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Detection of TYLCV in Ten Genotypes of Tomato (Solanum spp L.) using Serological and Molecular Techniques in a Coastal Savanna Zone of Ghana

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Abstract

Use of resistant varieties is a proven way of controlling the tomato yellow leaf curl virus (TYLCV), which causes a devastating disease of tomato in tropical and warm temperate regions, resulting in significant yield losses. However, breeding for resistant varieties is slow due to lack of effective methods of virus detection that will lead to efficient selection of desired varieties. A study was conducted to evaluate the susceptibility or otherwise of ten tomato genotypes to TYLCV under field conditions. The ten (10) tomato genotypes were planted in the field and left to natural infection by whitefly vectors. Leaf samples were collected from symptomatic as well as asymptomatic plants at five weeks after transplanting (5WAT) for analysis by serological and nucleic acid-based techniques. Triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) detected TYLCV in five out of ten genotypes while polymerase chain reaction using six primers (virus-specific and degenerate) detected the virus in eight out of ten genotypes. The polymerase chain reaction (PCR) technique detected the virus in three samples that tested negative to TAS-ELISA, implying its enhanced sensitivity. A correlation of symptom expression in the field to the presence of TYLCV in test samples led to an effective and unbiased selection of tolerant/resistant genotypes which can be used in further breeding programmes. The study highlights the need for a combination of two or more detection techniques in breeding to enhance the efficiency of selection of TYLC-resistant lines.

Keywords: Tomato yellow leaf curl virus, enzyme-linked immunosorbent assay, polymerase chain reaction, genotypes, resistant varieties, *Solanum lycopersicon*.

1. Introduction

Tomato (*Solanum lycopersicon* L.) is the second most widely grown vegetable crop in the world after potato (*Solanum tuberosum* L.) with a total production of around 152.9 million tonnes, valued at 74.1 billion dollars (US) on a cultivated area of 5 million hectares (FAOSTAT, 2009). It has received attention not only because of its nutritional and economic importance, but also because it is seen as a model crop in plant genetics, physiological and pathological studies (Heuvelink and Costa, 2006).

In Ghana, tomato is consumed on nearly daily basis by every household (Horna et al., 2006). It is used directly as raw vegetable in sandwiches, salads, assorted soups, sauces and other dishes (Osei et al., 2008). In general, the crop is susceptible to more than 200 diseases, out of which about 40 are caused by viruses (Lukyanenko, 1991). In addition, it is susceptible to a number of fungal and bacterial diseases (AVRDC, 1998; Antherton and Rudich, 1986).

Virus diseases affecting tomato include; Beet curly top virus (BCTV) disease, Tomato mosaic virus (TMV) disease, and the most devastating Tomato yellow leaf curl virus (TYLCV) disease (Kalloo, 1991; Alexander, 1971). TYLCV disease severely affects the biological function of the leaves, stems and roots, eventually reducing yield in terms of fruit size, weight and number (Pico et al., 1996). Ever since the discovery of TYLCV disease in 1940 (Pico et al., 1996) to date, it has become the most important limiting factor to tomato production in many tropical and subtropical regions of the world, causing up to 100% yield losses (Glick et al., 2009; Czosnek and Laterrot, 1997).

Average fruit yield of tomato in Ghana is low, typically less than 10t/ha (Robinson et al., 2010), attributed in part to crop failure due to disease and pest incidence. Homa et al. (2006) reported widespread incidence of the TYLCV disease causing severe yield losses. Osei et al. (2008) after testing tomato leaf samples collected from the Ashanti Region, including Akumadan, the main tomato producing area in the country,

identified three distinct begomoviruses associated with tomato leaf curl disease in Ghana. The observed leaf curl disease incidence in farmers' fields was approximately 75%. This provides some basis for initiating efforts towards developing resistant cultivars.

