Improved Diagnostic Techniques for Tomato Yellow Leaf Curl Virus in Tomato Breeding Programs

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ABSTRACT

Pico, B., Díez, M. J., and Nuez, F. 1999. Improved diagnostic techniques for tomato yellow leaf curl virus in tomato breeding programs. *Plant Dis.* 83:1006-1012.

Breeding for tomatoes resistant to tomato yellow leaf curl geminivirus (TYLCV) is difficult, mainly due to the lack of an accurate system for selecting resistant plants. The variable nature of the genotypes used (wild or wild-derived genotypes and advanced breeding lines) makes the development of a reproducible selection procedure necessary during the breeding program. Serological and nucleic acid-based diagnostic techniques for detecting TYLCV were evaluated for their sensitivity, reliability, and possibility of quantification in order to select the combination of techniques that provides the most rapid and accurate characterization of the resistance level of each item. Squash blot followed by the more sensitive polymerase chain reaction (PCR) detection of inconclusive samples is recommended for the screening of new resistant sources among asymptomatic *Lycopersicon* spp., as they accumulate extremely low viral concentrations that cannot be detected by serological methods. A triple antibody sandwich-enzyme-linked immunosorbent assay (TAS-ELISA), modified to reduce the background, can be used in large-scale field screening of advanced breeding lines if combined with the more sensitive squash blot, which is less affected by the age and state of the tissue. Hybridization methods were also more appropriate to assess viral distribution throughout the plant than TAS-ELISA or even PCR, which fails to reliably detect TYLCV in tissues like roots.

Additional keywords: *Bemisia tabaci*, *Lycopersicon chilense*, *L. esculentum*, *L. peruvianum*, screening for resistance, whitefly-transmitted geminivirus

Tomato yellow leaf curl geminivirus (TYLCV) is a whitefly-transmitted geminivirus that causes serious economic losses to tomato crops all over the world (26). Outbreaks of diseases caused by TYLCV and other closely related geminiviruses are being reported in new areas (6,9,18) and affecting new crops (30). In Spain, TYLCV has mainly affected tomato crops, but recently it has also been detected in bean fields (22) and infecting new weed hosts (3).

Cultural practices adopted to control TYLCV epidemics must be combined with use of tolerant or resistant tomato varieties in severe open-air attacks (17,27). Unfortunately, current breeding programs based on resistance found in wild *Lycopersicon* spp., mainly in *L. chilense*, are progressing slowly, partially due to the lack of effective means for accurate selection of resistant plants (32,33). In other viral diseases affecting tomato, marker-assisted selection (MAS) provides a convenient and rapid

assay for selection during breeding programs (4,10), allowing the molecular detection of resistance genes. Restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) markers have been reportedly linked to the major gene of TYLCV resistance, *Ty-1*, derived from *L. chilense*, and to a locus involved in quantitative resistance to TYLCV derived from *L. pimpinellifolium* (5,20,36). However, the apparently complicated genetics of the character, i.e., the existence of minor modifying genes and the incomplete dominance and partial breakdown of resistance, still make MAS not feasible. Therefore, selection for TYLCV resistance depends on the phenotypic expression of TYLCV resistance genes.

The mild symptoms of TYLCV infection exhibited by wild tomato relatives and wild-derived material prevent accurate management of segregating populations and advanced breeding lines when selection is based solely on symptom severity (27,33). Therefore, sensitive, rapid, and reliable TYLCV detection methods are needed to accelerate the development of tomato cultivars resistant to TYLCV.

Several techniques have been used to detect TYLCV and assess its accumulation in host tissues. Immunoassays, i.e., triple antibody sandwich-enzyme-linked im-

munosorbent assay (TAS-ELISA) and tissue-blot immunoassay (TBIA), employed in the diagnosis of other virus diseases have limitations to the specific detection of geminiviruses due to cross-reaction caused by the high homology among their coat proteins (31). This limitation has been used as an advantage to detect TYLCV by using heterologous antisera (2,14,19). Antisera against TYLCV have recently become available commercially (15).

Sequence analysis of different TYLCV isolates has resulted in three homology groups: TYLCV-Tha (Thailand), TYLCV-Isr (Israel), and TYLCV-Sar (Sardinia) (26). This has allowed the development of nucleic acid probes for squash, dot, and southern blotting hybridization (7,8,23,29,37) and the design of degenerate or specific primers for broad-range or for specific polymerase chain reaction (PCR)-based detection methods (12,21,24,34).

Use of different detection techniques may lead to noncomparable and contradictory results, making the progress of breeding programs difficult. Therefore, an appropriate combination of detection techniques is needed to better characterize resistance found in different genotypes, optimizing the selection process.

