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RESEARCH PAPERS

Evaluation under diverse conditions of a differential host reaction scale to *Tomato yellow leaf curl virus* in tomato

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Summary. *Tomato yellow leaf curl virus* (TYLCV) is the most widespread virus species causing Tomato yellow leaf curl disease. Accurate methods for assessment of resistance are requisite in breeding programs. Researchers at the Volcani Center (Israel) developed a scale of differential TYLCV reactions in seven non-segregating tomato host lines, with virus responses ranging from susceptible to highly resistant. In the present work, this scale has been evaluated with inoculation and assessment methods routinely used by two different research groups, in Spain and Cuba. Different TYLCV isolates, inoculation methods, plant ages, and environmental conditions were compared. Symptom scores of the tomato lines were generally lower in the conditions assayed in both locations than those originally described. Reaction ranking order of the standard tomato lines was more similar to the original description in the assays carried out in Spain. However, response of the lines to TYLCV obtained in both locations did not correspond to scale grades for most of the conditions. A large difference between symptom scores in the susceptible and the resistant lines was observed, with the range in the resistant lines being narrow. These results indicate that the number of standard lines used could be reduced, selecting the most susceptible and the most resistant lines, and one with intermediate resistance. All the factors evaluated affected symptom development. This highlights the importance of establishing a standard inoculation method, experimental conditions, evaluation period and appropriate resistance evaluation criteria, to ensure precise evaluation of genotype responses.

Key words: TYLCV, *Solanum lycopersicum*, whitefly inoculation, agroinoculation.

Introduction

Tomato yellow leaf curl disease (TYLCD) is caused by a complex of whitefly-transmitted virus species belonging to the genus *Begomovirus*, family *Geminiviridae* (King *et al*., 2011). This disease is one of the major limitations of tomato (*Solanum lycopersicum* L.) cultivation in tropical and subtropical regions (Hanssen *et al*., 2010). The most widespread species causing TYLCD is *Tomato yellow leaf curl virus* (TYLCV). Strategies to manage the disease mainly rely on controlling the insect vector populations (Lapidot *et al*., 2006). However, these measures are not completely effective, especially under high inoculum pressures or early infections. Breeding resistant tomato varieties is likely to be the best long-term approach to control the disease.

Several host genes conferring resistance to TYLCD have been described, derived from different wild tomato relatives (Caro *et al*., 2015). Some have been transferred to cultivated tomato, aiming to develop resistant cultivars. However, available commercial varieties are not a definitive solution, as they do not

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provide total resistance. Several research groups are still searching for new sources of resistance to TYLCD.

Availability of accurate methods for the assessment of the levels of disease resistance is an important requisite in plant breeding programmes. From a breeding perspective, the most important parameter to evaluate resistance is the yield loss caused by infections. It would be desirable to measure this effect in TYLCD-resistant materials, comparing yield and yield components in infected and uninfected plants (Lapidot *et al*., 1997, 2006). However, these tests are only affordable for advanced breeding lines due to the costs of their development (Lapidot and Friedmann, 2002). Accumulation of virus DNA has been used as an indicator of the resistance levels (Zakay *et al*., 1991; Fargette *et al*., 1996). However, the correlation between viral accumulation and the effects on yield is not valid for intermediate levels of resistance; so virus accumulation is not recommended as the sole indicator of relative resistance (Lapidot *et al*., 1997). The most directly observable criterion to evaluate resistance is symptom severity induced by infection, and most of the inheritance studies for resistance to TYLCD have been based on symptom development (Pérez de Castro *et al*., 2007). The threshold to consider a plant as resistant is variable. In most assays, only asymptomatic plants have been classified as resistant (Hassan *et al*., 1984; Kasrawi, 1989; Pilowsky and Cohen, 1990). In contrast, other authors have established the limit score in slight symptoms that would not produce appreciable yield losses (Kasrawi and Mansour, 1994; Pérez de Castro *et al*., 2007; Pérez de Castro *et al*., 2013). Nevertheless, several factors, such as plant age at the time of inoculation, inoculation pressure, and growing conditions can affect the responses to begomovirus infection (Picó *et al*., 1998; Lapidot *et al*., 2000; Lapidot *et al*., 2006; Levy and Lapidot, 2008). Moreover, the level of phenotypic expression of the distinct resistance genes is probably affected by environmental conditions and the virus isolate/species (Picó *et al*., 1998; Lapidot *et al*., 2000; Pérez de Castro *et al*., 2005; Pereira-Carvalho *et al*., 2015).

Lapidot *et al*. (2006) developed a scale for differentiating reactions to TYLCV in tomato hosts. The scale employs seven non-segregating host lines with reactions to TYLCV ranging from susceptible to highly resistant. One of the inbred lines, 'STY-1', is included as the susceptible standard, and the other six lines were derived from resistant material originating from different wild tomato relatives. The lines 'STY-2' to 'STY-5' are derived from resistant hybrids developed by different seed companies, while 'STY-6' and 'STY-7' are derived, respectively, from the Volcani Center breeding lines 'TY197' and 'TY172' (Lapidot *et al*., 1997). The scale was tested under different environmental conditions (greenhouse and open field) and using different plant ages. Symptom severity and virus accumulation were measured, and the yield loss induced by TYLCV infection was evaluated under field conditions. The ranking of the lines according to symptom scores was stable across the different experimental conditions, except for one genotype (Lapidot *et al*., 2006). This scale was recommended to determine and compare the level of resistance to TYLCD in evaluation assays. The scale has also been tested by whitefly-mediated inoculation in cages with an Australian isolate of TYLCV (Van Brunschot *et al*., 2010). However, the symptom scores obtained were systematically lower than in the assays carried out by Lapidot *et al*. (2006). These results demonstrate the need to test this set of standard tomato lines under different experimental conditions.