Breeding for resistance to Tomato yellow leaf curl virus (TYLCV) is difficult, due to lack of a reliable system for selecting resistant plants. The variable nature of plant genotypes used (wild or wild-derived genotypes and advanced breeding lines) makes the development of a reproducible selection procedure necessary during breeding programmes (Pico et al., 1999). In addition, like other begomoviruses, the identification and detection of TYLCV is primarily based on the visual observation of symptoms such as leaf yellowing, leaf curling, stunting of plant and death of plant (Malmrot, 2010; Rojas, 2004; Nono-Womdim et al., 1996).

However, this is difficult due to the great variation in symptom expression and the existence of different strains of the virus associated with the disease (Glick et al., 2009; Anfoka et al., 2008). Environmental factors such as climatic stress, soil nutrient deficiencies and water stress also produce symptoms which could be mis-diagnosed as viral infection. Symptomatology alone, therefore, cannot be used for definitive identification of TYLCV infection (Vidavsky et al., 1998).

Several techniques such as triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) and tissue blot immunoassay (TBIA) have been employed in the diagnosis of TYLCV. These techniques, however, have limitations on the specific detection of Geminiviruses due to cross-reaction resulting from high homology among their coat proteins (Thomas, 1986). Other techniques such as visualisation of nuclear inclusion bodies by light microscopy, ultrastructural localisation of virions in plant cell by transmission electron microscopy (Pico et al., 1999; Hunter et al., 1998), DNA hybridisation assays (Lotrakul et al., 1998), polymerase chain reaction (PCR) (Pico et al., 1999; Ghanem et al., 1998), and print-PCR (Navas-Castillo et al., 1998) have also been used with varying degrees of success and efficiency. To enhance the development of TYLCV-resistant tomato varieties, more rapid and effective detection techniques are needed. This study was aimed at using a combination of TAS-ELISA and PCR techniques to detect TYLCV in the ten genotypes of tomato grown under field conditions and left to natural infection.

2.0. Materials and Methods

2.1. Study Area

The study was conducted at the Biotechnology and Nuclear Agriculture Research Institute of the Ghana Atomic Energy Commission between November 2011 and October 2012. The study site is located about 20 km north of Accra (05º 40' 60 N and 0º 13' 0 W), with an elevation of 76 m above sea level. The vegetation is Coastal Savannah, and the area is characterised by a bimodal rainfall pattern with the major season falling between the months of March and June, and a minor rainy season in September/October. The mean annual rainfall is 810 mm distributed over less than 80 days, and temperatures are moderate with the maximum rarely exceeding 35 ºC while the minimum does not fall below 17 ºC.

2.2. Experimental Design and Sample Collection

Seedlings of the ten genotypes (Table 1) were transplanted in the field in December, 2011. The experiment was laid out in a randomised complete block design (RCBD) with three replications. Symptomatic leaf samples were collected five (5) weeks after transplanting (WAT) when disease symptoms were fully developed. Samples were carefully collected from freshly expanded young leaves from the uppermost parts of the plant. To avoid cross contamination, disposable gloves were changed between collection of different samples and pieces of leaf samples were plucked directly into Ziploc™ bags, using the lid as a cutting implement or sterilised forceps. The Ziploc™ bags were labelled and transported on ice to the laboratory.

Table 1: Tomato Genotypes used in the Study

Accession	Code	Status	Pedigree
S. pimpinellifolium	Wild	Local	-
Wosowoso	Woso	Local	-
Cherry Red	C-Red	Exotic	-
Roma	Roma	Exotic	-
Hyb-1	Hyb-1	Hybrid	Woso x Wild
Hyb-2	Hyb-2	Hybrid	Roma x Wild
Hyb-3	Hyb-3	Hybrid	C-Red x Wild
BC-1	BC-1	Backcross	Woso x (Woso x Wild)
BC-2	BC-2	Backcross	Roma x (Roma x Wild)
BC-3	BC-3	Backcross	C-Red x (C-red x Wild)

