CHAPTER 1

DETECTION METHODS FOR TYLCV AND TYLCSV

GIAN PAOLO ACCOTTO AND EMANUELA NORIS
Istituto di Virologia Vegetale, CNR, Strada delle Cacce 73, Torino-10135, Italy

1. OVERVIEW

The tomato yellow leaf curl disease (TYLCD) has been known for many years. The cause was, premature but with commendable intuition, put down to an entity named *Tomato yellow leaf curl virus* (TYLCV) (Cohen and Nitzany, 1966) although the viral etiology was recognized only in the late 1970s, and a virus with geminate morphology detected even later. Electron microscopic (EM) observations of thin sections from TYLCV-infected tomato leaves indicated that geminate particles were located in the nuclei of phloem parenchyma cells (Russo et al., 1980; Cherif & Russo, 1983), with intranuclear occurrence of fibrillar rings and small virus-like particles like those in the new virus group named "geminiviruses" (Goodman, 1981). In those times EM was therefore the only possible way to detect TYLCV.

However, what are considered "detection methods" for this virus complex had to wait for isolation of viral particles and demonstration that they are the causal agent of TYLCD. The virus was first isolated and purified in 1988 (Czosnek et al., 1988), and its association with the disease was demonstrated by membrane feeding of the whitefly vector on purified virus preparations. Since then, several detection methods for what is now recognized as a virus complex have been developed, both for mass screening and for more specific characterization.

In this review only methods for mass screening will be discussed, omitting specific applications, such as *in situ* hybridization and immuno-enzymatic methods for light or electron microscopy.

Depending on the kind of investigation, different questions can be asked, and no single detection method can fulfil all needs. Are the tomato plants which show yellowing and curling on leaves infected by TYLCV? Or are they infected by a different begomovirus? Which virus strain or variant is present? Are the plants infected by more than one begomovirus (mixed infection)?

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Difficulties arise mainly because there are several begomoviruses that cause similar symptoms in tomato (Fauquet et al., 2005) and they are not sufficiently different to allow easy and reliable discrimination using techniques such as ELISA, familiar to plant virologists and agricultural extension services.

2. SEROLOGICAL TECHNIQUES

Serological methods have not had much success in detecting TYLCV and whitefly-transmitted begomoviruses more generally. This is due both to difficulty in obtaining pure virus preparations and to the low immunogenicity of virus particles. Although the first purification procedure was described in 1988 (Czosnek et al., 1988) and later improved (Luisoni et al., 1995), the polyclonal antibodies obtained, while adequate for Western blotting, were not suitable for reliable detection of TYLCV by ELISA in field samples (Al-Bitar and Luisoni, 1995). Today some commercial ELISA reagents are available, detecting both TYLCV and the related but separate species *Tomato yellow leaf curl Sardinia virus* (TYLCSV) (Dalmon et al., 2000; Crescenzi et al., 2004). Some monoclonal antibodies (MAbs) raised against particles of *African cassava mosaic virus* (ACMV) have been reported to detect three isolates of TYLCSV, but were not tested against TYLCV in TAS–ELISA (Macintosh et al., 1992).

A different TAS–ELISA format has been described in a EWSN datasheet (Winter & Louro, 2000) and in an EPPO Bulletin (EPPO, 2005). It uses a polyclonal antibody prepared against ACMV for the coating step, followed by either a MAb that can detect TYLCV and TYLCSV isolates present in Europe (DSMZ AS-0546/2) or another MAb (DSMZ AS-0546/4) which does not react with TYLCSV. To differentiate the two virus species, a sample must be analysed with both MAbs: if it is positive only to the first, it is probably infected only by TYLCSV; if it is positive to both, certainly TYLCV is present, but it is impossible to conclude on the presence of single infection by TYLCV or mixed infection by both viruses. To resolve mixed infections molecular techniques are necessary.

Immunoblotting methods have also been reported, both in the form of tissue-printing (squash immunoassay) and dot-blotting (dot immunoassay) (Hajimorad et al., 1996; Pico et al., 1999; Dalmon et al., 2000). In comparison with ELISA, these methods suffer from a relatively high background, also found in healthy controls, that masks weak signals. Furthermore, they have not been tested for their ability to distinguish among similar species.

Recently some companies have introduced lateral flow assays for TYLCV (see www.neogeneurope.com; pdiag.csl.gov.uk). However, the sensitivity and ability of these assays to detect all or some species or isolates have not yet been tested thoroughly.