In this study, we evaluated the use of different detection techniques, e.g., serological methods (TAS-ELISA, squash, and dot immunoblotting) and nucleic acid-based techniques (squash blot, dot blot, and PCR), to search for new wild sources of TYLCV resistance, to screen wild-derived genotypes with different degrees of resistance, for field screening of advanced breeding lines, and for monitoring viral accumulation in different plant tissues, which is necessary for a better understanding of resistance mechanisms.

MATERIALS AND METHODS

Plant materials. Plant materials from different steps in a breeding program were used. Accessions of *L. peruvianum* and *L. chilense* were used in the screening of new wild resistance sources: *L. peruvianum* PI-126935 and PI-143679 are both asymptomatic carriers of TYLCV-Sar (27). The former has also been used in Israel as a resistance source against TYLCV-Isr to produce commercial tomato hybrids (28). These accessions were supplied by the Plant Introduction Station, USDA, Ames,

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Accepted for publication 19 July 1999.

Iowa. *L. chilense* LA 2884 and LA 1932 have different levels of resistance to TYLCV-Sar (27); and *L. chilense* LA-1969, which is the carrier of the TYLCV resistance gene *Ty-1* used in current breeding programs (20,35), was supplied by C. M. Rick, Tomato Genetics Resource Center, University of California, Davis.

Wild-derived materials were also employed: an interspecific hybrid obtained from the cross *L. esculentum* × *L. peruvianum* PI-143679 (F1-*per*) by using pollen mixture and embryo culture techniques, and a second backcross to tomato derived from the cross *L. esculentum* × *L. chilense* LA 1938 (BC2-*chil*).

In field screening, advanced tomato breeding lines derived from *L. chilense* LA 1932 and LA 1938 (5°-8° backcross) were also used.

In interspecific crosses, the tomato line FC, carrier of the recessive marker gene *c* (potato leaf), was used as a female parent. This line was also used as a TYLCV-susceptible control in all assays.

Whitefly maintenance and virus source. *Bemisia tabaci* colonies were reared on healthy plants of the tomato line FC. These plants were grown in muslin-covered cages. An isolate of TYLCV belonging to the TYLCV-Sar group was obtained from tomato plants from Murcia (a tomato producing region in southern Spain) and maintained in plants of the tomato line FC by whitefly transmission, renewing the virus stock every month.

Inoculation procedures. Two artificial whitefly-mediated inoculation methods, cage and mass inoculation, were used to inoculate wild and wild-derived genotypes, essentially as described in Pico et al. (27).

Cage inoculation. *B. tabaci* adults were given a 48-h acquisition access period on TYLCV-infected tomato source plants. Whiteflies were then moved to fourth-leaf-stage seedlings of each genotype grown in trays (25 × 15 cm) that were individually exposed to 15 to 25 viruliferous whiteflies per plant for 4 days in whitefly-protected cages. After inoculation, plants were sprayed with imidacloprid (Confidor) and held in an insect-proof greenhouse for symptom development and sampling.

Mass inoculation. Plants of different genotypes were grown in pots in mesh-protected greenhouses. *L. esculentum* plants showing severe TYLCV symptoms and maintaining a high population of *B. tabaci* were used as an inoculum source (one infected source plant per five tested plants). Plants were periodically agitated to obtain a uniform vector distribution. No chemicals were applied during the assays.

Natural infection. Natural infection was monitored in advanced tomato breeding lines derived from *L. chilense* in summer-autumn growing cycles in commercial fields in Murcia (southern Spain).

Symptom monitoring and sampling. Symptom severity was assessed for each

plant and graded on a scale of 0 (symptomless) to 4 (severe symptoms). In all cases, individual plants were tested for the presence of TYLCV virions and/or viral DNA on different days postinoculation (DPI). To evaluate different diagnostic techniques, three sets of samples were collected from each tissue per plant. One set of samples was used for serological assays, a second was squashed onto nitrocellulose or nylon membranes for serological and/or nucleic acid-based detection, and the other was extracted for dot blot and PCR analyses.

Serological assays. For TYLCV detection, commercial polyclonal and monoclonal antisera against TYLCV (ADGEN Diagnostic Systems, Scotland) were used. Serological techniques, including TAS-ELISA, and squash and dot immunoassays (TBIA) were tested for the detection of TYLCV.

