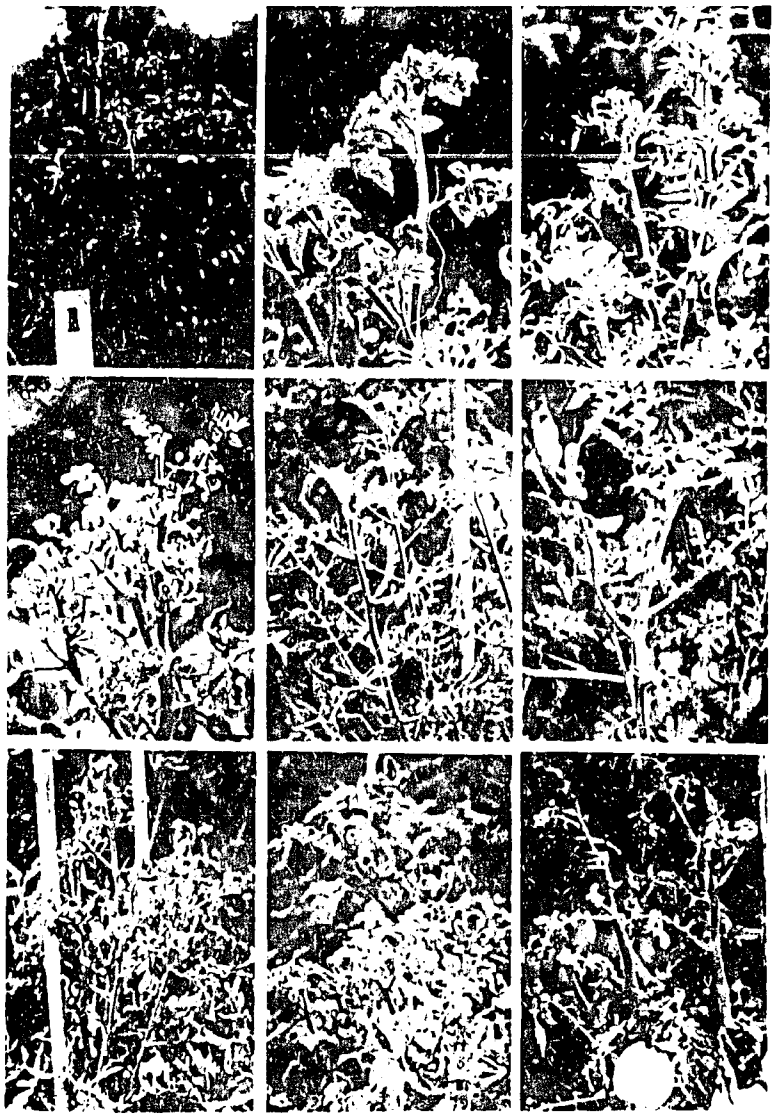
AID/SCI PSTC PROJECT No. 7314



Immunization of Tomato Seedlings to Protect Against the Whitefly Transmitted Tomato Yellow Leaf Curl Virus

Final Report July, 1995

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Final Report

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Pre-immunization of tomato seeds to protect against the whitefly transmitted Tomato Yellow Leaf Curl Virus

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EXECUTIVE SUMMARY

The project entitled "Pre-immunization of tomato seeds to protect against the whitefly transmitted tomato yellow leaf curl virus" was aimed at the control of tomato yellow leaf curl disease in Thailand. The approach taken was to develop a mild strain of this virus to protect tomato seedlings from infections by severe strains.

The virus was purified and its protein and nucleic acid were characterized in great detail. Viral ssDNA was cloned and DNA probe was developed to detect the virus in infected tomato plants and other hosts with high degrees of sensitivity and accuracy.

The whitefly *Bemisia tabaci* was shown to be the only vector of TYLCV. Its biology, epidemiology and relationship with TYLCV were substantially investigated. The study clearly demonstrated that the whitefly could acquire the virus or other viral components through membrane feeding. The virus could persist in the whitefly and also pass through eggs.

Tomato yellow leaf curl virus was found to infect some wild hosts. Seven mild strains of the virus were identified from naturally infected tomato. Tomato seedlings could be mass inoculated with this virus by regenerating plantlets from TYLCV infected tissues or by the feeding of viruliferous whiteflies. Plants infected with mild strain performed well in the field as compared with those infected by severe strain. However symtom suppression by mild strain was shown to be inconsistence presumable due to the instability or mixed population of mild strain being used.