The objective of the present study was to evaluate the levels of resistance exhibited by the standard tomato lines of Lapidot *et al*. (2006) under experimental conditions employing inoculation and assessment methods routinely used by two different research groups (in Spain and Cuba). Different TYLCV isolates, inoculation methods, plant ages, and environmental conditions were compared.

Materials and methods

Plant material

The seven STY lines (provided by Lapidot, Volcani Center, Israel) that comprise the standards of the TYLCV resistance scale developed by Lapidot *et al*. (2006) were evaluated using the inoculation and assessment methods routinely used in two different research institutions in Spain and in Cuba (Table 1). Four assays (assay 1 to assay 4) were carried out at the 'Instituto de Conservación y Mejora de la Agrodiversidad Valenciana' (Valencia, Spain), and three assays (assay 5 to assay 7) were carried out at the 'Instituto Nacional de Ciencias Agrícolas' (San José de las Lajas, Cuba). The first two assays were developed in 2008, in, respectively, spring-summer (assay 1) and autumn-winter season (assay 2). Assays 3 and 4

Table 1. Environmental conditions, inoculation method and disease assessment used in the different assays described in this study.

Assay	Location	Season[c]	Isolate	Control	Inoculation	Assessment[d]
1	Spain[a]	SS	TYLCV-Mld[ES:72:97]	'Fortuna C'	Whitefly cage inoculation	S
2		AW	TYLCV-Mld[ES:72:97]	'Fortuna C'		S/A
3		SS	TYLCV-Mld[ES:72:97]	'Fortuna C' and 'TY52'		S
4		SS	TYLCV-Mld[ES:72:97]	'Fortuna C' and 'TY52'		S
5	Cuba[b]	SS	TYLCV-IL[CU]	'Campbell 28' and 'TY52'	Whitefly mass inoculation	S/A
6		AW	TYLCV-IL[CU]	'Campbell 28' and 'TY52'	Whitefly mass inoculation	
7		S	TYLCV-IL[CU]	'Campbell 28' and 'TY52'	Agroinoculation	

[a] 'Instituto de Conservación y Mejora de la Agrodiversidad Valenciana' (Valencia, Spain).
[b] 'Instituto Nacional de Ciencias Agrícolas' (San José de las Lajas, Cuba).
[c] SS, spring-summer; AW: autumn-winter; S: summer.
[d] S, Symptom evaluation; A: Viral accumulation.

were carried out simultaneously in 2012, during the spring-summer season, but used inoculated plants of different ages. In these four assays, ten plants per line were employed in each of the different experimental conditions. Ten to 20 plants of the breeding line 'Fortuna C' ('FC') were used as susceptible controls in all assays. Ten plants of the introgression line 'TY52', carrying the *Ty-1* gene (Michelson *et al*., 1994), were included as resistant controls in the two assays carried out in 2012. Assays 5 and 6 were conducted in 2009, in, respectively, spring-summer and autumn-winter seasons. Assay 7 was carried out in the summer of 2011. In assays 5, 6 and 7, ten plants per host line were inoculated. Also, ten plants each of cultivar 'Campbell 28' and the introgression line 'TY52' were used, respectively, as the susceptible and resistant controls.

Inoculation methods

Inoculation was carried out as routinely done in each of the research institutions. Whitefly-mediated inoculations (under growth chamber conditions) using muslin-covered cages were used in the four assays in Spain. Conditions in the growth chamber were: 25ºC, relative humidity 60 to 65% (day) and 95 to 99% (night), irradiance 34 $\mu Em^{-2}s^{-1}$ and a 16 h light 8 h dark daily cycle. The whitefly population used was classified as *Bemisia tabaci* biotype Q (supplied by F. Beitia, Instituto Valenciano de Investigaciones Agrarias, IVIA, Valencia, Spain) and the insects became viruliferous by feeding on tomato plants infected with the Spanish isolate of TYLCV-Mld[ES:72:97] (accession No. AF071228). Stage for inoculation varied depending on the assay. In assays 1 and 2 plants were inoculated at the three true leaf stage. To test applicability of the scale with plant inoculation at different host stages, plants were inoculated 14 days after sowing (das) in assay 3 and 28 das in assay 4. In all cases, plants were randomly distributed inside cages, with 50 whiteflies per plant for seven days. During the inoculation period plants were shaken daily to ensure the uniform distribution of the whiteflies. After the inoculation period, plants were sprayed with imidacloprid (Confidor, Bayer). Two days later, plants were transplanted to 12 L capacity pots filled with coconut fibre, transferred to an insect-proof greenhouse, and organized in randomized block experimental design. Plants were cultivated under controlled temperature and light conditions, and fertirrigated with the doses and frequency recommended for standard tomato cultivation.