TAS-ELISA. Two TAS-ELISA procedures were used. (i) Standard TAS-ELISA (S-TAS): This procedure was conducted essentially as reported in Pico et al. (27). Polyclonal antiserum against TYLCV was diluted 1:1,000 in carbonate buffer (pH 9.6) to coat wells in standard 96-well microtiter plates and incubated for 2 h at 34°C. Tissue from infected plants and healthy controls was extracted in 1:10 (wt/vol) extraction buffer as described by Macintosh et al. (19). Monoclonal antiserum was used diluted 1:1,000 in phosphate buffered saline (PBS), and rabbit anti-mouse globulin/alkaline phosphatase conjugate was diluted 1:1,500 in PBS. Absorbances ($A_{405\text{ nm}}$) after overnight incubation at 4°C with substrate (*p*-nitrophenyl phosphate) were scored as follows: 0 ($\leq$ twice that of the healthy control); 1 ($>$ twice that of the healthy control but $\leq$ 0.6); 2 ($>$ 0.6 but $\leq$ 1.2); 3 ($>$ 1.2 but $\leq$ 1.8); 4 ($>$ 1.8) (19).

(ii) Modified TAS-ELISA (M-TAS): The previous procedure was modified by blocking the plates with PBS + 4% BSA (bovine serum albumin) for 1 h after polyclonal coating, and by adding 1% BSA to the extraction buffer, the monoclonal antiserum buffer, and the globulin/alkaline phosphatase conjugate buffer (kindly suggested by I. Hita, CIDA-Murcia, Spain).

Immunoblotting. For squash immunoassays, 0.5 cm of stem, leaf, and root sections were cut and pressed onto a nitrocellulose membrane. For dot immunoassays, 5 μ l of crude extract prepared as for TAS-ELISA was spotted onto membrane. Single whiteflies reared on infected plants of the tomato line FC were directly squashed onto the membrane.

The technique described by Abou Jawdah et al. (2) was followed with minor modifications. Nitrocellulose membranes were treated for 1 h with blocking buffer (Tris buffered saline (TBS), pH 7.4, containing 2% gelatin and 2% Triton). After blocking, membranes were incubated with monoclonal antiserum (ADGEN) diluted

1:1,000 in TBS + 0.1% BSA for 1 h followed by rabbit anti-mouse globulin/alkaline phosphatase conjugate diluted 1:1,000 in TBS + 0.1% BSA for 1 h. Finally, the blots were incubated for 10 to 15 min in the substrate solution. TYLCV was detected in tissue blots by the formation of a black-purple precipitate.

Nucleic acid-based assays. Viral DNA was detected by squash and dot blotting. For squash blotting, sections of 0.5 cm of different plant tissues were directly squashed onto a nylon membrane. Single whiteflies were spotted onto membranes as described for immunoblotting.

Total nucleic acids and sap extracts were obtained for dot blots from both infected plants and healthy controls according to the following four extraction procedures. In all cases, plant tissue was frozen in liquid nitrogen and ground into a fine powder.

Total nucleic acids extracts.

(i) Extraction procedure 1 (EP1) (8): Frozen tissue was crushed in boiling extraction buffer (2% CTAB, 100 mM Tris-HCl [pH 8], 10 mM EDTA, 1.4 M NaCl, 1% 2- β -mercaptoethanol) and incubated for 5 min at 55°C. Supernatants were extracted after washing with 1 vol of phenol:chloroform:isoamyl 25:24:1. Total nucleic acids were recovered by precipitation with absolute ethanol. The pellets were washed with 70 and 100% ethanol, dried, and resuspended in TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 7.5]).

(ii) Extraction procedure 2 (EP2) (modified from Dellaporta et al. [11] according to Crespi et al. [7]): Frozen tissue was crushed in extraction buffer (100 mM Tris-HCl [pH 8], 50 mM EDTA, 500 mM NaCl, 10 mM 2- β -mercaptoethanol, and 1% sodium dodecyl sulfate [SDS]) and incubated at 65°C for 5 min. Then 0.25 vol of 5 M potassium acetate (pH 4.8) was added and samples were incubated on ice for 10 min. After centrifugation for 10 min, supernatants were precipitated with isopropanol and processed as in EP1.

Sap extracts.

(iii) Extraction procedure 3 (EP3) (15): Frozen tissue was crushed in extraction buffer (50 mM Tris-HCl, 1 mM EDTA [pH 8]), and 1 vol of 1 M NaOH was then added. Supernatants were recovered after incubation at room temperature for 30 min and 10 min centrifugation.

(iv) Extraction procedure 4 (EP4) (modified from Rom et al. [29]): Frozen tissue was crushed in 0.4 M NaOH. Supernatants were recovered after 2 min centrifugation.