A different approach has also been used for trapping virus particles: the coating step of an ELISA is performed using GroEL protein rather than antibodies to the virus. Indeed, GroEL is much more potent in binding TYLCV than commercial anti-TYLCV antibodies (Akad et al., 2004). The method

exploits the strong interaction between the GroEL protein of the whitefly *Bemisia tabaci* and the coat protein of TYLCV and other begomoviruses (Morin et al., 1999).

3. MOLECULAR HYBRIDIZATION

The use of labelled DNA probes for detecting TYLCV dates back to 1988 (Czosnek et al., 1988; Navot et al., 1989; Nakhla et al., 1993), when a cDNA clone representing part of the viral genome was radiolabelled and used in Southern blots to detect the different viral DNA forms present in infected plants. The same probe was also employed on leaf squashes obtained by tissue printing, where it showed very good specificity (no reaction with the other viruses tested and no reaction with healthy plants). These characteristics and the ease, with which nylon membranes can be prepared, even in field conditions, immediately indicated the potential of molecular hybridization assays for mass screening and diagnosis. Interestingly, TYLCV can also be efficiently detected in squashes of single whiteflies. The method was proposed for large-scale epidemiological studies and for use in breeding programmes for virus resistance (Lapidot et al., 1997, 2001). One step of the molecular hybridization, however, made it impossible for many diagnostic laboratories to run the assay: probes were radioactively labelled.

To overcome this problem non-radioactive labelling techniques were explored. The most popular, even today, is based on digoxigenin (Crespi et al., 1991; Abou Jawdah et al., 1995). Probes are labelled with digoxigenin–dUTP, and the signal is detected with an anti-digoxigenin antibody conjugated with alkaline phosphatase, followed by incubation with an enzyme substrate. The first substrates were chromogenic, producing a pink–violet colour. Sensitivity was comparable with that of radiolabelled probes (Crespi et al., 1991; Quiñónez et al. 2004). Chemiluminescent substrates brought a further improvement. This allowed: (a) visualization of the signal on a film, therefore permitting optimal exposures and better interpretation of results, especially in cases where a weak hybridization signal is superimposed on the colour of the tissue print, and (b) more important, easy removal of the probe for a second hybridization. Reprobing membranes is particularly useful when mass screening is performed in areas where TYLCD can be caused by more than one virus species (see below). Non-radioactive hybridization was also successfully used for quantitative determination of TYLCV in dot blots of plant and whitefly extracts (Caciagli. & Bosco, 1996, 1997).

Probes, radioactively labelled or not, able to recognize the entire viral genome or selected portions of it can easily be produced, and proved very useful in organizing the taxonomy of the numerous whitefly-transmitted geminiviruses affecting tomato cultures in many tropical and subtropical regions. Hybridization tests with two DNA probes derived from a cloned isolate of TYLCV from Israel have been used to assess the affinities of viruses in naturally

infected tomato plants with yellow leaf curl or leaf curl symptoms from 25 countries (Czosnek & Laterrot, 1997). By a careful choice of probes and hybridization conditions, it was possible to conclude that samples from countries in the Middle East, Cuba, or the Dominican Republic were closely related to TYLCV from Israel, whereas samples from nine countries in the western Mediterranean area, Africa, or SouthEast Asia were more distantly related and probably represent one or more additional geminivirus species; a further group of samples contained very distantly related geminiviruses. The great number of geminivirus sequences available today have confirmed that TYLCD is caused by a number of geminiviruses whose genomes share a wide range of similarity levels (Fauquet et al., 2005).

The versatility of probes – and the need to make the best choice for everyone's purpose – is demonstrated by the cases of Spain and Italy, where TYLCSV strains were present and well established when TYLCV strains appeared in 1997 in Spain (Navas-Castillo et al., 1997) and 2002 in Italy (Accotto et al., 2003). For each species several strains have been described and their DNA sequences determined. Between the two species the nucleotide identity is about 74%, while among strains within each species it is above 90%. Sequence similarities are not uniformly distributed along the genome, so probes can be designed that will cross-react to different degrees with heterologous sequences. For example, probes based on the intergenic region (IR), the less conserved portion of genome, are the most specific. For example, an IR-specific probe made on the Sardinia isolate of TYLCSV does not recognize TYLCV and gives little or no reaction with other TYLCSV strains, so is not suited for most screening purposes. On the other hand, a probe designed on a more conserved region, such as the CP gene, will reliably detect all strains of the viral species from which it was designed, and can therefore be considered a good choice for mass screening (Accotto et al., 2000a). It should be noted, however, that samples giving weak signals may contain a low concentration of the homologous virus or a high concentration of the heterologous. Hybridization of the same membrane with a second probe, specific for the CP region of the other virus species, will generally help in final interpretation, but a definitive answer can only be given by a PCR-based method, such as PCR/RFLP described below. One remedy for the excessive specificity of IR-specific probes is to use an artificial mixture of them (Accotto et al., 2000b), and to use less stringent conditions during post-hybridization washing.