Two different inoculation methods were used in the assays carried out in Cuba. Whitefly mediated mass inoculation was conducted in assays 5 and 6. Air temperature in the greenhouse was 25 ± 2ºC. Whiteflies were *B. tabaci* and they became viruliferous by feeding on tomato plants infected with the isolate TYLCV-IL[CU] (accession No. AJ223505). Plants were inoculated at the two true leaf stage,

with high inoculum pressure (≈100 whiteflies/plant), for 2 days. During the inoculation period, plants were shaken three times each day to ensure the uniform distribution of the whiteflies. After the inoculation period, plants were sprayed with imidacloprid (Confidor, Bayer) and then transplanted to beds in open field in a completely randomized experimental design. Agroinoculation was used in assay 7. The bacterial strain was LBA 4404 of *Agrobacterium tumefaciens* transformed with a dimeric copy of the viral genome of TYLCV-IL[CU] (Fuentes et al., 2006). Bacterial cultures were grown for 48 h at 28ºC in YEB medium supplemented with 50 mg L^{-1} rifampicin, 100 mg L^{-1} streptomycin, and 30 mg L^{-1} kanamycin. Cells were concentrated by centrifugation, suspended in MS liquid medium containing 0.2% acetosyringone, and immediately used for inoculation. Plants were inoculated at the two true leaf stage by injection of the bacterial suspension into the leaf axils, and were then maintained in a greenhouse at 28ºC-24ºC (day/night) and 80 ± 10% relative humidity.

Disease assessments

The disease assessments were carried out according to the protocols developed by each research institution. The scale used for all the assays was that proposed by Friedmann *et al*. (1998), with scores ranging from 0 (asymptomatic plant, inoculated plants show the same growth and development as non-inoculated plants) to 4 (very severe stunting and yellowing, and pronounced leaf cupping and curling; plant ceases to grow). Intermediate scores (0.5, 1.5, 2.5, or 3.5) were also used to obtain more precise evaluations. For assays 1 to 4, symptom development was scored at 15, 25, 35, 45, and 55 days post-inoculation (dpi), while for assays 5 to 7, the assessments were at 15, 30 and 45 dpi. The statistical significance of the different factors applied in the assays was calculated by ANOVA, and mean severity scores were compared using Fisher LSD at P=0.05.

In assay 2, virus accumulation was measured at each evaluation date by molecular hybridization, as described in Pérez-de-Castro *et al*. (2013). In brief, DNA was isolated and 2.5 µL of each sample and a 10-fold dilution were denatured and blotted on to nylon membranes. The probe (supplied by E.R. Bejarano, Universidad de Málaga, Spain) contained the intergenic region of the TYLCV-Mld[ES:72:97] isolate and was digoxigenin-labelled by PCR. Hybridization was carried out following manufacturer's instructions (Roche Molecular Biochemicals). Detection was carried out with CSPD by exposure to a CCD camera for approx. 1 h (Intelligent Dark Box-II, Fujifilm, Tokyo, Japan). Quantification of viral ssDNA was achieved by comparison with a standard curve of TYLCV dotted on the same membrane (ranging from 10 ng to 1 pg). Fluorimetry was used to quantify total plant DNA extracted (Hoefer DyNA Quant 300 fluorometer, according to manufacturer's instructions). Accumulation was expressed as ng of virus DNA μg^{-1} of total DNA isolated.

In assays 5, 6 and 7, virus accumulation was also measured by dot-blot molecular hybridization, as described by Fuentes *et al*. (2006). In brief, an extract was prepared from five plants of each genotype, and 3 µL of the extract were denatured and blotted on to nylon membranes. The probe contained a fragment of the intergenic region of the TYLCV-IL[CU] isolate and was random prime labelled with α-[^{32}P]-dCTP. Hybridization was conducted as described by Martínez *et al*. (2001). Detection was carried out by exposure to X-ray films (Kodak, mod. X-Omat AR) for 4 h at -70ºC. A standard curve of TYLCV-IL [CU] (ranging from 10 ng μL^{-1} to 0.01 ng μL^{-1}) was used to visually estimate the concentration of viral DNA in each sample.

Results

Assays 1 and 2

These two assays carried out in Spain with the same experimental conditions, differed only for the season in which the evaluation was carried out: assay 1 was during spring-summer and assay 2 during autumn-winter. The rate of symptom development was affected by the different seasons, as this factor was statistically significant at the 15, 25 and 35 dpi assessments (Table 2). The interaction genotype × season was only significant at 15 dpi. Symptom scores were higher in assay 2 at 15 dpi for the two most susceptible genotypes ('FC' and 'STY-1'), while they were similar in both seasons for the remaining genotypes (Figure 1a). Symptom scores tended to be higher in assay 2 for the first three evaluation dates. However, no significant differences were found at 45 and 55 dpi. The effect of the genotype was highly significant for all assessment periods. Variability among differ-

Table 2. P-values from ANOVA tests of the effects of experimental factors and their interactions, on symptom scores at the different evaluation dates, in assays 1-2 and 3-4.