Five μ l of each sample corresponding to about 5 mg of fresh tissue and serial aliquots were spotted onto nylon membranes for dot blot hybridization.

Hybridization. Both dot and squash samples blotted on membranes were UV-crosslinked and overnight hybridized at 65°C with TYLCV-DNA probe at approximately 10 ng/ml in buffer containing

phosphate buffer (pH 7.2), 1 mM EDTA, and 7% SDS and washed once in 2× SSC-2% SDS (10 min) at room temperature, and then twice in 0.1× SSC-0.1% SDS (15 min) at 60°C. The probe routinely employed (kindly provided by E. R. Bejarano, Universidad de Málaga, Spain) represented the entire genome (2.77 kb) of a Spanish TYLCV isolate (TYLCV-Alm) belonging to the TYLCV-Sar group, and was ³²P-dCTP radiolabeled by random-priming. After hybridization, membranes were exposed to a phosphorimager screen (Bioimaging analyzer, BAS-1500, Fujifilm, Tokyo). The amount of viral ssDNA in each dot was quantified according to a standard curve of TYLCV DNA (ranging from 10 ng to 1 pg) dotted on the same membrane. Densitometric squash blotting results were scored from 0 (no reaction) to 4 (strong reaction = reaction of the susceptible control).

PCR. The two primers were designed based on a common sequence of TYLCV-Sar and TYLCV-Isr (sense 5'-CGC CCG TCT CGA AGG TTC-3' and antisense 5'-GCC ATA TAC AAT AAC AAG GC-3') (kindly supplied by J. L. Cenis, CIDA, Murcia, Spain). Five µl of each sample was used in a total volume of 25 µl in the following PCR mixture: 2.5 µl of 10 mM of each primer, 2 µl of 2.5 mM of each dNTP, 1.5 µl of 25 mM MgCl₂, 2.5 µl of 10× Taq buffer, and 2 units of Taq DNA polymerase (Boehringer Mannheim, GmbH, Germany). The cycling profile was 10 min of melting at 95°C followed by 35 cycles of melting, annealing, and DNA extension of 1 min at 95°C, 30 s at 53°C, and 2 min at 72°C, respectively. The predicted length of the amplified fragment is approximately 670 bp. PCR products were electrophoresed in 1% agarose gels and photographed after ethidium bromide staining.

Comparison of sensitivity. Crude M-TAS-ELISA extracts of susceptible and partially resistant genotypes were serially diluted to evaluate the sensitivity of this serological method. Serial dilutions of EP2 extracts derived from susceptible and partially resistant materials were used for comparison of sensitivity of hybridization and PCR detection methods.

Statistical analysis. Statistical analyses were carried out by means of both one-way and multifactor analysis of variance, and by a Newman-Keuls multiple range test for mean comparisons (Statgraphics Plus, Statistical Graphics Corp.).

RESULTS

TYLCV detection by serological methods. TYLCV was detected using polyclonal and monoclonal antisera by the three serological procedures, TAS-ELISA, squash, and dot immunoblotting, in susceptible controls. Two TAS-ELISA procedures, S-TAS and M-TAS, were used. Analysis of variance indicated significant differences in the readings of healthy controls with both procedures (Table 1). The mean background value was higher when using S-TAS-ELISA than that obtained with M-TAS-ELISA.

S-TAS-ELISA values were erratic, with false negatives in susceptible genotypes (FC) showing clear TYLCV symptoms and unexpectedly high readings in some samples from highly resistant genotypes (*L. chilense* LA 1932) in which a reduced viral accumulation would be expected (27). M-TAS-ELISA allowed for a more effective differentiation among susceptible geno-

types, positives, and highly resistant materials, negatives. The most discriminatory results were obtained with samples derived from apex tissues, where viral accumulation is expected to be higher. Due to the reduced background and the consistency of the results, M-TAS-ELISA was selected for the latter assays.

Comparison of TAS-ELISA and immunoblotting. M-TAS-ELISA was successfully employed to study the pattern of TYLCV distribution in susceptible genotypes (FC) (Fig. 1), whereas both squash and dot immunoblotting detected TYLCV only in tissues in which viral accumulation was significantly higher (apex and higher stem sections). The weak signals obtained in the other tissues were masked by a high background, also found in healthy controls, probably caused by poor blocking of the membrane. Neither procedure, M-TAS-ELISA or immunoblotting, was found suitable to accurately study the pattern of TYLCV distribution in the partially resistant *L. peruvianum* PI-126935.

TYLCV detection by nucleic acid-based assays. Viral TYLCV ssDNA was successfully detected in direct squashes of tissues and in sap or nucleic acid extracts by using the described TYLCV-Sar probe.