Both in Spain and Italy epidemiological studies on TYLCSV and TYLCV were conducted using tissue printing on membranes that were consecutively hybridized with two species-specific probes (Sanchez-Campos et al., 1999; Davino et al., 2006). Molecular hybridization employing non-radioactive probes, in spite of some limitations, definitely remains the best choice for simultaneous analysis of hundreds of samples: it does not require expensive equipment or a laboratory authorized for radioisotopes, and tissue-blotted membranes can be prepared in the field, with no extraction steps, and then stored for long

periods or sent to diagnostic laboratories. Furthermore, digoxigenin-labelled non-radioactive probes are stable for years, and can be reused at least five times without loss of sensitivity (Accotto et al., unpublished).

4. PCR-BASED METHODS

Numerous PCR-based methods have been reported for detecting whitefly-transmitted geminiviruses, using specific or degenerate primers, or combining PCR with tissue printing, immunoblotting, restriction enzyme digestion, molecular hybridization, etc. Navot et al. (1992) developed primers for detecting TYLCV in plants and whiteflies; for one of the primer combination tested, they estimated that a single infected whitefly could be detected in a bulk sample of 1,000.

Several publications describe degenerate primers for detecting whitefly-transmitted begomoviruses. One of the most cited uses primers PAL1v1978 and PAR1c713 to amplify a 1.4 kbp fragment (Rojas et al., 1993). Further analysis of this DNA provides information on the begomovirus under investigation. This approach to a suspected geminivirus infection in tomato can be found in many "first reports" in the literature. Other protocols have been proposed for detecting all begomoviruses (Deng et al., 1994; Wyatt & Brown, 1996), but not all of them have been tested on TYLCV and TYLCSV isolates, and might be unsuitable in some cases.

As mentioned above, a more challenging situation happens when there is need to rapidly and reliably detect and differentiate two related begomoviruses, that infect the same host (tomato) producing the same symptoms. For identification and differentiation of the two species infecting tomato in Europe, TYLCV and TYLCSV, the EPPO standard (EPPO, 2005) describes a PCR/RFLP protocol (Accotto et al., 2000a, c), that utilizes two degenerate primers – TY1(+) and TY2(–) – to amplify a 580 bp fragment from both species, followed by digestion with AvaII, which produces one pattern for TYLCV isolates and a clearly different one for those of TYLCSV. In another study (Martinez-Culebras et al., 2001), following extensive sequence alignments of several begomoviruses, two primer pairs were designed and successfully tested, one for detection of both TYLCV and TYLCSV, the other for TYLCSV only. A duplex PCR was also reported, that can detect either TYLCV or TYLCSV in a single step: However this protocol cannot distinguish the two viruses when mixed infections are present.

Print-capture PCR protocols have been successfully employed for several purposes. For example, squashes of plant or whitefly tissue on small pieces of nylon membranes were directly used in amplification reactions (Atzmon et al., 1998) to study TYLCV acquisition and transmission. This approach was modified by Navas-Castillo and coworkers (1998), who used pieces of Whatman 3 MM paper instead of nylon, with similar results. An important advantage consists in omitting the DNA extraction step needed before standard PCR.

Immunocapture-PCR, when applied to transmission studies, showed that the capsid protein of TYLCV was present in the insect organs at the same

time as DNA, suggesting that at least part of the virus circulates as virions within the insect (Ghanim et al., 2001). However, this technique is not widespread in diagnostics.

Finally, it is worth mentioning a new technique, named loop-mediated isothermal amplification (LAMP), that has been applied to TYLCV detection (Fukuta et al., 2003). It amplifies DNA with high efficiency under isothermal conditions without being significantly influenced by co-presence of non-target DNA, and its detection limit is a few copies, being comparable to that of PCR. Although LAMP applications are today mostly in clinical microbiology, its future development in plant virology cannot be excluded.