This research project has accomplished most of its objectives. Research results could contribute to the better and more efficient control program for tomato yellow leaf curl disease. The support provided by AID/SCI PSTC research project has tremendous impact on research individuals, laboratories and Kasetsart University to improve their research capabilities and strengthen collaborations with leading researchers and institutions in developed countries.

RESEARCH OBJECTIVES

The tomato yellow leaf curl disease was observed in Thailand since 1973, but reported to be a nutritional deficiency. The disease became one of the most destructive virus disease affecting tomato production in this country. Up to now, there is no resistant tomato variety found or developed through the existing breeding program to be used for disease control. Tomato varieties reported to be resistant to this disease elsewhere were susceptible to tomato yellow leaf curl virus in Thailand (Fig. 1). There should be an alternative method to control this leaf curl disease in tomato.

Tomato yellow leaf curl virus (TYLCV) is transmitted naturally by the whitefly *Bemisia tabaci*. The whitefly vector has very wide host range and can reproduce abundantly in the tropical environment. Highly toxic insecticides are applied to control *B. tabaci* on tomatoes and shown not to be an effective mean to control this disease. Therefore, instead of controlling the vector, inoculation of tomato seedlings with a mild strain of TYLCV to pre-immunize seedling by the whitefly is subjected to this investigation. If this method has proven to be effective, a method for the massive inoculation of tomato seedlings with a mild strain of TYLCV by using the whitefly vector will be developed.

Tomato yellow leaf curl virus is a small single stranded DNA virus. However, very little is known on the physical and biochemical properties of TYLCV. The better understanding on some important properties of this virus could lead to the better control strategy.

In summary, the specific objectives of this project are as follows;

1. to develop a mild strain of TYLCV to cross protect against severe strain of this virus

2. to produce *in vitro* tomato seedlings which is immune to tomato yellow leaf curl disease

3. to physically and biochemically characterize this virus

4. to develop a method for the massive inoculation of tomato seedlings with a mild strain of TYLCV by using the whitefly *B. tabaci*

Figure 1 Symptoms of tomato yellow leaf curl disease on different tomato varieties a) FC104395, b) Latyle No. 1, c) Latyle No. 3-3, d-f) Hirsutyle No. 1, 1-1, 3, 4-1, 6-2 and 6-3, respectively

METHODS AND RESULTS

1. Virus Isolation and Characterization

1.1 Virus Isolation : Tomato yellow leaf curl virus was purified from infected tomatoes var. Seeda by the method modified from Sequiera and Harrison (1982). One kilogram of infected tissues was homogenized in 0.1 M tris buffer, pH 8.4, containing 1% mercaptoethanol. Crude extract was clarified by adding chloroform to 10%, stirred and centrifuged at 10,000 g. The virus in supernatant was collected by 8% polyethylene glycol precipitation and resuspended in 0.01 M tris buffer, pH 8.0, containing 0.005 M EDTA. The supernatant obtained from low speed centrifugation of resuspended virus was subjected to high speed centrifugation at 270,000 g for 2 hours. The pellet was resuspended and centrifuged in cesium sulfate gradient solution (1.48 g/cm^3). Virus fraction was collected and dialyzed in 0.01 M tris buffer, pH 8.0 for 48 hours before used.

Result : The virus was successfully purified from infected tomato tissues yielding 80 µg virus/kg tissue. Virus yield varied from sample to sample and was less in old infected tissues. We have never observed a sharp band of virus after cesium sulfate density gradient centrifugation. However, purified virus preparation was clean and most of particles remained intact. Single and geminate particles with the average sizes of 18 and 18 x 38.8 nm were observed in negatively stained preparations (Fig. 2).

