as already suggested by Pérez-Benlloch et al. (2001) and Leiva-Brondo et al. (2006).

Interestingly, the hybrid with *S. caripense* has shown a general good performance against all diseases, indicating that it may be a good material for introgression breeding for resistance or

tolerance to multiple diseases. Also, given that hybrids of solanaceous crops generally are heterotic for vigor traits (Kumari et al., 2020), this hybrid might be of interest for being used as rootstock (Spanò et al., 2020). The moderate resistance of the hybrid suggests incomplete dominance for the resistance or tolerance to the pathogens assessed, although further studies with segregating populations are needed to confirm the genetic control of these phenotypes. Although we did not find any cultivated pepino accession of resistant or tolerant to all pathogens, some of them (Mur2, Mur4 and Mur6) have shown good behavior against all four, so they could be interesting candidate materials to start breeding programs. Simultaneously, it will be worth investigating if the broad spectrum of tolerance of some materials to more than one pathogen has a common genetic cause or is provided by the combination of multiple genes, which often occur in clusters (Wiesner-Hanks & Nelson, 2016).

However, further studies will be required to dissect the genetic patterns of the tolerance and resistance identified in the materials screened. By linkage analysis and synteny, it may be possible to find out if the pepino genomic regions involved in the defence mechanisms are syntenic with the tomato ones and if the genes are orthologs and conserved in *Solanum* crops (Rinaldi et al., 2016). The results obtained have made it possible to identify materials with tolerance or resistance to some of the main potential pepino pathogens in Mediterranean climates. It is worth pointing out that even though the results presented here came from single experiments for each of the four pathogens, they interaction of each of them with pepino constitute pathosystems characterized by an efficient infection of their easy transmission in the host by the pathogen. Therefore, the likelihood of identifying false-positive resistant plants due to the lack of infection is considered as low. However, in future studies, new pathogens strains and races should be tested in order to investigate if the tolerances and resistances found are broad or strain/race specific.

Our results suggest that by hybridizing materials that complement to each other for resistance or tolerance for the four diseases, it may be possible to develop multi-resistant varieties of pepino. These materials can contribute to the development of multi-resistant varieties for pepino and consequently to the expansion of this crop in Mediterranean regions.

Conflict of interest

The authors have no conflict of interest to declare.

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Tables

Table 1. Accessions and controls assessed in this study and their respective study code, species, geographical origin and number of plants evaluated for each pathogen.

Accession	Code	Species	Origin	FOL	VE	PepMV	ToMV
				n	n	n	n
Cultivated							
37-A	Mur1	*S. muricatum*	Ecuador	10	5	5	10
Sweet Round	Mur2	*S. muricatum*	Spain	10	10	9	10
Valencia	Mur3	*S. muricatum*	Spain	10	10	5	7
OV-8	Mur4	*S. muricatum*	Chile	9	3	8	6
Virú	Mur5	*S. muricatum*	Peru	10	12	10	10
Vetas Verdes	Mur6	*S. muricatum*	Ecuador	10	12	5	6
Wild relatives							
EC-40	Car1	*S. caripense*	Ecuador	7	7	9	10
PI-243342	Car2	*S. caripense*	Ecuador	6	3	10	10
BIRMS1034	Car3	*S. caripense*	Ecuador	7	7	9	10
E-34	Trac	*S. trachycarpum*	Ecuador	10	10	5	10
E-80	Cati	*S. catilliflorum*	Peru	10	8	6	10
E-62	Perl	*S. perlongysttilum*	Peru	10	7	7	10
PT-084	Base	*S.basendopogon*	Peru	10	7	10	10
BIRMS1975	Cane	*S. canense*	Panama	7	6	6	10
BIRMS1978	Frax	*S. fraxinifolium*	Costa Rica	3	5	10	10
Interspecific hybrid							
F1 (Sweet Long x EC-40)	Hb	*S. muricatum* x *S. caripense*	Spain	7	11	9	10
Susceptible controls							
Mallorquin	MLL	*S. lycopersicum*	Spain	10	10	18	18
Valenciano	VLC	*S. lycopersicum*	Spain	10	9	-	-

Table 2. Mean maximum symptoms index (MMSI, ± SE) for symptoms severity registered at any of the dates where measurements were performed (7, 15, 21, 30) days for FOL and VE and (15, 30, 45, 60) days for PepMV and ToMV, percentage of plants with symptoms and reaction classification for the pathogens evaluated in this study. Resistant (R), tolerant (T), moderately tolerant (MT), and susceptible (S).