5. COMPARISONS AMONG METHODS

Not much effort has been devoted to compare the different detection methods. It is assumed that PCR is more sensitive than hybridization, which is more sensitive than serological methods. But sensitivity does not always go together with reliability. In the most comprehensive study, Pico et al. (1999) compared TAS–ELISA, squash immunoassay, dot immunoassay, squash and dot blot hybridization, and PCR for their sensitivity, reliability, and possibility of quantification, in order to measure resistance levels in tomato breeding lines. The squash and dot immunoassay were neither sensitive nor reliable. TAS–ELISA can be used in large-scale field screening, but hybridization methods are generally more appropriate, and, in the dot blot version, are quantitative. PCR, in principle the most powerful technique, sometimes fails to detect TYLCV reliably. A good protocol could be a squash blot followed by the more sensitive PCR applied to inconclusive samples. Dalmon et al. (2000) confirmed the superiority of squash and dot hybridization methods, because of their reliability and low cost. PCR gave some false negatives, probably due to the presence of inhibitors in the plant extract.

6. CONCLUDING REMARKS

Detection of begomoviruses causing TYLCD is best achieved by integrating two or more methods, as shown by the two following examples. When a new disease is suspected to be caused by a begomovirus, PCR with degenerate primers (better more than one combination) is the first step, followed by sequencing the amplified DNA. Comparing the new sequence with the DNA databases will give a strong indication on the nature of the begomovirus. In cases where mass screening is required, such as epidemiological studies, high-throughput is necessary; the best choice is squash blot hybridization, which allows analysis of hundreds of plants or insects on every membrane, without extraction steps, and with the option of reprobing the same membrane with different probes. For samples where results are not clear, PCR can then be applied.

However, some situations cannot be resolved with a simple strategy; virus populations are dynamic, and recombinant viruses have been shown to evolve

and spread in the parental population (Monci et al., 2002). If the new recombinant virus is present together with one or both parents, results of hybridization can easily be misinterpreted. In these cases, unfortunately, PCR using carefully selected primers is probably the only way to study the population dynamics. Rolling circle amplification (RCA), a method which utilizes a DNA polymerase from a bacteriophage, has recently been proposed for diagnosis of geminiviruses (Haible et al., 2006). Although it is probably the best way to face the challenge of characterizing populations of viruses with single-stranded DNA genomes in situations where mixed infections, recombinants, or new viruses are present or expected, RCA will hardly substitute the other techniques, at least in the near future, in diagnostic laboratories.

7. REFERENCES

Abou Jawdah, Y., Shebaro, W. A., & Soubra, K. H. (1995). Detection of tomato yellow leaf curl geminivirus (TYLCV) by a digoxigenin-labelled DNA probe. *Phytopathol. Mediterr*. **34**, 52–57.

Accotto, G. P., Navas-Castillo, J., Noris, E., Moriones, E., & Louro, D. (2000a). Typing of tomato yellow leaf curl viruses in Europe. *Eur. J. Plant Pathol*. **106**, 179–186.

Accotto, G. P., Moriones, E., Janseen, D., and Winter, S. (2000b). TYLCV-Sar and TYLCV-Is, a Tissue-Print Method for Detection. EWSN datasheet, Part A1, p. 2.

Accotto, G. P. and Moriones, E. (2000c). TYLCV-Sar and TYLCV-Is, a PCR-Based Method for Identification and Typing. EWSN datasheet, Part A1, p. 1.

Accotto, G. P., Bragaloni, M., Luison, D., Davino, S., & Davino M. (2003). First report of tomato yellow leaf curl virus (TYLCV) in Italy. *Plant Pathol*. (New Disease Reports), **52**, 799.

Akad, F., Dotan, N., & Czosnek, H. (2004).Trapping of tomato yellow leaf curl virus (TYLCV) and other plant viruses with a GroEL homologue from the whitefly *Bemisia tabaci. Arch. Virol*. **149**, 1481–1497.

Al-Bitar, L. & Luisoni, E. (1995). Tomato yellow leaf curl geminivirus: serological evaluation of an improved purification method. *EPPO Bull*. **25**, 269–276.

Atzmon, G., v. Oss, H., & Czosnek, H. (1998). PCR-amplification of tomato yellow leaf curl virus (TYLCV) DNA from squashes of plants and whitefly vectors: application to the study of TYLCV acquisition and transmission. *Eur. J. Plant Pathol*. **104**, 189–194.