1.2 Isolation of TYLCV Nucleic Acid : Viral nucleic acid was isolated from the purified virus suspension as described by Stanley and Townsend (1985). Purified virus adding with 0.1% SDS was extracted with 1:1 (v:v) phenol-chloroform solution. The extraction was repeated twice. Nucleic acid in the aqueous phase was precipitated with 2.5 volume of absolute ethanol containing 0.3 M sodium acetate, pH 4.8. After low speed centrifugation at 10,000 rpm for 10 min, the pellet was washed in 70% ethanol, vacuum dried and resuspended in TE buffer (0.1 M tris, 0.01 M EDTA) for further uses. Isolated nucleic acid was subjected to various tests for physical and biochemical studies.

Results : Nucleic acids extracted from purified TYLCV preparation showed typical absorbency profile of nucleic acid with A 260:280 ratios of 1.85. Diphenylamine test of the isolated

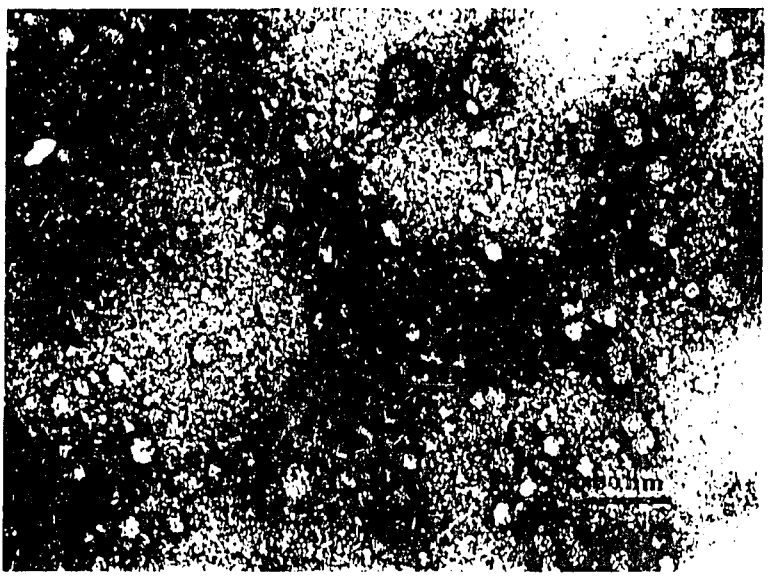


Figure 2 Electron micrograph of TYLCV from purified preparation showing single and geminate particles. The virus was negatively stained with uranyl acetate.

nucleic acid gave pale blue color after reaction indicating that it was DNA. Treatment of isolated nucleic acid with RNAse and DNAse showed that RNAse had no effect on TYLCV while DNAse completely digested TYLCV nucleic acid. The effects of heat and formaldehyde treatment on isolated nucleic acid were studied. It was found that heating one ml of isolated nucleic acid for 10 min at various temperatures resulted in hyperchromicity shift about 12% as compared to 53 % of double stranded calf thumus DNA (Fig. 3). Formaldehyde treatment of isolated nucleic acid increased the maximum absorption at 260 nm to 11.3% at 257 nm and caused the shifting of the minimum absorption at 233 to 235 nm (Fig. 4). These results confirmed that TYLCV has a single stranded DNA as its genome.

Electrophoresis of extracted TYLCV nucleic acid on 0.7-0.8% agarose gels revealed two distinct bands with the estimated molecular weights of 1.19×10^6 daltons for the upper band and 0.6×10^6 daltons for the lower band (Fig. 5). Electron microscopic observation of the eluted DNAs showed that the upper band was linear ssDNA and the lower band was circular DNA with the contour length of 717 nm (Fig. 6). It was estimated that TYLCV-DNA contained 2001 nucleotides.

1.3 Analysis of viral associated proteins : Efforts were made to analyze viral associated proteins from purified virus preparation and from systemically infected plants. It is hoped not only to gain information on physical and biochemical properties of TYLCV, but to develop criteria to differentiate mild and severe strains of TYLCV. Analysis of proteins was made by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970) and Conejero and Semancik (1977). The preparation was made as follows;

A. Separating gel : A separating gel solution contains 10% (w/v) acrylamide, 375 mM Tris-HCl (pH 8.8) and 0.1% (w/v) sodium dodecylsulfate (SDS). 0.025% (w/v) of ammonium persulfate and 0.05% (v/v) of N,N,N,'N'-tetramethylethylene diamide (TEMED) were mixed immediately and poured into a gel apparatus to about comb foot. The surface of the gel was then layered with water-saturated n-butonal and allowed to polymerize to produce a 0.75 mm thick slab gel. The top layer was drawn and the gel surface was washed with water once.