Accession code	FOL			VE			PepMV			ToMV		
	MMSI	% of plants with symptoms	Reaction	MMSI	% of plants with symptoms	Reaction	MMSI	% of plants with symptoms	Reaction	MMSI	% of plants with symptoms	Reaction
Cultivated												
Mur1	**0.15 ± 0.08**	100	**T**	2.00 ± 0.00	100	S	2.00 ± 0.00	100	S	1.15 ± 0.08	100	S
Mur2	**0.00 ± 0.00**	**0**	**R**	**0.50 ± 0.00**	100	**T**	**1.00 ± 0.00**	100	**MT**	1.80 ± 0.08	100	S
Mur3	**0.10 ± 0.07**	100	**T**	3.00 ± 0.00	100	S	2.00 ± 0.00	100	S	1.57 ± 0.07	100	S
Mur4	**0.06 ± 0.06**	100	**T**	**0.50 ± 0.00**	100	**T**	**1.00 ± 0.00**	100	**MT**	2.92 ± 0.69	100	S
Mur5	**0.15 ± 0.08**	100	**T**	**0.50 ± 0.00**	100	**T**	2.00 ± 0.00	100	S	3.15 ± 0.24	100	S
Mur6	1.15 ± 0.25	100	S	1.58 ± 0.15	100	S	**0.50 ± 0.00**	100	**T**	1.25 ± 0.11	100	S
Wild relatives												
Car1	**0.00 ± 0.00**	**0**	**R**	3.00 ± 0.00	100	S	**0.00 ± 0.00**	0	**R**	2.75 ± 0.08	100	S
Car2	**0.00 ± 0.00**	**0**	**R**	**1.00 ± 0.17**	100	**MT**	**0.95 ± 0.27**	100	**MT**	2.30 ± 0.23	100	S
Car3	**0.57 ± 0.57**	100	**MT**	1.07 ± 0.17	100	S	1.28 ± 0.09	100	S	1.00 ± 0.00	100	**MT**
Trac	**0.05 ± 0.05**	100	**T**	3.00 ± 0.00	100	S	**1.00 ± 0.00**	100	**MT**	1.50 ± 0.00	100	S
Cati	**0.50 ± 0.00**	100	**T**	2.00 ± 0.00	100	S	2.00 ± 0.00	100	S	1.70 ± 0.08	100	S
Perl	**0.10 ± 0.07**	100	**T**	3.00 ± 0.00	100	S	**0.79 ± 0.18**	100	**MT**	1.50 ± 0.00	100	S
Base	**0.75 ± 0.08**	100	**MT**	2.14 ± 0.26	100	S	**1.00 ± 0.00**	100	**MT**	1.00 ± 0.00	100	**MT**
Cane	1.50 ± 0.00	100	S	2.33 ± 0.21	100	S	3.00 ± 0.00	100	S	3.00 ± 0.00	100	S
Frax	**0.17 ± 0.17**	100	**T**	1.40 ± 0.22	100	S	3.00 ± 0.00	100	S	3.00 ± 0.00	100	S
Hb	**0.06 ± 0.06**	100	**T**	**0.55 ± 0.08**	100	**MT**	**0.50 ± 0.00**	100	**T**	1.00 ± 0.00	100	**MT**
Susceptible controls												
MLL	3.80 ± 0.20	100	S	3.00 ± 0.00	100	S	3.03 ± 0.07	100	S	2.69 ± 0.09	100	S
VLC	3.10 ± 0.46	100	S	3.00 ± 0.00	100	S	-	-	-	-	-	-

Table 3. Mean maximum absorbance (MMA, ± SE) at any of the dates where measurements were performed (15, 30, 45, 60) days for PepMV and ToMV, viral accumulation index and total percent of plants with systemic infection measured with DAS-ELISA for the viruses PepMV and ToMV after mechanical inoculation. Samples were considered infected (positive) when absorbance was greater than the threshold value of 0.174 for PepMV and 0.123 for ToMV.