2.3. TAS-ELISA Technique for Detection of TYLCV

A complete TAS-ELISA kit with a known TYLCV-infected *Nicotiana benthamiana* positive control was obtained from DSMZ Plant Virus Collection, Braunschweig, Germany. Tomato leaf samples of the ten genotypes, each weighing 1.0 g, were homogenised in 10 ml of 0.05 M Tris containing 0.06 M sodium sulphite, (pH 8.5) using a sterilised mortar and pestle. The crude extracts were then kept at 4^{o}C for subsequent analysis. The protocol according to Clark & Adams (1977) was followed. Uninfected plants of Wild tomato grown in cages were included in the test as negative controls.

The absorbance values were read at 405 nm using a spectrophotometer (Multiskan Ascent VI.25-Version 1.3.1) at the Radiological and Medical Science Research Institute (RAMSRI) of the Ghana Atomic Energy Commission (GAEC). The ELISA test was considered positive if the absorbance of the sample was more than two times the absorbance of the healthy (negative) control.

2.4. PCR Technique for Detection of TYLCV

Total DNA was extracted from very young, fresh leaf tissues of the ten tomato genotypes using the CTAB method described by Lohdi et al. (1994). The quality of the DNA was maintained on 1% agarose gel stained with ethedium bromide and then stored at -20 ^{o}C for subsequent experiments. DNA amplification was carried out using degenerate, as well as virus-specific primers (Table 2), in a 25 ul reaction mix containing 2.5 ul 10 x Reaction buffer, 0.2 ul of 10 mM dNTPs, 1.5ul of 25mM MgCl2, 1 ul each of 10 uM forward and reverse primers, 0.5 units of Taq DNA polymerase and 1 ul of DNA template. DNA samples from healthy and symptomatic plants were used as negative and positive controls, respectively.

DNA amplification was performed in an iCycler™ Thermal Cycler (Bio-rad, USA). The DNA amplification protocol used for primer pairs PAR1c496/PAL1v1978, AV494/AC1048, TYLCGHv F/R and TYLCKv F/R were, an initial denaturation step at 94 ^{o}C for 1 minute followed by 35 cycles of denaturation at 94 ^{o}C for 30 seconds, primer annealing at 50 ^{o}C for 1 minute and elongation at 72^{o}C for 3minutes. This was followed by a final elongation step at 72 ^{o}C for 7 minutes. For primer pairs PTYv787/PTYc 1121 and TYLCVMv F/R, the PCR conditions were 30 cycles of denaturation for 1 minute at 94 ^{o}C, primer annealing for 1 minute at 58 ^{o}C, and primer extension for 2 minutes at 72 ^{o}C, with an initial denaturation at 94 ^{o}C for 1minute and a final extension for 10 minutes at 72 ^{o}C. After amplification, 7 µl aliquot of the PCR products mixed with a 2 µl loading dye (Bromophenol blue - Sigma) were electrophoresed using 1X TAE (Tris-Acetic-EDTA) buffer at 80 Volts for 1 hr. 30 mins on 1% agarose gel stained with EtBr (0.5 µl ml-1). The gels were visualised and photographed under UV light using a BioDoc-It™ Imaging System (UVP, U.S.A).

2.5. Data Analysis

Data were evaluated for statistical significance using a one-way analysis of variance (ANOVA) and means separated by Duncan's Multiple Range Test (Statgraphics 12 Edition).