Fig. 1. Detection of tomato yellow leaf curl geminivirus (TYLCV) by modified triple antibody sandwich–enzyme-linked immunosorbent assay (M-TAS-ELISA) and tissue immunoblotting in samples of different plant tissues from susceptible tomato and a partially resistant wild genotype. DPI: Days post cage whitefly-mediated inoculation. Absorbances (A_{405nm}) were recorded 24 h after addition of substrate and represent averages of four wells. APEX: Shoot apex. S-1: Stem section at the apex region. S-2: Stem section in middle of plant. S-3: Basal stem section. R: Roots. (+) represents visual rating of both squash and dot immunoblotting. (+/-) indicates inconclusive diagnosis due to high background.

Table 1. Tomato yellow leaf curl virus (TYLCV) detection by using standard and modified triple antibody sandwich–enzyme-linked immunosorbent assays (S- and M-TAS-ELISA) in susceptible tomato and a highly resistant *Lycopersicon chilense* accession

Genotype	Young leaf tissue		Shoot apex tissue		Symptoms ^b
	S-TAS ^a	M-TAS	S-TAS	M-TAS	
<i>L. esculentum</i> FC (30 DPI) ^c	0.5	1.25	1.5	2.75	4
<i>L. esculentum</i> FC (10 DPI)	1.5	0.75	1	1.25	1
<i>L. chilense</i> LA 1932 (30 DPI)	0.75	0.25	2.25	0.25	0
Healthy control– <i>L. esculentum</i> FC	0.257 ± 0.113 ^d	0.098 ± 0.0254 ^d	0.211 ± 0.0474 ^d	0.07 ± 0.0175 ^d	0
	$P = 0.0340$		$P = 0.014$		

^a Absorbances were taken 24 h after addition of substrate and represent means of four wells. Numbers indicate ELISA readings (A_{405nm}) score as follows: negative = 0 (≤twice the absorbance on the healthy control); 1 (>twice the healthy control but ≤0.6); 2 (>0.6 but ≤1.2); 3 (>1.2 but ≤1.8); 4 (>1.8).

^b Mean scores of symptoms for each genotype: 0 (symptomless) to 4 (severe symptoms).

^c DPI: days post cage whitefly-mediated inoculation.

^d Analysis of variance (ANOVA) results: Mean, standard deviation, and *P* values for significant differences between S-TAS-ELISA and M-TAS-ELISA readings.

The detection limit, 1 to 5 pg, was estimated by using a calibration curve constructed with samples of known concentration of purified TYLCV DNA. Similar sensitivity was obtained for both dot blot and squash blot procedures. Hybridization with healthy controls was not observed in any case.

Extraction procedures. The four extraction procedures allowed us to distinguish between susceptible and partially resistant genotypes (Table 2). The relative order of the different genotypes based on the level of viral accumulation was consistent using the four EP, and was positively correlated with symptomatology for the most susceptible (FC) and the most resistant genotypes (*L. peruvianum* PI-126935 and *L. chilense* LA 1932). However, differences in ssDNA accumulation were not directly correlated with symptom severity in wild-derived materials with intermediate levels of resistance (F1-*per* and BC2-*chil*).

The analysis of variance indicated significant differences in the concentration of TYLCV ssDNA extracted by the four methods in each genotype (Table 2). EP2, EP3, and EP4 were effective for extraction of detectable TYLCV DNA in spots corresponding to as little as 5 µg of plant tissue from susceptible FC, 10 to 20 µg of plant tissue from moderately resistant materials (F1-*per* and BC2-*chil*), and 0.1 mg of plant tissue in partially resistant wild sources (*L. peruvianum* PI-126935). The amount of viral ssDNA obtained by the EP1 procedure was 2 to 20 times lower than that obtained with the other EP, probably because of DNA losses during the purification procedure. Thus, EP1 did not yield detectable amounts of viral DNA from less than 1 mg of tissue in the partially resistant

L. peruvianum PI-126935. Viral DNA was only barely detected in some single samples of EP2, EP3, and EP4 from extracts corresponding to 5 mg of plant tissue of *L. chilense* LA 1932.

TYLCV detection by PCR. Since dot blotting failed to consistently detect TYLCV in highly resistant genotypes, the four EP extracts were used as templates for PCR amplification. Only EP1 and EP2 yielded DNA of quality to be directly used in PCR (Table 2). Only erratic amplifications (+/-) were obtained with the fast sap extraction procedures EP3 and EP4, even after serial dilutions, probably due to the existence of sap contaminants that may inhibit *Taq* DNA polymerase amplification. These extracts should be purified with resins or columns before their use for PCR amplification. EP2 was selected for further assays as being more effective than EP1 in extracting high-quality TYLCV DNA.