Accession code	PepMV			ToMV		
	MMA	Viral accumulation index	Total % of plants with systemic infection	MMA	Viral accumulation index	Total % of plants with systemic infection
Cultivated						
Mur1	2.28 ± 0.55	0.74	100	1.27 ± 0.07	0.68	100
Mur2	1.43 ± 0.37	0.47	100	1.12 ± 0.15	0.60	100
Mur3	1.99 ± 0.48	0.65	100	1.15 ± 0.07	0.62	100
Mur4	1.61 ± 0.42	0.52	100	1.11 ± 0.34	0.60	100
Mur5	2.54 ± 0.40	0.82	100	1.08 ± 0.23	0.58	100
Mur6	0.30 ± 0.10	0.10	100	1.44 ± 0.04	0.77	100
Wild relatives						
Car1	0.28 ± 0.04	0.09	100	1.54 ± 0.21	0.83	100
Car2	1.51 ± 0.45	0.49	100	2.04 ± 0.07	1.10	100
Car3	0.44 ± 0.10	0.14	100	1.85 ± 0.10	0.99	100
Trac	1.63 ± 0.51	0.53	100	1.76 ± 0.08	0.94	100
Cati	3.22 ± 0.14	1.04	100	2.18 ± 0.07	1.17	100
Perl	1.63 ± 0.50	0.53	100	2.18 ± 0.06	1.17	100
Base	0.60 ± 0.16	0.19	100	1.10 ± 0.08	0.59	100
Cane	3.50 ± 0.00	1.14	100	2.54 ± 0.02	1.37	100
Frax	3.44 ± 0.06	1.12	100	2.55 ± 0.12	1.37	100
Hb	0.41 ± 0.07	0.20	100	2.06 ± 0.08	1.11	100
Susceptible controls						
MLL	3.08 ± 0.34	1.00	100	1.86 ± 0.08	1.00	100