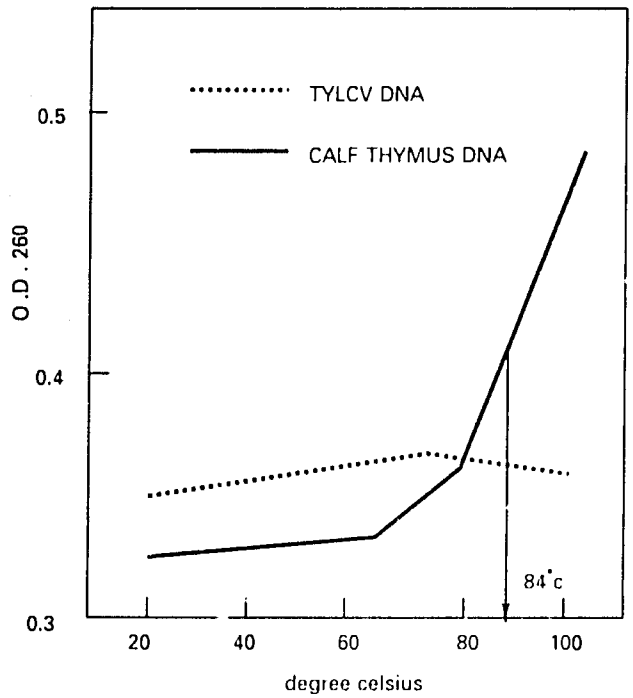


Figure 3 Hyperchromicity shift after heat denaturation (20-100°C) of TYLCV-DNA in 1x SSC buffer

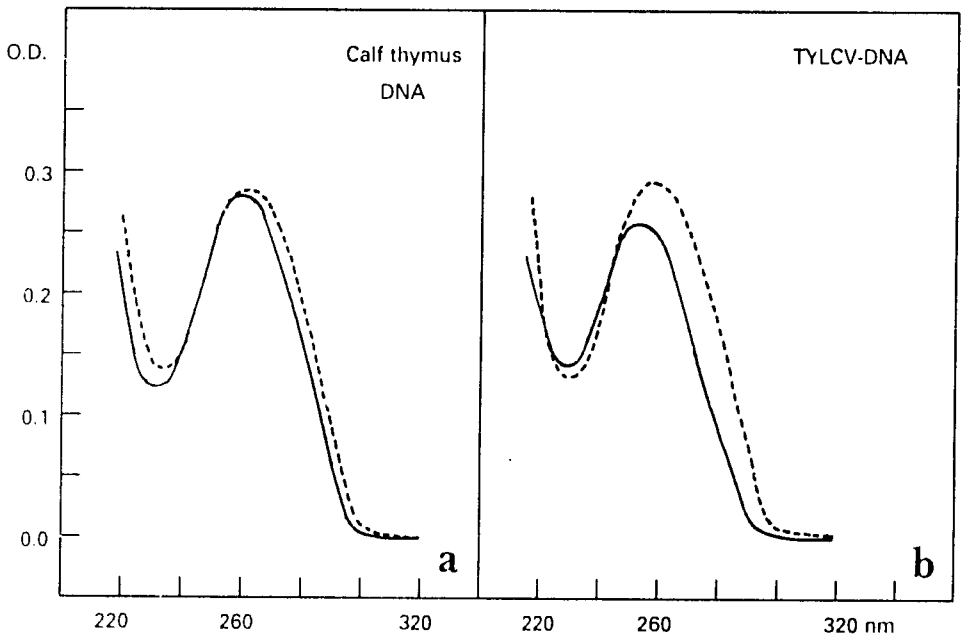


Figure 4 Effect of formaldehyde treatment on the ultraviolet absorbance of calf thymus DNA (a), and TYLCV-DNA (b).