3.0. Results and Discussion

3.1. ELISA Detection of TYLCV in Leaf Extracts of Ten Tomato Genotypes

The results of ELISA detection of TYLCV in leaf extracts of the ten tomato genotypes, together with the positive and negative controls are shown in Table 2. TYLCV-infected *N. benthamiana* (positive control) recorded an absorbance value of 0.3830 ± 0.0050 whilst the healthy *S. pimpinellifolium* (Wild Tomato, used as negative control) gave an absorbance value of 0.1748 ± 0.0050. The results indicate TYLCV infection in Woso, Hyb-1, BC-1, Roma and BC-2. The highest positive absorbance was recorded by Roma (1.3585 ± 0.00463) whilst the least was recorded by Hyb-2 (0.1860 ± 0.02409). The test failed to detect TYLCV in Wild tomato, Hyb-3, Hyb-2, BC-3 and C-Red despite the expression of TYLCV-like symptoms in these genotypes in the field. This suggests that the expression of TYLCV-like symptoms in the field is not exclusively due to infection by the virus. However, severe disease symptoms were associated with genotypes in which the virus was detected by ELISA.
Pico et al. (1999) reported unreliable detection of TYLCV using TAS-ELISA in early as well as late stages of infection. Similar results have been reported by several other workers (Abou et al., 1996; Fargette et al., 1996; Konate et al., 1995; Noris et al., 1995). The virus was not detected in the Wild tomato and its derivatives (BC-3, Hyb-3 and Hyb-2) probably due to low levels of viral particle accumulation in these genotypes, as similarly reported by Pico et al. (1999).

Table 2: TAS-ELISA Detection of TYLCV in Ten Genotypes on the Field

Genotypes	Plant no.	Absorbance @ 405nm ± SE	ELISA reaction (+ or -)
S. pimpinellifolium	1	**0.2140 ± 0.02949**	-
	2	**0.2715 ± 0.02475**	-
	3	**0.2085 ± 0.02591**	-
	4	**0.2130 ± 0.01927**	-
Woso	1	0.6423 ± 0.04077	+
	2	0.4980 ± 0.02552	+
	3	0.9335 ± 0.30924	+
	4	0.3820 ± 0.10871	+
C-Red	1	**0.2830 ± 0.02593**	-
	2	**0.3400 ± 0.02015**	-
	3	0.6050 ± 0.02662	+
	4	**0.2548 ± 0.01863**	-
Roma	1	1.3585 ± 0.00463	+
	2	0.3525 ± 0.02861	+
	3	0.3553 ± 0.3353	+
	4	**0.2735 ± 0.2735**	-
Hyb-1	1	1.2410 ± 0.07773	+
	2	0.7420 ± 0.04363	+
	3	1.1693 ± 0.02950	+
	4	**0.2260 ± 0.02065**	-
Hyb-2	1	0.7260 ± 0.06138	+
	2	**0.3395 ± 0.03037**	-
	3	**0.1860 ± 0.02409**	-
	4	**0.3338 ± 0.01300**	-
Hyb-3	1	**0.3032 ± 0.03151**	-
	2	**0.2868 ± 0.02401**	-
	3	**0.3335 ± 0.01683**	-
	4	**0.2448 ± 0.03990**	-
BC-1	1	0.365 3± 0.03676	+
	2	0.3613 ± 0.01879	+
	3	**0.01879±0.02155**	-
	4	0.6353 ± 0.01496	+
BC-2	1	0.9615 ± 0.04398	+
	2	0.5768 ± 0.02489	+
	3	0.5992 ± 0.08169	+
	4	0.7072 ± 0.09288	+
BC-3	1	**0.2505 ± 0.02141**	-
	2	0.4295 ± 0.01252	+
	3	**0.3112 ± 0.00861**	-
	4	**0.3165 ± 0.07284**	-
Healthy (negative control)	1	**0.1748 ± 0.0050**	-
+ve control (infected *N. benthamiana* from DSMZ)	1	0.3830 ± 0.0050	+

Means are averages of four tests. Key: (+) Positive, (-) Negative. Values in bold are negative.

3.2. PCR Detection of TYLCV in Ten Tomato Genotypes

The specific primer TYLCGHVF/R could not detect the virus in any of the ten tomato genotypes. Together, the other five primer pairs (Table 3) were able to detect the virus in eight out of the ten tomato genotypes (Table 4). The virus was, however, not detected in two backcross breeding lines BC-1 and BC-3.