Assays 1-2	15 dpi	25 dpi	35 dpi	45 dpi	55 dpi
Genotype	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Season	<0.0001	0.0077	0.0201	NS	NS
Interaction	<0.0001	NS	NS	NS	NS
Assays 3-4	**15 dpi**	**25 dpi**	**35 dpi**	**45 dpi**	**55 dpi**
Genotype	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Plant age	0.0135	0.5990	0.4252	NS	<0.0001
Interaction	0.0347	<0.0001	0.0027	NS	0.0063

ent plants of the same line was found for the most susceptible genotypes in both seasons, especially at the initial assessments. Symptom scores increased with assessment period for most of the genotypes. However, some genotypes such as 'STY-3' showed maximum symptom expression at early assessments but displayed subsequent recovery in both seasons (Figure 1a). Moreover, as previously stated, symptom intensification was delayed in assay 1 when compared to the assay 2, especially for the subgroup of susceptible genotypes. The final assessment date (i.e. 55 dpi, Figure 1a) was selected to classify the response to infection of each STY line. Three groups could be established, which displayed some consistency across seasons. The first group included only line 'STY-1'. This genotype was classified as susceptible. The response was similar to that found in line 'FC', which is the susceptible control routinely used in our inoculation assays. The second group included lines with intermediate levels of resistance. The lines 'STY-2' and 'STY-4' were classified within this group in both seasons, as they differed significantly from 'TY-1' and from lines with the highest levels of resistance. The highly resistant group comprised the lines 'STY-3', 'STY-6', and 'STY-7' in both assays. The line 'STY-5' showed instable disease reaction, being resistant in the autumn-winter assay, but of intermediate resistance in the spring-summer assay.

Virus accumulation was measured in the autumn-winter assay (assay 2). The differences among genotypes were clearer from 35 dpi than earlier, corresponding to higher virus accumulation (Figure 2). The highest values were detected at 55 dpi for most of the genotypes. At this date, virus accumulation in line 'STY-1' was higher than for the rest of the STY lines and comparable to the levels found in the susceptible control 'FC'. 'STY-1' was the only STY line classified as susceptible in our conditions according to symptom scores. Virus accumulation in 'STY-2' and 'STY-4' (intermediate resistance) was slightly higher than in 'STY-3', 'STY-5', 'STY-6', or 'STY-7' (classified as highly resistant). Considering all plants of the STY lines included in the assay, there was a moderate positive correlation, although highly significant, between symptom development and virus accumulation for each sampling date: values for correlation were 0.37 (P=0.0015) for 15 dpi, 0.38 (P=0.0012) for 25 dpi, 0.39 (P=0.0009) for 35 dpi, 0.37 (P=0.0015) for 45 dpi and 0.55 (P<0.0001) for 55 dpi.

Assays 3 and 4

Assays 3 and 4 were carried out simultaneously in the same season (spring-summer 2012). The inoculation conditions for both assays were the same, except for the age of the plants: inoculation was done at 14 days after sowing (das) in assay 3 and 28 das in assay 4.

The interaction of genotype × age for symptom development was statistically significant, except at 45 dpi (Table 2). The effect of age was significant at 15 and 55 dpi, with higher average symptom scores in plants of inoculated at 14 das. The genotype effect was highly significant for all disease assessments. Results in these assays were similar to those from assays 1 and 2 with respect to variability of the reaction