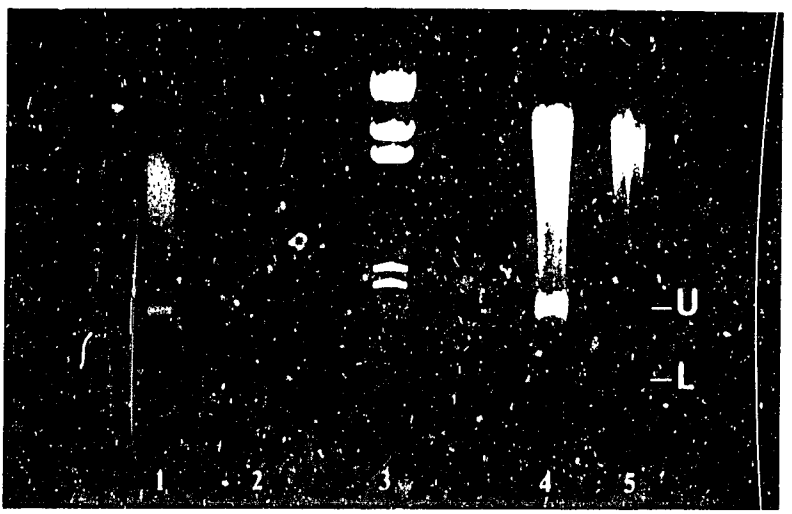


Figure 5 Agarose gel electrophoresis of TYLCV-DNA in TBE buffer. Lane 1,4 : TYLCV-DNA, Lane 2 : TYLCV-DNA treated with DNase, Lane 5 : TYLCV treated with RNase, Lane 3 : Lambda DNA digested with Hind III as markers. U = upper band L = lower band

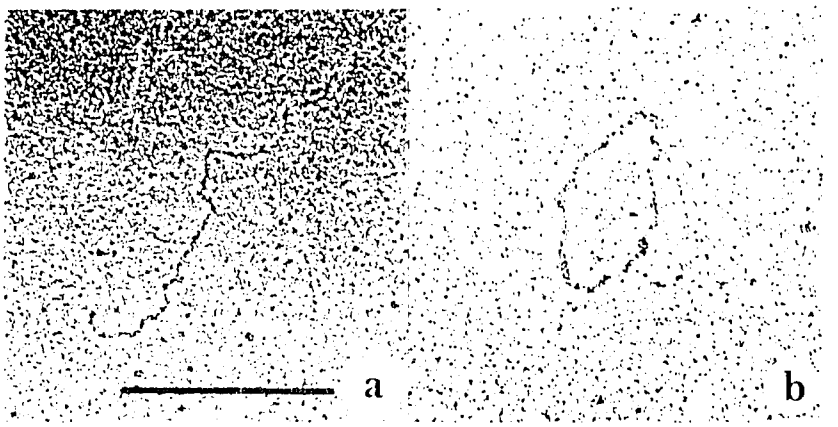


Figure 6 Electron micrograph of TYLCV-DNA eluted from agarose gel after electrophoresis.

a, linear form of DNA eluted from upper band.

b, circular form of DNA eluted from lower band.

Bar = 500 nm.

B. Stacking gel : Stacking gel solution contains 4% (w/v) acrylamide, 125 mM Tris-HCl (pH 6.8), and 0.1% (w/v) SDS. 0.05% (w/v) ammonium persulfate and 0.2% (v/v) TEMED were added immediately before pouring into the separating gel. Comb was inserted immediately and allowed for polymerization.

C. Sample preparation : Protein samples were boiled for 5 min in 62.5 mM Tris-HCl, pH6.8, 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, and 10% (v/v) glycerol and then placed samples on ice. In some cases, samples were clarified to remove insoluble proteins by low speed centrifugation.

D. Electrophoresis : Electrophoresis was performed in 10 C water-circulated electrophoresis buffer contained 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 0.1% (w/v) SDS at a constant 150 voltage. The gel was stained either with coomassie brilliant blue R-250 in water without fixing or with silver staining with fixing (See and Jackowski, 1989)

E. Silver staining : The gel was fixed overnight with 50% ethanol and 10% glacial acetic acid. The fixed gel was then soaked in 10% glutaraldehyde for 30 min, followed by rinsing with water for 30 min and then soaked in 15% ethanol with at least three changes of ethanol for 10 min each. The gel was transferred to a clean glass dish containing 19.2 mM sodium hydroxide, 1.4% ammonium hydroxide, and 45.7 mM silver nitrate for 10 min. The stained gel was washed with water for 6 min, and transferred to developing reagent containing 2.6 mM citric acid, 50 µl of 37% formaldehyde and 100 mM of 95% ethanol. After bands became visible, the gel was rinsed with distilled water and kept in 4% glycerol and 10% ethanol solution.