Comparison of sensitivity of serological and nucleic acid-based methods. The sensitivity of M-TAS-ELISA and PCR was compared with that of the hybridization methods by quantifying the ssDNA concentration in each EP2 sample by dot blot according to the calibration curve constructed with known amounts of purified TYLCV DNA (Table 3). Using this reference, M-TAS-ELISA was estimated to detect TYLCV in samples with viral concentration of >100 pg of viral ssDNA. PCR was, as expected, more sensitive than hybridization methods, detecting TYLCV in samples with less than 1 pg of ssDNA, that is, in less than 1 µg and 0.1 mg of fresh tissue from susceptible and highly resistant material, respectively.

TYLCV detection in whiteflies. The two membrane methods (squash immuno-

blotting and squash blotting) were successfully used for rapid and reliable detection of TYLCV and TYLCV-ssDNA, respectively, in squash blots of individual virus-carrier whiteflies. Both methods were equally effective in detecting the first viruliferous adults in a healthy whitefly population reared on infected FC tomato plants before the appearance of symptoms. Therefore, both could be used to score inoculum pressure in whitefly population in field conditions.

Screening for new wild resistance sources. M-TAS-ELISA, squash blotting, and PCR were compared to search for new resistant *Lycopersicon* spp. sources. TYLCV detection percentages, compared with the total plants inoculated, were lower with M-TAS-ELISA (10 to 40%) than with squash blot or PCR (20 to 100%) (Table 4). The latter allowed a more accurate differentiation among resistance levels of different *Lycopersicon* spp., placing them into two groups. The first group includes the accessions *L. peruvianum* PI-126935 and PI-143679, and *L. chilense* LA 2884, which are asymptomatic TYLCV carriers exhibiting high percentages of infected plants (>85%). Although PCR is more sensitive than squash blot, the latter allows for a rough quantification of viral DNA accumulation (VA) that successfully differentiates between the resistance levels of the two accessions of *L. peruvianum* (VA = 1.4 in PI 143679 > VA = 0.8 in PI 126935) with similar infection percentages. The second group includes the accessions *L. chilense* LA 1932 and LA 1969, with lower percentages of infection (<30%) and reduced virus content (VA < 0.2 in both cases).

Field assaying of advancing tomato breeding lines. The three diagnostic tech-

Table 2. Detectable tomato yellow leaf curl virus (TYLCV) ssDNA by hybridization with a radiolabeled probe and polymerase chain reaction (PCR) in samples from fresh shoot apex tissues using four extraction procedures

Genotype	EP1 ^a	EP2	EP3	EP4	Symptoms ^b
<i>L. esculentum</i> FC	0.38 ^c A 15 ^d + ^e	2.2B 5 +	2 B 5 -	3 B <i>P</i> = 0.0000 5 +-	4
BC2- <i>chil</i>	0.12A 50 +	0.71B 10 +	0.32A 20 -	0.62B <i>P</i> = 0.0004 10 +-	2
F1 <i>per</i>	0.045A 110 +	0.72B 10 +	0.53B 10 -	0.75B <i>P</i> = 0.0000 10 -	1
<i>L. peruvianum</i> PI-129365	0.005A 1,000 +	0.06B 100 +	0.076B 100 -	0.073B <i>P</i> = 0.0000 100 -	0.5
<i>L. chilense</i> LA 1932	ND ^f +	NCD +	NCD -	NCD -	0

^a EP: extraction procedures.

^b Mean scores of symptoms for each genotype: 0 (symptomless) to 4 (severe symptoms).

^c Viral ssDNA pg/µg fresh tissue (30 DPI: days post cage whitefly-mediated inoculation). Analysis of variance (ANOVA) results: Data are means of six replicates. *P* values for significant differences among EP. Different capital letters indicates significant differences in viral ssDNA accumulation obtained for each genotype by using different EP (Newman Keuls test).

^d Minimal tissue amount (µg) in which TYLCV ssDNA could be detected considering a detection limit of 5 pg of TYLCV DNA.

^e Results of PCR detection in three serial 1/10 dilutions (+ = detected, - = not detected, +- = variable response).