Two primer pairs, TYLCMVF/R and TYLCKVF/R detected the virus in one and two genotypes respectively. However, AV494/AC1029 and PALIv787/PARIc496 as well as PTYvy787/PTYc1121 were able to detect the same virus in six and five genotypes indicating their superior sensitivity compared to the first two. Primer pairs AC1048/AV494, PTYv787/PTYc1121and PALv1978/PARc496 gave expected PCR products, of size ~ 550bp, 500bp and 1.1 kb respectively (Fig. 2 and 3). Similar product sizes were obtained by several workers (Alba, 2003; Zhou et al., 2008; Deng et al., 1994; and Rojas et al., 1993) in PCR detection of begomoviruses. The specific primer TYLCVKVF/R amplified the viral DNA in only two of the ten genotypes

with a product size of ~20kb while TYLCMV F/R showed amplification in only Woso with a product size of ~2.5 (Table 3). These primers have been used in earlier studies to detect two strains of TYLCV in Ghana (Osei et al., 2008).

Table 3: Oligonucleotide Primers used in the Detection of TYLCV

Primer name	Primer Code	Type	Source
Tomato yellow leaf curl Ghana virus (F/R)	TYLCGHVF/R	Specific	Osei et al., 2008
Tomato yellow leaf curl Kumasi virus (F/R)	TYLCKVF/R	Specific	Osei et al., 2008
Tomato yellow leaf curl Mali virus (F/R)	TYLCMVF/R	Specific	Osei et al., 2008 /Zhou et al., 2008
PAR1c496/PAL1v1978		Degenerate	Rojas et al., 2003
AV494/ AC1029		Degenerate	Wyatt and Brown, 1996
PTYv787/ PTYc1121		Degenerate	Rojas et al., 2003

Table 4: Amplification of TYLCV DNA Fragments in Leaf Samples at Five Weeks after Transplanting (WAT) using Polymerase Chain Reaction.

Genotypes	Symptoms observed	TYLCGHV F/R	TYLCKV F/R	TYLCMV F/R	PAL1v1978 / PAR1c496	AV494/ AC1029	PTYv787/ PTYc1121
S. pimpinellifolium	**(0 - 1)**	-	-	-	-	+	+
Woso	**(1- 4)**	-	-	+	+	+	+
C-Red	**(1 - 3)**	-	+	-	+	+	+
Roma	**(1 - 4)**	-	+	-	+	+	+
Hyb-1	**(1 - 2)**	-	-	-	+	-	-
Hyb-2	**(1 - 2)**	-	-	-	+	+	-
Hyb-3	**(1 - 2)**	-	-	-	-	-	+
BC-1	**(1- 2)**	-	-	-	-	+	-
BC-2	**(1 - 2)**	-	-	-	-	-	-
BC-3	**(1 - 2)**	-	-	-	-	-	-
Total		**0/10**	**6/10**	**5/10**	**0/10**	**5/10**	**2/10**

Symptoms key: (0) No visible symptom; (1) Slight yellowing of margins of apical leaflets; (2) Moderate yellowing and slight curling of leaflet tips; (3) Extensive leaf yellowing, curling and cupping with some reduction in leaf size; (4) Very severe stunting of plant and leaf yellowing, pronounced cupping and curling of leave (+) Positive and (-) Negative