Caciagli, P. & Bosco, D. (1996). Quantitative determination of tomato yellow leaf curl geminivirus DNA by chemiluminescent assay using digoxigenin-labeled probes.. *J. Virol. Methods* **57**, 19–29.

Caciagli, P., & Bosco, D. (1997). Quantitation over time of tomato yellow leaf curl geminivirus DNA in its whitefly vector. *Phytopathology* **87**, 610–613.

Cherif, C. & Russo, M. (1983). Cytological evidence of the association of a geminivirus with tomato yellow leaf curl disease in Tunisia. *Phytopathology* **108**, 221–225.

Cohen, S., Nitzany, F. E. (1966) Transmission and host range of tomato yellow leaf curl virus. *Phytopathology* **56**, 1127–1131.

Crescenzi, A., Comes, S., Napoli, C., Fanigliulo, A., Pacella, R., & Accotto, G. P. (2004). Severe outbreaks of tomato yellow leaf curl Sardinia virus in Calabria, Southern Italy. *Commun Agric. Appl. Biol. Sci*. **69**, 575–580.

Crespi, S., Accotto, G. P., Caciagli, P., & Gronenborn, B. (1991). Use of digoxigenin-labeled probes for detection and host range studies of tomato yellow leaf curl geminivirus. *Res. Virol*. **142**, 283–288.

Czosnek, H. & Laterrot, H. (1997). A worldwide survey of tomato yellow leaf curl viruses. *Arch. Virol*. **142**, 1391–1406.

Czosnek, H., Ber, R., Antignus, Y., Cohen, S., Navot, N., & Zamir, D.(1988). Detection of tomato yellow leaf curl virus in lysates of plants and insects by hybridization with a viral DNA probe. *Plant Dis*. **72**, 949–951.

Dalmon, A., Cailly, M., & David, C. (2000). Comparison of serological and molecular techniques for detection of tomato yellow leaf curl begomovirus. *Bull. OEPP* **30**, 457–462.

Davino, S., Napoli, C., Davino, M., & Accotto, G. P. (2006). Spread of tomato yellow leaf curl virus in Sicily: partial displacement of another Geminivirus originally present. *Eur. J. Plant Pathol.* **114**, 293–299.

Deng, D., McGrath, P. F., Robinson, D. J., & Harrison, B. D. (1994). Detection and differentiation of whitefly-transmitted Gemini-viruses in plants and vector insects by the Polymerase chain reaction with degenerate primers. *Ann. Appl. Biol*. **125**, 327–336.

EPPO. (2005). Tomato yellow leaf curl and tomato mottle begomoviruses. *Bull. OEPP/EPPO Bull.* **35**: 319–325.

Fauquet, C. M., Mayo, M. A., Maniloff, J., Desselberger, U., & Ball, L. A. (2005). *Virus Taxonomy: VIIIth Report of the International Committee on Taxonomy of Viruses*. London: Elsevier/Academic Press.

Fukuta, S., Kato, S., Yoshida, K., Mizukami, Y., Ishida, A., Ueda, J., Kanbe, M., & Ishimoto, Y. (2003). Detection of tomato yellow leaf curl virus by loop-mediated isothermal amplification reaction. *J. Virol. Methods* **112**, 35–40.

Ghanim, M., Morin, S., & Czosneck H. (2001). Rate of tomato yellow leaf curl virus translocation in the circulative transmission pathway of its vector, the whitefly *Bemisia tabaci. Phytopathology* **91**, 188–196.

Goodman, R. M. (1981). Geminiviruses. In E. Kurstak (Ed.), *Handbook of Plant Virus Infection and Comparative Diagnosis*. New York: Elsevier/North Holland Biomedical Press, pp. 879–910.

Haible, D., Kober, S., & Jeske, H. (2006). Rolling circle amplification revolutionizes diagnosis and genomics of geminiviruses. *J. Virol. Methods* **135**, 9–16.

Hajimorad, M. R., Kheyr-Pour, A., Alavi, V., Ahoonmanesh, A., Bahar, M., Rezaian, M. A., & Gronenborn, B. (1996). Identification of whitefly transmitted tomato yellow leaf curl geminivirus from Iran and a survey of its distribution with molecular probes. *Plant Pathol*. **45**, 418–425.