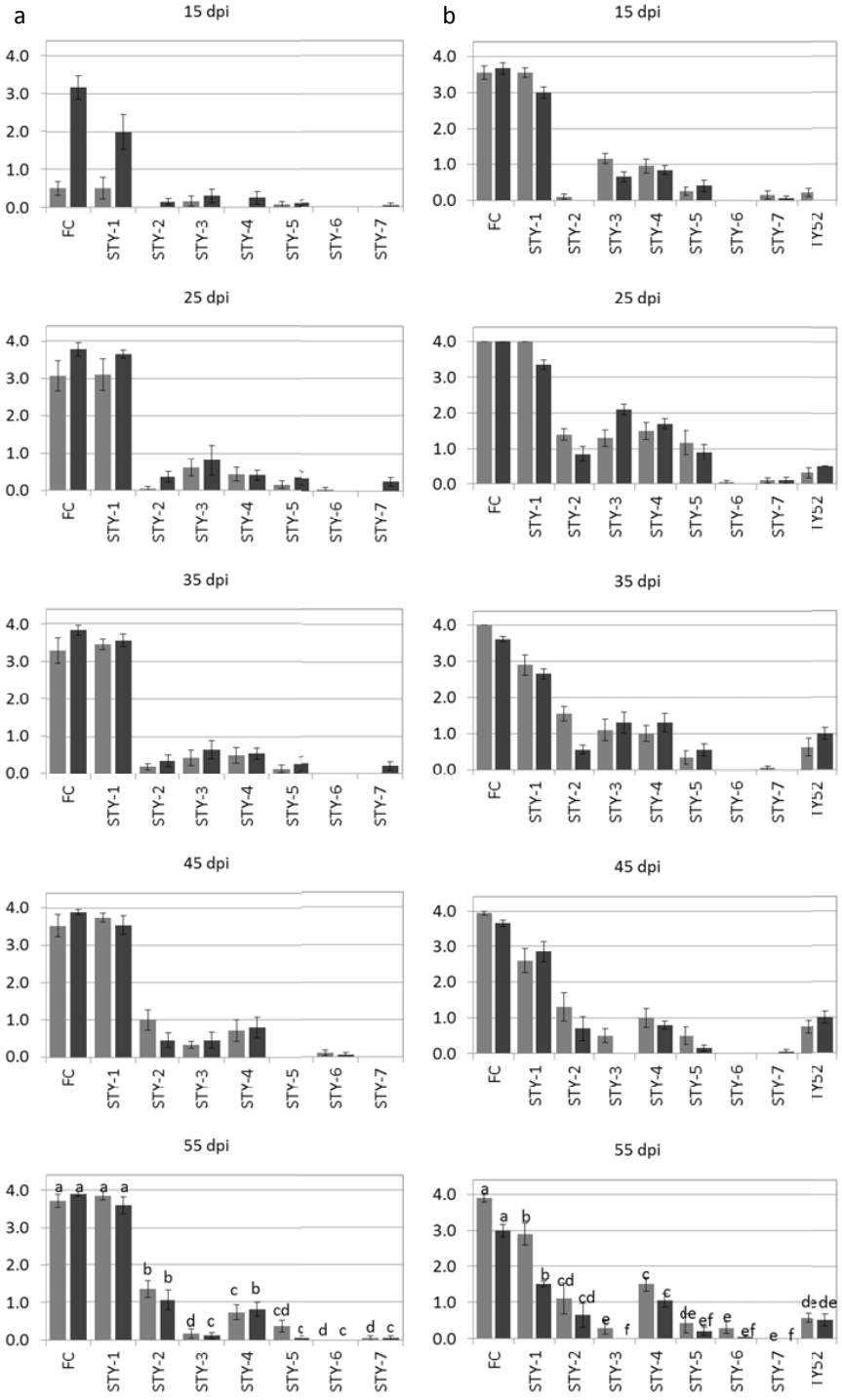

Figure 1. Average symptom score (± standard error) at each evaluation date (dpi: days post inoculation). Symptom scores were evaluated using a scale from 0 (symptomless) to 4 (severe symptoms). At 55 dpi, different letters for genotypes in the same assay indicate statistically significant differences (LSD, $P<0.05$). a) Assays 1 (light grey) and 2 (dark grey): STY lines and the susceptible control 'Fortuna C' (FC). b) Assays 3 (light grey) and 4 (dark grey): STY lines, the susceptible control 'Fortuna C' (FC) and the resistant control 'TY52'.

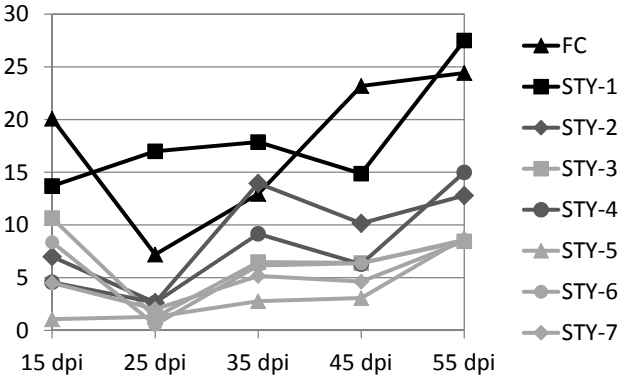

Figure 2. Virus accumulation (ng virus DNA/μg total DNA extracted) in assay 2 at different days post-inoculation (dpi) with *Tomato yellow leaf curl virus* of the STY lines and the susceptible control 'Fortuna C' (FC). Genotypes are classified according to symptom development: susceptible genotypes in black, intermediate resistance in dark grey and highly resistant in light grey.

to TYLCV among plants of the same line. Symptom development was earlier in assays 3 and 4 than in assays 1 and 2 (Figure 1b), but at the final assessment date (55 dpi) symptom scores were similar or even lower than in assays 1 and 2. In assays 3 and 4, for most of the genotypes, the maximum average symptom score occurred at 25 or 35 dpi, with a subsequent recovery of some of the plants at later assessments. Differences in symptom scores between plants inoculated at 14 and 28 das were significant for some of the genotypes. At 55 dpi, symptom scores were significantly higher in the most susceptible genotypes ('FC' and 'STY-1') for plants inoculated at younger development stages than those inoculated later. The susceptibility groups established in assays 1 and 2 were well conserved in assays 3 and 4. 'STY-1' was the line with highest symptom scores. However, considering symptom scores at 55 dpi in the assay conducted with plants inoculated at 28 das, the line 'STY-1' was classified as resistant (average symptom score = 1.5). Scores were higher at earlier assessment dates, but some plants recovered later. In any case, in assays 3 and 4, symptoms were more severe in the line 'FC' (the susceptible control routinely used by us), which was classified as susceptible. The lines 'STY-2' and 'STY-4' were included in the intermediate resistance group in assays 1 and 2. Both lines did not differ in assays 3 and 4, but differed significantly from 'STY-1'. The difference with respect to the previous assays was that 'STY-2' did not differ from some of the lines in the resistant group in assay 3. This group comprised lines 'STY-3', 'STY-5', 'STY-6', and 'STY-7'. The resistant control 'TY52' showed lower level of resistance than lines 'STY-6' and 'STY-7' when inoculated at 28 das, and was not significantly different from 'STY-2', classified as intermediate resistant.

Assays 5 and 6

Assays 5 and 6 were carried out in Cuba, each employing the same experimental conditions and differing only for the season of the evaluation: assay 5 was carried out in the spring-summer season and assay 6 in autumn-winter. Effects of the season, genotype, and the season × genotype interaction were significant for all evaluation dates. At 15 dpi, symptom scores were higher in spring-summer for the most susceptible genotypes, while for most of the other genotypes higher scores were observed in the autumn-winter assay. (Figure 3a). Variability was observed at 30 and 45 dpi. The symptom scores were similar in both seasons for some of the genotypes, although important differences occurred for others, such as 'STY-7'.