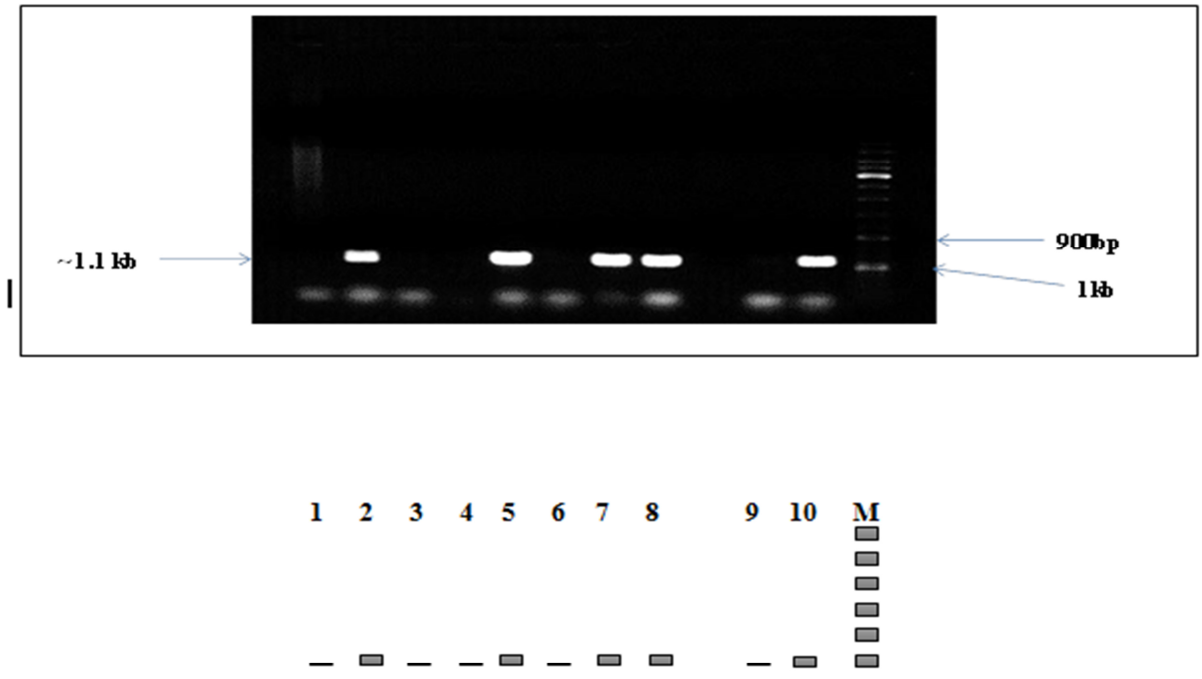

Fig. 1 Gel electrophoresis of PCR products generated with primer pair PAL 104/PAR 108: lane 1 = Wild, lane 2 = BC-3, lane 3 = Hyb-3, lane 4 = C-Red, lane 5 = BC-1, lane 6 = Hyb-1, lane 7 = Woso, lane 8 = BC-2, lane 9 = Hyb-2, lane 10 = Roma, Lane M = 100bp DNA ladder