Figures

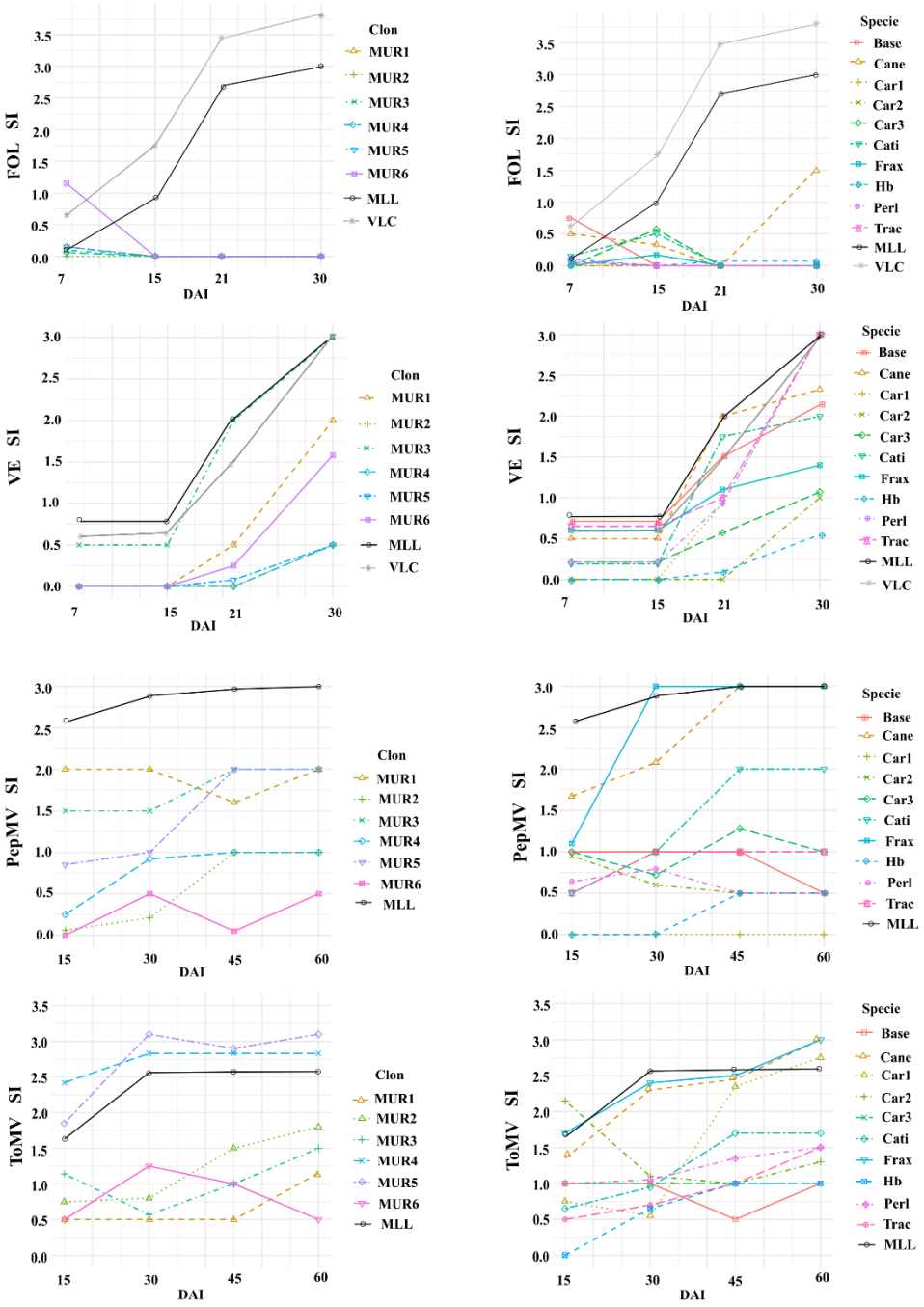

Figure 1. Evolution of the average symptoms index (SI) at 7, 15, 21, 30, 45 and 60 days of cultivated pepino (left) and wild relatives (right) accessions plus tomato controls (MLL and VLC) after inoculation with FOL, VE, PepMV and ToMV.