Variability in severity scores was low among plants of the same line in both assays. Symptoms scores either increased with date or were stabilized from 15 or 30 dpi for most of the genotypes. The line 'STY-5' was an exception, as recovery was observed in assay 6 (Figure 3a). The STY lines were classified according to the symptom scores at 45 dpi. The lines 'STY-1' and 'STY-2' were classified as susceptible in both assays, although symptom scores were significantly lower for 'STY-2', especially in the autumn-winter assay. 'STY-1' was comparable to the susceptible control 'Campbell 28'. The line 'STY-7' also displayed a susceptible reaction in the spring-summer assay. The rest of the lines were classified as resistant in this assay, divided in two subgroups: the line 'STY-3' and 'STY-6' showed intermediate resistance, while 'STY-4' and 'STY-5' showed higher levels of resistance, similar to the resistant control 'TY52'. In the autumn-winter assay, the line 'STY-7' was among the resistant lines. Symptom scores among all the resistant lines in this assay were equal to 1, except for 'STY-5', that showed the highest level of resistance.

Virus accumulation was measured for each line at the three sampling dates in both seasons. The maxi-

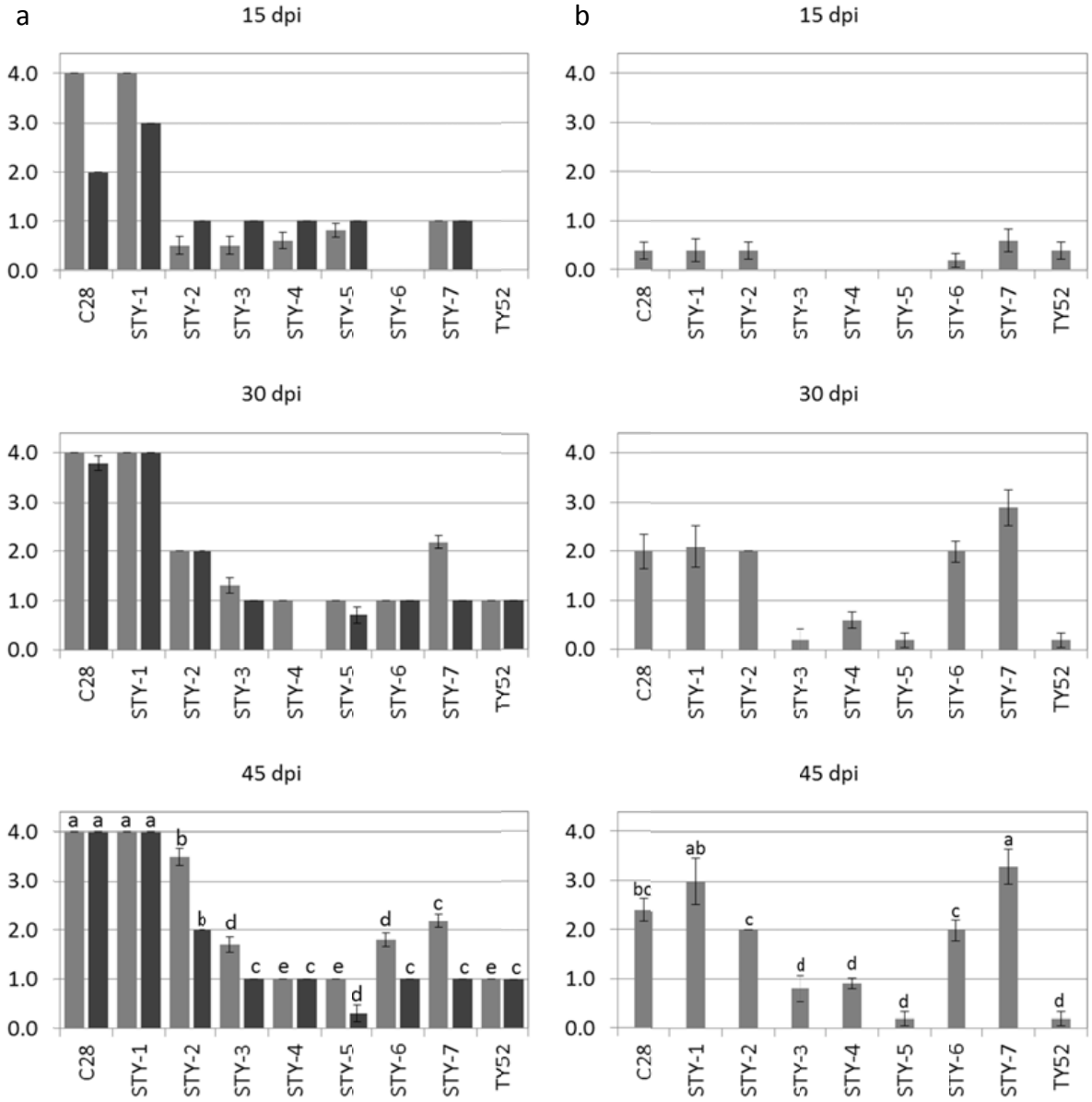

Figure 3. Average symptom score (± standard error) at each evaluation date (dpi: days post inoculation) in the STY lines, the susceptible control 'Campbell 28' (C28) and the resistant control 'TY52'. Symptom scores were evaluated using a scale from 0 (symptomless) to 4 (severe symptoms). At 45 dpi, different letters for genotypes in the same assay indicate statistically significant differences (LSD, $P<0.05$). A) Assays 5 (light grey) and 6 (dark grey). B) Assay 7.

mum accumulation was detected in the susceptible control 'Campbell 28' and the lines 'STY-1' and 'STY-2'; however there was no clear relationship between symptom scores and virus load for the rest of the genotypes.