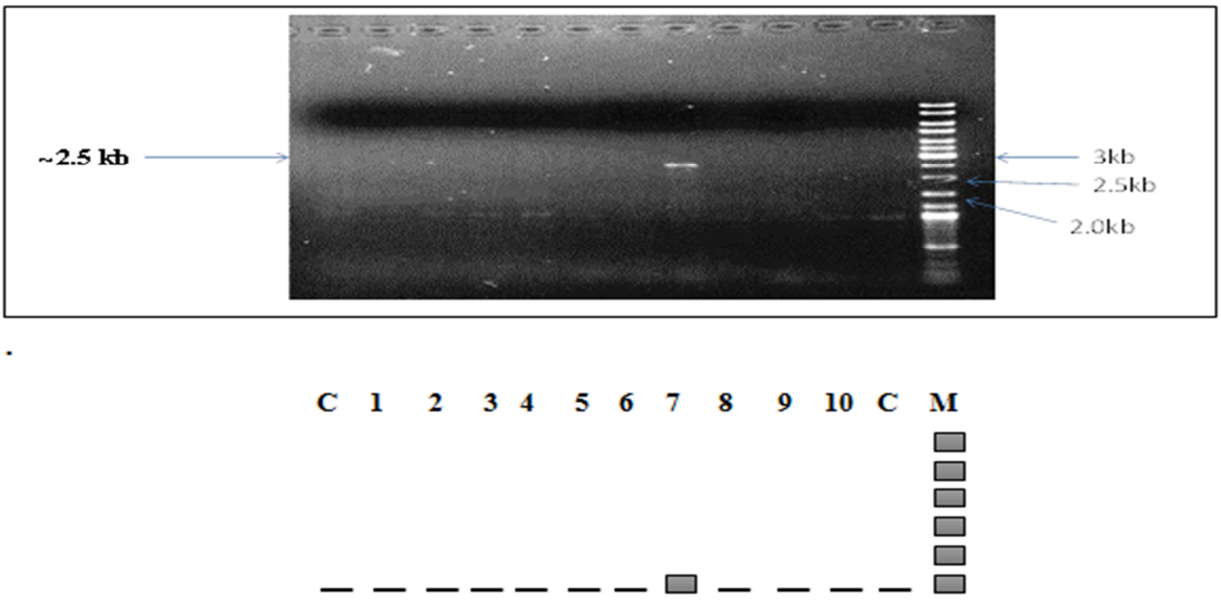

Fig. 2 Gel electrophoresis of PCR products generated with primer TYLCMV F/R: lane C = water control, lane 1 = Wild, lane 2 = BC-3, lane 3 = Hyb-3, lane 4 = C-Red, lane 5 = BC-1, lane 6 = Hyb-1, lane 7 = Woso, lane 8 = BC-2, lane 9 = Hyb-2, lane 10 = Roma, Lane M = GeneRuler DNA ladder Mix

3.3. Detection of TYLCV using TAS-ELISA and PCR

TAS-ELISA detected TYLCV in five tomato genotypes while PCR detected the same virus in eight genotypes. The virus was detected by both PCR and TAS-ELISA in four genotypes (Table 4). PCR technique was able to detect the virus in four samples that tested negative to TAS-ELISA, but could not detect the presence of the virus in two backcross lines (BC-2 and BC-3) even though the ELISA techniques detected the virus in BC-2. This confirms the enhanced sensitivity of the PCR technique (Martin, 1998), making it the preferred method for the detection and characterisation of viral infections that are difficult to detect and diagnose by serological methods (Seal and Coates, 1998).

Table 4: Comparison of PCR and TAS-ELISA Test in the Detection of TYLCV

Genotype	ELISA	PCR					
		TYLCGHV F/R	*TYLCKV F/R*	*TYLCMV F/R*	*PAL1v1978/ PAR1c496*	*AV494/ AC1029*	*PTYv787/ PTYc1121*
S. pimpinellifolium	-	-	-	-	-	+	+
Woso	+	-	-	-	+	+	+
C-Red	-	-	+	-	+	+	+
Roma	+	-	+	-	+	+	+
Hyb-1	+	-	-	+	+	-	-
Hyb-2	-	-	-	-	+	+	-
Hyb-3	-	-	-	-	-	-	+
BC-1	+	-	-	-	-	+	-
BC-2	+	-	-	-	-	-	-
BC-3	-	-	-	-	-	-	-
% Detection	50%	Nil	20%	10%	50%	60%	50%

Conclusions

In this study, TAS-ELISA detected TYLCV in five out of ten tomato genotypes investigated, namely, Wosowoso, Hyb-1 (Woso x Wild), BC-1 [Woso x (Woso x Wild)], Roma and BC-2 [Roma x (Roma x Woso)]. The PCR technique, on the other hand, detected the virus in eight genotypes including the four identified by TAS-ELISA. This confirms the superior sensitivity of the PCR technique as a detection method of TYLCV, compared to TAS-ELISA. The combined usage of the two techniques resulted in detection of the virus in a total of nine genotypes. Wild tomato, which served as negative control for TAS-ELISA detection of the virus, proved to be susceptible using the PCR techniques. It is thus a tolerant genotype. However, using both detection

methods, the virus was not detected in BC-3, suggestive of its potential value in breeding for TYLCV-resistant varieties.

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