Results :

1. From infected plants; Healthy and infected plants showed similar protein patterns (in SDS-PAGE) except four minor additional proteins at Rf 0.307, 0.435, 0.507, and 0.874, were observed in infected plants as shown in Fig. 7. Based on molecular weight markers, molecular weight of these proteins were estimated to 51 kD (Rf = 0.307), 39 kD (Rf = 0.435), 33.5 kD (Rf = 0.507) and 14 kD (Rf = 0.874).

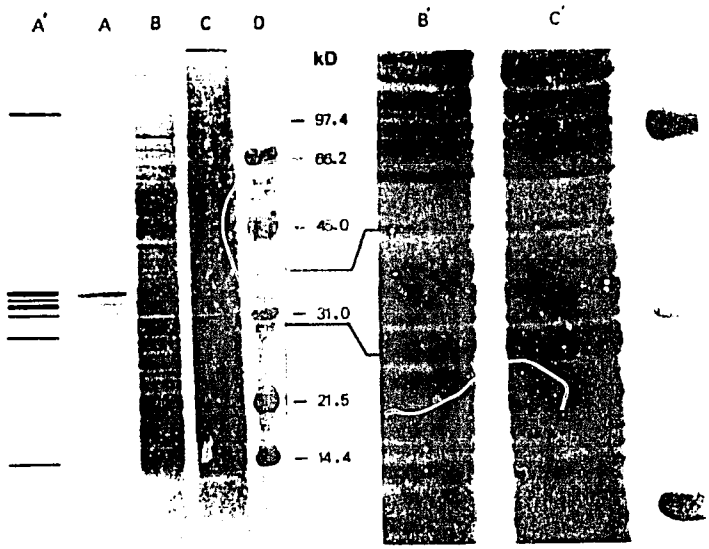


Figure 7 Electrophoretic patterns of [illegible] purified proteins
A) partially purified [illegible] [illegible] zymogram of lane [illegible]
B) crude sap of healthy tomato B' lane B expanded
C) crude sap of infected tomato C' lane C expanded

2. From partially purified virus preparation; When partially purified TYLCV was subjected to 10% SDS-PAGE, 7 protein bands were observed at Rf 0.135, 0.500, 0.508, 0.518, 0.535, 0.588 and 0.846). Six of them were found to be similar to those of both healthy and infected plants. One protein (Rf 0.508), specific to infected plants, co-migrated with the 33.5 kD protein (Rf 0.507) found in crude sap from infected plants (Fig. 8). The function of this protein is still unknown. The 33.5 kD protein is still in the range of previously reported geminiviral coat proteins such as 27.4 kD for BGMV (Goodman *et al.*, 1977), 28 kD for chloris striate mosaic virus (Francki. *et al.*, 1979) and 34 kD for cassava latent virus (Bock. *et al.*, 1977).

Results obtained from the analysis of virus associated proteins from infected plants and partially purified virus preparation were not sufficient to develop any criteria for strain differentiation in TYLCV.

2. Detection of TYLCV and Mild Strain Selection

2.1 Detection by using immunological techniques

A. Antibody production : Antiserum against TYLCV was produced by using the purified TYLCV as antigen. The rabbit was immunized by intramuscularly injecting a mixture of 0.5 ml virus suspension (50 µg/ml) and 0.5 ml Freund's complete adjuvant. The next three injections were done intravenously each with 0.5 ml of virus suspension at one week interval. The serum was collected weekly after the last injection up to four weeks and its titer was determined by microprecipitin test. Before use, antiserum was cross-absorbed with tomato leaf powder to minimize non-specific antibody.

Results : The antisera obtained has the highest titre of 1/128 and the lowest of 1/16 for the second and fourth bleed, respectively. However, agar gel diffusion test of the produced antiserum against sap from infected tomato always showed non-specific antibody reaction.