Assay 7

This assay used the agroinoculation method. An overall delay in symptom development in this assay was observed when compared to the whitefly-mediated inoculation assays (Figure 3b). At 15 dpi,

average symptom score was below 1 for all the lines and the controls. Some of the genotypes reached the highest score at 30 dpi and this was maintained until 45 dpi. For the rest of the genotypes, the highest symptom score occurred at the final evaluation date. The line 'TY52' was the only exception: some of the plants scored 1 at 15 dpi and remained symptomless later. Response of some of the genotypes differed from the previous assays. The susceptible control 'Campbell 28', 'STY-1' and 'STY-2' were classified as susceptible. In the case of 'Campbell 28' and 'STY-1', symptom scores were markedly lower than in assays 5 and 6. The lines 'STY-6' and 'STY-7' were classified as susceptible after agroinoculation. Symptom score for 'STY-7' was not significantly different from that of 'STY-1'. The three remaining lines ('STY-3', 'STY-4', and 'STY-5') were classified as highly resistant. Average symptom scores in these lines were in all cases below 1 and comparable to the resistant control 'TY52'. No clear relationship was observed between symptom scores and viral accumulation.

Discussion

This study has evaluated the scale developed by Lapidot *et al*. (2006), to check its applicability in a diverse range of experimental conditions, in seven assays conducted in two geographic locations (Cuba and Spain), with different TYLCV isolates, inoculation methods, plant ages, and environmental conditions.

The results obtained were not comparable to those described by Lapidot *et al*. (2006), probably due to differences in the inoculation methods and to environmental influences in the level of host resistance. In addition, the viral isolates used were not the same. In the first place, the overall symptom scores were generally lower in the conditions assayed in both locations with respect to the results described by Lapidot *et al*. (2006). The results obtained did not correspond to a scale of graded levels of resistance; instead, it was only possible to establish three reaction groups of susceptible, intermediate resistant and highly resistant among the standard and control genotypes. For most of the conditions (assay 5 was the only exception), large differences were observed among the susceptible and the resistant lines, while the range for the symptom scores in the two resistant groups was narrow. Therefore, the possibility for establishing a reliable gradation of the levels of susceptibility and resistance in the plant material evaluated together with the standard lines was reduced with respect to the original purpose of the scale (Lapidot et al., 2006). Similar results were found when the STY scale was tested with the Australian TYLCV-IL[Au:Bri3:06] isolate using whitefly-mediated inoculation in cages. Similarly, the observed symptom scores were lower for each line compared with results reported by Lapidot *et al*. (2006), and a clear-cut grade scale was not obtained (Van Brunschot, 2010).

Regarding the ranking order of the lines in the scale, coincidence with the original description was higher in the assays carried out in Spain. Symptom scores in the 'STY-1' line where higher than in the other lines in assays 1 to 4. Lines 'STY-2' and 'STY-4' followed 'STY-1', although their order varied depending on the experimental conditions. 'STY-3' showed lower scores than expected, especially in assays 3 and 4. 'STY-3' was the only line for which the order was affected by the different conditions evaluated by Lapidot *et al*. (2006); moreover, the TYLCV-induced yield reduction in this line was lower than expected according to symptom severity. 'STY-3' was derived from a semideterminate hybrid, while 'STY-2', 'STY-4', 'STY-5', and 'STY-6' were derived from determinate hybrids. The difference in vegetative vigour may explain the high level of resistance observed in the line 'STY-3' in conditions more similar to those occurring in normal tomato production systems, such as those of used in assay 4 (with plants being inoculated later than in other assays).

The ranking order of lines 'STY-5', 'STY-6', and 'STY-7' was quite conserved in the Spanish assays. However, the results in the assays carried out in Cuba differed. The ranking order of the lines 'STY-1' to 'STY-5' was almost maintained with respect to the original scale. However, the lines 'STY-6' and 'STY-7' were not as resistant as in the experimental conditions reported by Lapidot *et al*. (2006). These lines showed variable responses depending on the season and the inoculation method. When the inoculation was carried out with whiteflies, the season was an important factor determining the host reactions. In assay 5, with warm temperatures, both lines showed symptom scores close to 2, with 'STY-7' being classified as susceptible, while in assay 6 both lines were scored with grade 1. Moreover, in assay 7, warm temperatures combined with agroinoculation intensified symptoms in 'STY-7' compared to assays 5 and 6.

Lines 'STY-2' to 'STY-5' are derived from resistant hybrids developed by different seed companies