^f ND: nondetected; NCD: nonconsistently detected.

niques were also used for large-scale detection of TYLCV in advanced breeding lines grown in commercial fields in Murcia (southern Spain). Initial sampling by using TAS-ELISA allowed identification of a high number of positives. However, a high number of misleading results was also obtained, mainly in plants with slight symptoms or in plants suspected to be infected with other viruses such as TSWV or ToMV. Sixty-three percent of the M-TAS-ELISA misleading samples were clearly positive with molecular hybridization, while only 40% were positive by PCR, probably due to the advanced developmental stage of plants that yield extracts with high levels of contaminants.

Selecting the appropriate tissue and time of sampling. TYLCV accumulates mainly in dividing tissues, i.e., apex and roots after inoculation. The time at which viral accumulation begins and the accumulation level could be reliable indicators of resistance. Therefore, we compared the reliability of M-TAS-ELISA, molecular hybridization, and PCR in detecting and quantifying TYLCV in different plant tissues, shoot apex and roots, at different DPI in susceptible (FC) and partially resistant material (BC2-*chil*) (Fig. 2).

The earliest detection (4 DPI) was by PCR in shoot apex tissues, reaching 100% detection after a week in both FC and BC2-*chil*. The effectiveness of PCR detection decreased in advanced states of infection (30 DPI), when plants exhibited severe symptoms and the level of contaminants increased the erratic results. This could also explain the low percentages of PCR detection when using root samples.

Hybridization procedures were more effective than PCR in distinguishing between susceptible and partially resistant material. Both squash and dot blotting detected

DNA in shoots and roots from 4 and 8 DPI on. The greatest differences among genotypes were found at 12 to 16 DPI.

A high correlation was found with M-TAS-ELISA and squash blot with the symptomatology in shoot apex samples in both genotypes (Fig. 2). However, M-TAS-ELISA failed to reliably detect TYLCV in early and late stages of infection, resulting in a high number of inconclusive results. M-TAS-ELISA gave unexpectedly high values in roots samples, which do not allow accurate infection monitoring and do not differentiate between the resistance levels of FC and BC2-*chil* as accurately as squash blot.

DISCUSSION

Breeding programs aimed at developing tomato cultivars resistant to TYLCV have progressed slowly, mainly due to the heterogeneous conditions in which they are conducted, i.e., the variability among TYLCV isolates and/or *B. tabaci* biotypes, the use of different inoculation techniques, and especially the lack of an accurate sys-

tem for selection during the breeding process.

Although the last evaluation of the level of resistance must correspond to the effect of infection on total yield and yield components (17), during the breeding program, the percentage of infected plants and the level of viral accumulation can serve as indicators of resistance. A combination of serological and nucleic acid-based detection methods can be successfully used to characterize the level of resistance of genotypes.

M-TAS-ELISA allows for an accurate differentiation between highly susceptible and highly resistant genotypes. Similar results were found with serological techniques in other studies using other heterologous antisera (2,13,16,25). However, the low sensitivity of TAS-ELISA precludes this technique from differentiating degrees of resistance found in wild accessions with low levels of viral accumulation (*L. peruvianum* and *L. chilense*). Although this method possesses the ideal properties for use in large-scale analysis, we have

Table 4. Comparative effectiveness of serological and nucleic acid-based tomato yellow leaf curl virus (TYLCV) detection techniques in screening for new wild resistance sources

Accession	M-TAS-ELISA ^a (%)	Squash blot (%)	PCR (%)	VA ^b
<i>Lycopersicon peruvianum</i>				
PI-143679	33 ^c	88 ^c	90 ^c	1.4
PI-126935	40	85	90	0.8
<i>L. chilense</i>				
LA 2884	35	90	100	1.5
LA 1932	15	30	20	0.15
LA 1969	10	20	20	0.2

^a Modified triple antibody sandwich–enzyme-linked immunosorbent assay.

^b VA: Viral accumulation as estimated by scoring densitometric squash blot results ranging from 0 (no signal) to 4 (strong signal of the susceptible control).

^c Percentage of samples in which TYLCV was detected (60 DPI) with each technique (based on the total number of plants inoculated).

Table 3. Comparative sensitivity of modified triple antibody sandwich–enzyme-linked immunosorbent assay (M-TAS-ELISA), dot hybridization, and polymerase chain reaction (PCR) for tomato yellow leaf curl virus (TYLCV) detection