B. Immune electron microscopy (IEM) for virus detection : The method used was trap-decoration as described by Milne and Luisoni (1977). Membrane coated grids were floated on drops of 1:10 diluted antisera for 5 min, washed with distilled water and floated on drops of infected plant sap for 15 min. Grids were then washed with 0.1 M. phosphate buffer, pH 0.7 followed by floating for 15 min. on diluted

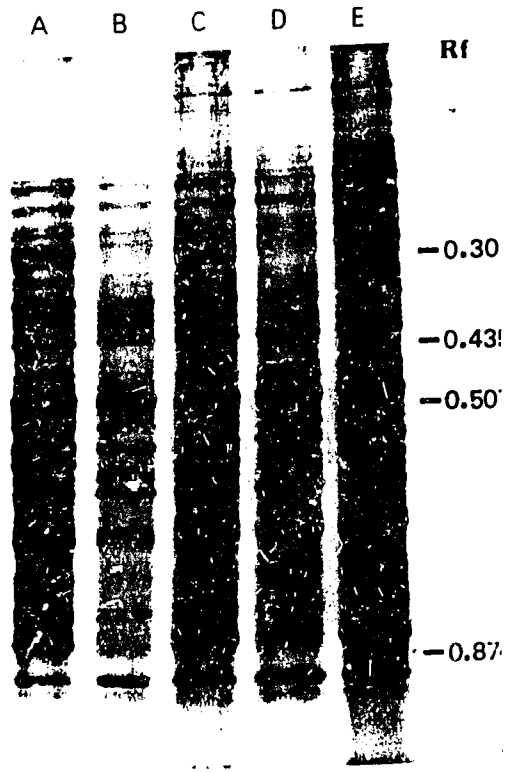


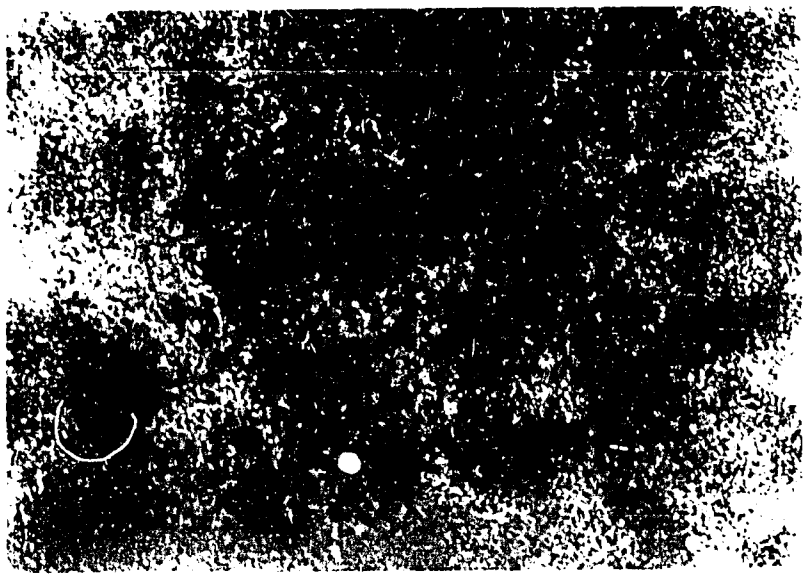
Figure 8 SDS-PAGE showing patterns of proteins from TYLCV infected (A, C, E) and healthy (B, D) crude saps of tomato tissues.

(1:100) absorbed-antiserum to allow virus particles being decorated by the antiserum. Grids were washed with several drops of distilled water before staining with 2% uranyl acetate for 1-2 min. and virus particles were observed under a transmission electron microscope.

Results : Observations of TYLCV particles in sap from infected tomato gave unsatisfactory result. It was very difficult to distinguish decorated virus particles from cell components and other impurities. In addition very few particles were detected due to low virus concentration in plant (Fig. 9). This method was very time consuming both for sample preparation and virus observation. It was not practical to use for the detection of the virus from hundreds of plant samples.