Lapidot, M., Friedmann, M., Lachman, O., Yehezkel, A., Nahon, S., Cohen, S., & Pilowsky, M. (1997). Comparison of resistance level to tomato yellow leaf curl virus among commercial cultivars and breeding lines. *Plant Dis*. **81**, 1425–1428.

Lapidot, M., Friedmann, M., Pilowsky, M., Ben-Joseph, R., & Cohen, S. (2001). Effect of host plant resistance to tomato yellow leaf curl virus (TYLCV) on virus acquisition and transmission by its whitefly vector. *Phytopathology* **91**, 1209–1213.

Luisoni, E., Milne, R. G., & Vecchiati, M. (1995). Purification of tomato yellow leaf curl geminivirus. *New Microbiol*. **18**, 253–260.

Macintosh, S., Robinson, D. J., & Harrison, B. D. (1992). Detection of three whitefly transmitted geminiviruses occurring in Europe by tests with heterologous monoclonal antibodies. *Ann. App. Bio*. **121**, 297–303.

Martinez-Culebras, P. V., Font, I., & Jorda, C. (2001). A rapid PCR method to discriminate between tomato yellow leaf curl virus isolates. *Ann. App. Biol*. **139**, 251–257.

Monci, F., Sanchez-Campos, S., Navas-Castillo, J., & Moriones, E. (2002). A natural recombinant between the geminivirus tomato yellow leaf curl Sardinia virus and Tomato yellow leaf curl virus exhibits a novel pathogenic phenotype and is becoming prevalent in Spanish populations. *Virology* **303**, 317–326.

Morin, S., Ghanim, M., Zeidan, M., Czosnek, H., Verbeek, M., & van den Heuvel, J. F. (1999). A GroEL homologue from endosymbiotic bacteria of the whitefly *Bemisia tabaci* is implicated in the circulative transmission of tomato yellow leaf curl virus. *Virology* **256**, 75–84.

Nakhla, M. K., Mazyad, H. M., & Maxwell, D. P. (1993) Molecular characterization of four tomato yellow leaf curl virus isolates from Egypt and development of diagnostic methods. *Phytopathol. Mediterr*. **32**, 163–173.

Navas-Castillo, J., Díaz, J. A., Sánchez-Campos, S., & Moriones, E. (1998). Improvement of the print-capture polymerase chain reaction procedure for efficient amplification of DNA virus genomes from plants and insect vectors. *J. Virol. Methods* **75**, 195–198.

Navas-Castillo, J., Sánchez-Campos, S., Díaz, J. A., Sáez-Alonso, E., & Moriones, E. (1997). First report of tomato yellow leaf curl virus-Is in Spain: coexistence of two different geminiviruses in the same epidemic outbreak. *Plant Dis*. **81**, 1461.

Navot, N., Ber, R., & Czosnek, H. (1989). Rapid detection of tomato yellow leaf curl virus in squashes of plants and insect vectors. *Phytopathology* **79**, 562–568.

Navot, N., Zeidan, M., Pichersky, E., Zamir, D., & Czosnek, H. (1992). Use of polymerase chain reaction to amplify tomato yellow leaf curl virus DNA from infected plants and viruliferous whiteflies. *Phytopathology* **82**, 1199–1202.

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

Quiñónez, M., Fonseca, D., et al. (2004). Comparison of nucleic acid hybridization methods for the diagnostic of tomato yellow leaf curl virus (TYLCV) in Cuba. *Revista de Protección Vegetal*. **19**, 26–32.

Rojas, M. R., Gilbertson, R. L., Russell, D. R., & Maxwell, D. P. (1993). Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses. *Plant Dis*. **77**, 340–347.

Russo, M., Cohen, S., & Martelli, G. P. (1980). Virus-like particles in tomato plants affected by the yellow leaf curl disease: *J. Gen. Virol*. **49**, 209–213.

Sanchez-Campos, Navas-Castillo, J., Camero, R., Saria, C., Diaz, J. A., & Moriones, E. (1999). Displacement of tomato yellow leaf curl virus-Sr by TYLCV-Is in tomato epidemics in Spain. *Phytopathology*, **89**, 1038–1043.

Winter, S. & Louro, D. (2000). TYLCV Species. Identification and differentiation with TAS-ELISA. EWSN datasheet, Part A1, p. 4.

Wyatt, S. D. & Brown, J. K. (1996). Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction. *Phytopathology* **86**, 1288–1293.