(Lapidot *et al*., 2006). According to the results obtained in these assays, resistance in these host lines is effective against different isolates of TYLCV and under different inoculation and environmental conditions. Among the resistance sources for these four lines, the hybrid Tyking (source of the line 'STY-5') is the most studied (Giordano *et al*., 2005; Garcia-Cano *et al*., 2008; Hutton *et al*., 2012; Pereira-Carvalho *et al*., 2015). The resistance to TYLCV in 'Tyking' is conferred by the recessive gene *ty-5* (Hutton *et al*., 2012). This hybrid has been used worldwide, providing high levels of resistance in breeding programmes against different begomoviruses (Pereira-Carvalho *et al*., 2010; 2015; Hutton *et al*., 2012). In the conditions employed here, 'STY-5' also showed high levels of resistance, with low symptom scores and low virus accumulation as previously reported (Pereira-Carvalho *et al*., 2015). This was not the case for the lines 'STY-6' and 'STY-7'. These lines are derived from the breeding lines 'TY197' and 'TY172', respectively, which incorporate resistance from *S. peruvianum* (Lapidot *et al*., 1997; Friedmann *et al*., 1998). High levels of resistance to TYLCV have been reported in 'TY197' and 'TY172' by other authors using different inoculation techniques (including agroinoculation), different isolates and different conditions (Pérez de Castro *et al*., 2005; Anbinder *et al*., 2009), even against Cuban isolates of TYLCV (Dueñas *et al*., 2008; Piñón, 2009). Moreover, these two lines have showed good levels of resistance against other begomoviruses (Santana *et al*., 2001; Mejía *et al*., 2005; Bian *et al*., 2007). However, contradictory results have been obtained in some cases. For example, Bian *et al*. (2007) reported 'TY172' with symptoms scores of 2 at 30 days after agroinoculation. According to the results obtained in the present work, resistance in these lines can be partially overcome by the Cuban TYLCV isolate, especially under high temperature conditions. Moreover, in the case of 'STY-7', agroinoculation intensified the manifestation of the viral symptoms.

Evolution of symptoms varied depending on the experimental conditions and the tomato lines. Differences were found when comparing similar inoculation conditions in different seasons. In assays 1 and 2, there was a clear delay in symptom appearance in the assay carried out in the spring-summer season with respect to the autumn-winter assay. Temperatures at the inoculation time could be a possible explanation: higher temperatures in assay 2 produced a rapid progression in symptom development when compared to the assay 1.

In all cases, disease symptoms increased with sampling dates, and the classification of the genotypes at the final sampling date (55 dpi) was not affected by differences in symptom development. Assays 5 and 6 were also conducted with the same inoculation conditions but in different seasons. In this case, temperatures were lower at the beginning of the assay carried out during the autumn-winter season (assay 6, the most appropriate season for tomato cultivation in Cuba), which could explain the delay in symptom evolution in the most susceptible genotypes, compared with the assay during the spring-summer season. Unlike the results from assays 1 and 2, differences were found regarding classification of the genotypes in assays 5 and 6. The different conditions led to the classification of line 'STY-7' as susceptible under high temperature conditions (assay 5) and resistant in the autumn-winter season (assay 6). Other studies have reported differences in symptom development after inoculation with TYLCV related to differences in temperature, with higher symptom scores being reported under higher temperatures (Lapidot *et al*., 2006; García-Cano *et al*., 2008).

The effect of plant age at inoculation was also studied in assays 3 and 4. Symptom scores were lower for some of the genotypes, especially the susceptible lines, when older plants were inoculated. Lapidot *et al*. (2006) validated the scale inoculating 10 and 21 days old plants, and their results were slightly different. Symptom scores also tended to be lower in plants inoculated later, but they found no differences in the most susceptible genotype, 'STY-1', while differences were higher than in our assays for some of the other genotypes. Levy and Lapidot (2008) reported no effect of plant age on symptom scores in the susceptible genotypes, and only minor effects on resistant varieties, when using whitefly-mediated TYLCV inoculation on plants at 14, 28 and 45 das.

Discrepancies among the different assays can be due to differences in the environmental conditions and/or the virus isolates used. In the assays 3 and 4 there was a recovery in symptom expression for later evaluation dates for most of the genotypes. This recovery was especially important in susceptible lines when inoculated 28 das. The line 'STY-1' was classified as resistant at the end of the assay 4 (inoculation 28 das). This was probably a consequence of the more advance developmental stage (older plants) during the inoculation procedure. Reduction in viral accumulation, and in yield losses, in plants inoculated at

an older stage has also been related to the developmental stage and the higher vegetative vigour of these plants. Levy and Lapidot (2008) discussed the best time to inoculate tomato plants when evaluating resistance to TYLCV. Their proposal was to inoculate at early stages in screening assays aiming to select plants with the highest levels of resistance. However, in trials to evaluate resistance in commercial tomato lines, they recommended inoculation at later stages (approx. 28 das), to represent the commercial situation, where nurseries are well protected against the disease and infection occurs mainly after transplanting.

The results obtained in the work presented here provide useful information about the effects of different experimental conditions on the results obtained using the Lapidot *et al*. (2006) scale for evaluation of tomato reaction to TYLCV. In the first place, the fact that response obtained in the conditions assayed here do not correspond to the original grade scale, suggests the possibility of reducing the number of standard lines used. Our recommendation is to select the two genotypes with extreme responses (the most susceptible and the most resistant), as well as a line with intermediate resistance. Moreover, some genotypes with resistance derived from distinct genetic sources could be incorporated, to provide clear representation of the distinct levels of resistant responses. As an example, in the conditions assayed in Spain, 'STY-1' could be the susceptible control, 'STY-4' the standard line for intermediate resistance, and 'STY-7' could be the resistant control. To increase the discrimination capacity, it would be advisable to incorporate more lines with lower resistance, such as the ones derived from the wild species *S. pimpinellifolium* (Pérez de Castro *et al*., 2007). Besides that, the variability in symptom evolution, depending on the line and on the season, highlights the importance of a standardised inoculation method and appropriate selection of criteria for the evaluation of the resistance. This will ensure that the experimental conditions and the evaluation period will provide more consistent genotype responses.

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