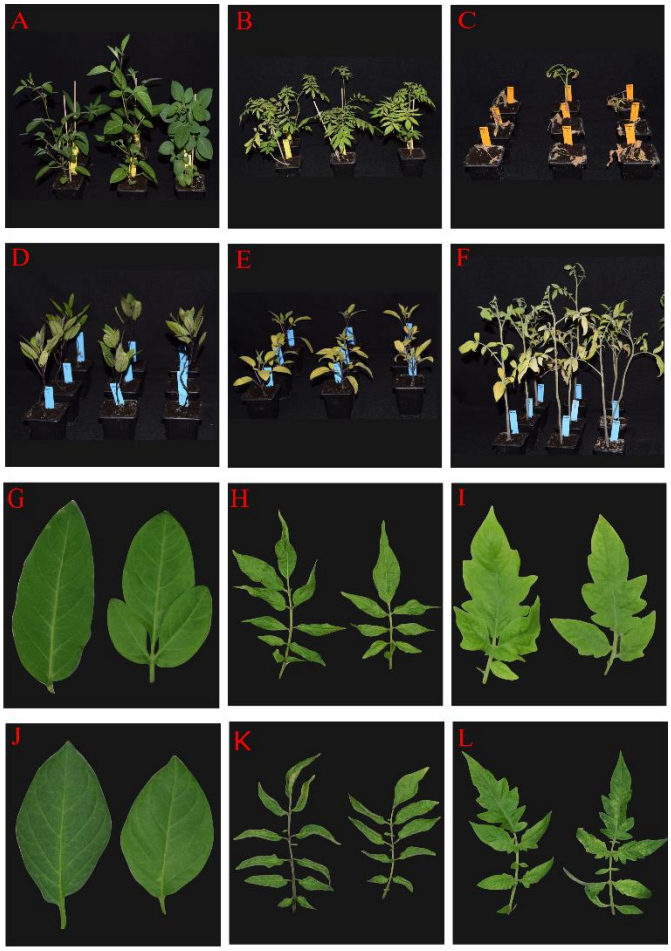

Figure 2. Foliar symptoms in plants at the end of each experiment. A, B, C; plants infected with FOL, Car2 accession plants showing no damage (A), generalized chlorosis in leaves of the Cane accession (B), dead plants of the tomato susceptible control MLL (C). D, E, F; plants infected with VE, Mur5 accession plants showing no damage (D), follicular chlorosis in leaves of the Mur3 accession (E), generalized chlorosis in tomato plants of the susceptible control VLC (F). G, H, I; plants infected with PepMV, Mur6 accession leaves showing no damage (G), crushing in leaves of the Frax accession (H), mild chlorosis and crushing in leaves of the susceptible tomato control MLL (I). J, K, L; plants infected with ToMV, Hb accession leaves showing no damage (J), generalized severe curling and chlorosis at the ends of the leaves of the Frax accession (K), generalized curling and chlorosis in tomato leaves of the susceptible control MLL (L).