Genotype Diagnostic technique	Fresh tissue weight ^a						
	EP2 = 5 mg	D1 = 1 mg	D2 = 100 µg	D3 = 10 µg	D4 = 1 µg	D5 = 100 ng	D6 = 10 ng
Viral ssDNA ^b							
<i>Lycopersicon esculentum</i> FC	10 ng	2 ng ^c	200 pg	20 pg	2 pg	0.2 pg	0.02 pg
PCR	– ^d	–	+	+	+	+	+
Dot blot	+	+	+	+	–	–	–
ELISA	+	+	+	–	–	–	–
<i>L. peruvianum</i> PI-126935	250 pg	50 pg	5 pg	0.5 pg			
PCR	–	+	+	+			
Dot blot	+	+	–	–			
ELISA	+	–	–	–			
<i>L. chilense</i> LA 1932	5 pg	1 pg	0.1 pg				
PCR	–	+	+				
Dot blot	+	–	–				
ELISA	–	–	–				

^a Weight of fresh tissue corresponding with 5 µl of EP2 extraction and one 1/5 (D1) and five 1/10 dilutions (D2 to D6).

^b Viral ssDNA in pg or ng, 30 DPI: days post cage whitefly-mediated inoculation, in 5-µl spots of EP2 extraction of each genotype estimated by using the calibration curve constructed with known amounts of purified TYLCV DNA. Data are means of six replicates.

^c Estimated viral ssDNA in serial dilutions.

^d + = detected, – = not detected, ± = variable response.

successfully used it only in preliminary monitoring of field advanced breeding lines because of its high dependence on the nature and the state of the tissue.

Hybridization procedures, although more complex, overcome serological limitations. These have a higher sensitivity, and unlike serological and nonradioactive hybridization methods (digoxigenin-labeled or chromogenic probes) (1,7,37), detection with radioactive probes is less affected by background limitations, because it is less dependent on the origin and nature of the sample assayed.

The similar sensitivity found in squash blot and dot blot hybridization assays makes the simpler squash assay preferred as a preliminary test. The easy sampling method that can be conducted in the field by nonspecialized personnel makes this procedure the most appropriate to confirm TYLCV presence in samples with inconclusive results from TAS-ELISA when assaying field advanced breeding lines.

Dot blot is more informative than squash blot because it allows direct quantification of the virus by densitometric evaluations of the spots. However, the DNA extraction procedures, which are time-consuming, reduce the acceptance of dot blotting in large-scale analysis (8,22). Simple extraction procedures requiring the minimum of purification steps (EP3 and EP4) detected TYLCV in dot blots and distinguished levels of resistance among genotypes. These procedures can be used to accelerate the treatment of samples by combining equal amounts of tissue from several plants, as they allow TYLCV detection in a minimal amount of tissue.

However, when testing highly resistant genotypes, which accumulate low viral concentrations, it is advisable to use a detection strategy based on dot blot, followed by PCR to confirm inconclusive results (<1 pg viral ssDNA).

The most sensitive PCR is adequate for early detection of TYLCV in young tissues (23). It also is suitable for testing young plantlets in seedbeds; by using small amounts of fresh tissue, thousands of plants can be rapidly tested in order to release virus-free plants. However, a disadvantage of this technique is the difficulty of obtaining high-quality purified preparations from field plants.

Our results provide the information necessary to establish a more adequate selection strategy at each stage of the breeding program by combining the advantages of each detection technique.

ACKNOWLEDGMENTS

The authors thank E. R. Bejarano (University of Malaga, Spain) for kindly providing the DNA TYLCV probe, and I. Hita and J. L. Cenis (CIDA, Murcia, Spain) for advice about the TAS-ELISA procedures and for kindly providing the specific primers for PCR amplification. This research was supported by the project PB-94-0530-C03-02 of the DGICYT program.

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Fig. 2. Tomato yellow leaf curl geminivirus (TYLCV) accumulation detected by modified triple antibody sandwich–enzyme-linked immunosorbent assay (M-TAS-ELISA), polymerase chain reaction (PCR), and squash blotting in shoot apex and roots of tomato line FC and backcross BC2-*chil* several times after cage-whitefly artificial inoculation. Correlation with symptomatology. DPI: Days post cage whitefly-mediated inoculation. Symptoms: Mean scores of symptoms for each genotype: 0 (symptomless) to 4 (severe symptoms). M-TAS-ELISA: Absorbances (A_{405nm}) were recorded 24 h after addition of substrate and represent averages of four wells scored as follows: negative = 0 ($\leq$ twice the absorbance on the healthy control); 1 ($>$ twice the healthy control but $\leq$ 0.6); 2 ($>$ 0.6 but $\leq$ 1.2); 3 ($>$ 1.2 but $\leq$ 1.8); 4 ($>$ 1.8). Squash blot: viral accumulation as estimated by scoring densitometric squash blot results ranging from 0 (no signal) to 4 (strong signal as the susceptible control). Numbers above data points are percentages of PCR-positive plants at each sampling date.

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