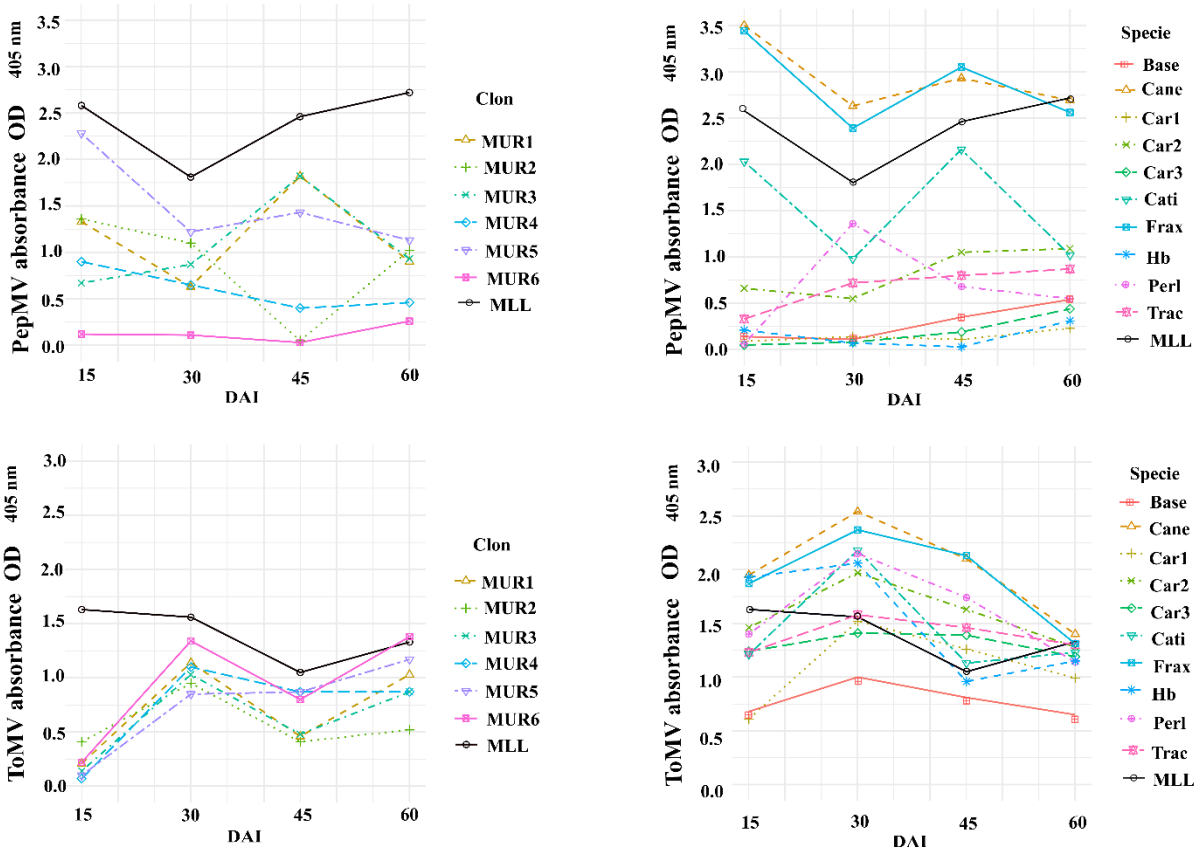

Figure 3. Evolution of mean absorbances of cultivated pepino (left) and wild relatives (right) accessions plus a tomato control (MLL) at 15, 30, 45 and 60 days after inoculation regarding PepMV and ToMV mechanical inoculation. Samples were considered infected (positive) when absorbance was greater than the threshold value of 0.174 for PepMV and 0.123 for ToMV.

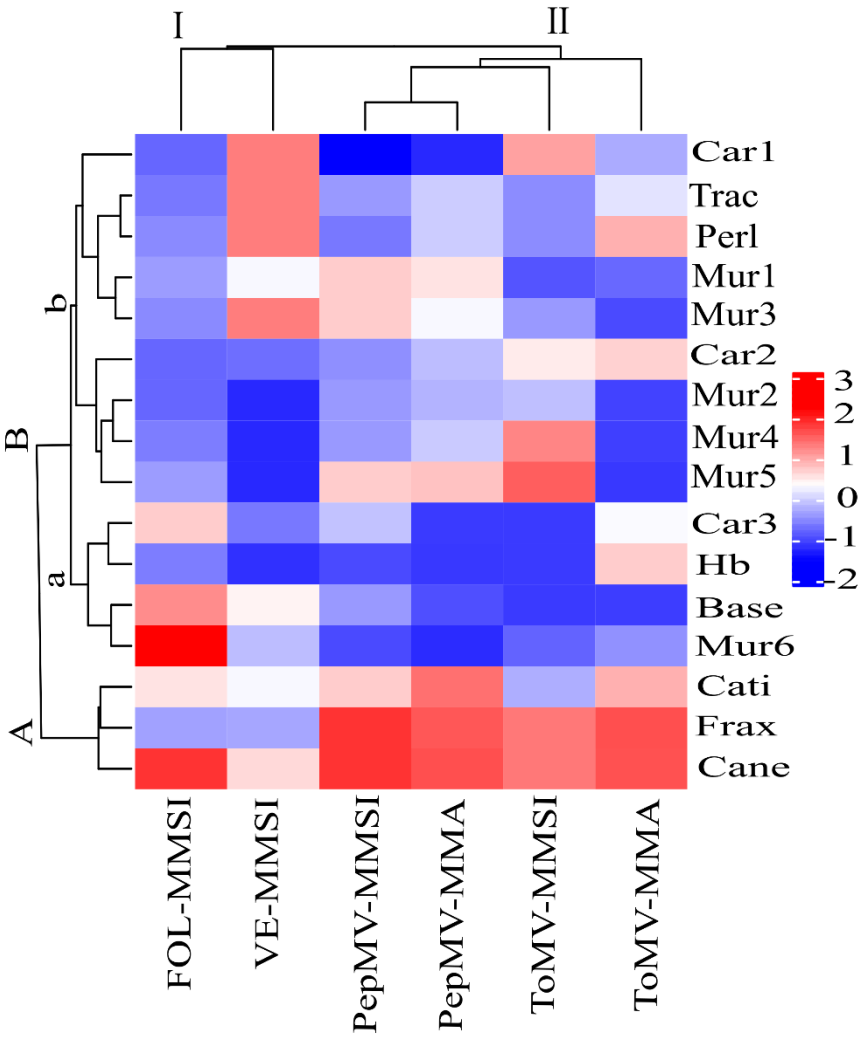

Figure 4. Heatmap of genotypes and disease traits mean maximum symptoms index (MMSI), mean maximum absorbance (MMA) in plants infected with FOL, VE, PepMV and ToMV. The colors of the clusters indicate the severity of the disease, the blue being the least severe, the white having an intermediate value and the red the most severe.