C. Virus dectection by dot immuno-binding assay (DIBA) : The detection by dot immuno-binding procedure used was modified from that described by Hibi and Saito (1985), and sample preparation method described by Yoshikawa *et al.*, (1986). The antiserum against TYLCV was cross-absorbed with dried leaf powder of healthy tomato tissue before used, and was called 'first' antibody. For virus detection, a 2 x 2 mm. leaf tissue was mixed and crushed with 10 µl of sample buffer (TBST: 20 m M tris-HCl, 0.5 M NaCl, pH 7.5, 0.05% Tween 20) on a parafilm sheet. Two microlitres of crude sap was withdrawn and spot onto nitrocellulose membrane. After air drying, the nitrocellulose sheet was immersed for 20 min. in blocking agent (TBST with 2% polyvinyl-pyrrolidone, PVP, and 2% Bovine serum albumin, BSA). After rinsing with distilled water and briefly blotting, the sheet was covered with 'first' antibody for 20 min. in a moist chamber at 37 C. The sheet was then washed with antibody buffer (TBST with 2% PVP and 0.2% BSA) for 10 min, followed by incubation with 'second' antibody (alkaline phosphatase conjugated-goat anti-rabbit IgG, Sigma Co.) for 20 min in moist chamber at 31 C. The sheet was again washed with antibody buffer, blotted briefly, and incubated for 10 min. in substrate solution (0.1% napthol AS-MX phosphate mixed with equal volume of 6 mg/ml Fast Red TR salt in 0.2 M tris-HCl, pH 8.2). Results were evaluated from pink color development as positive reaction.

Results : Dot immuno-binding assay was shown to be more effective method to detect TYLCV in crude sap than the IEM technique. It can detect ten nanograms of purified TYLCV, and 1-1/2 dilutions of plant sap. Working dilutions of 'first' and 'second' antisera were 1:100



[illegible] microscopic [illegible] particles [illegible] sap [illegible]
[illegible] dolomite [illegible] sap [illegible] [illegible]
[illegible] and [illegible] [illegible]

and 1:1,000, respectively. Tomato plant samples showing yellow leaf curl symptom gave positive pink color while the healthy tissues did not (Fig. 10). However, it was found that some of leaf-curl tissues did not give positive pink color and the reading was often interfered with brown green color of plant sap. There were some disadvantages when using DIBA for TYLCV detection in crude sap. First, green color of concentrated crude sap interfered with color development in positive reaction. Second, DIBA required a large amount of serum per test. To overcome these problems and to make TYLCV detection more reliable, nucleic acid hybridization was introduced to be a method of choice.

2.2 Nucleic acid probe and hybridization of TYLCV detection

A. DNA template and probe production : The single stranded (ss) DNA extracted from purified TYLCV particles was used as template DNA. Double stranded (ds) DNA was synthesized by annealing ssDNA template with synthesized oligonucleotide primers containing base sequences similar to hairpin loop region to tomato golden mosaic virus DNA (Hamilton *et al.*, 1984). The completion of dsDNA synthesis was obtained by Klenow polymerase filling-in reaction. The dsDNA was subsequently cloned into pBluescript plasmid at EcoRI site. Analysis of cloned DNA by agarose gel electrophoresis and restriction enzyme mapping revealed two components, A and B of TYLCV-DNA with nearly the same size of 2.7 kilobasepairs (Fig. 11.) Full length cloned DNA of the component A was selected to be used for probe production.

B. Radioactive labeling of TYLCV-DNA probe : The component A of TYLCV-DNA was excised from the pBluescript vector at EcoRI site. Linearized DNA was oligonucleotide primed and labeled with ^{32}P following the method of Hodgson and Fisk (1987). In the reaction mixture, 300 ng of dp(N) random primer was combined with 500 ng of template DNA in 10 µl of TE buffer (10 mM tris, pH 7.5, 1 mM EDTA). After immersing a reaction tube in a 80 C waterbath for 2 min and left to cool down at room temperature, the mixture was added with 4 µl of 10x oligolabeling buffer (0.5 M tris, pH 6.9, 0.1 M magnesium sulfate, 1 mM dithiothreitol), 0.6 mM each of dCTP, dGTP, and dTTP, 4 µl (40 µCi) of -^{32} P-dATP (3000 µCi/m mole) , and 5-10 units of Klenow polymerase, to make 40 µl final volume. After 30 min at 37 C, the reaction was added with 160 µl of STE (TE with 0.1 m NaCl) to bring the volume to 200 µl. The oligolabeling mixture was purified by using spun-column chromatography (Maniatis *et al.*, 1982), The eluent was collected

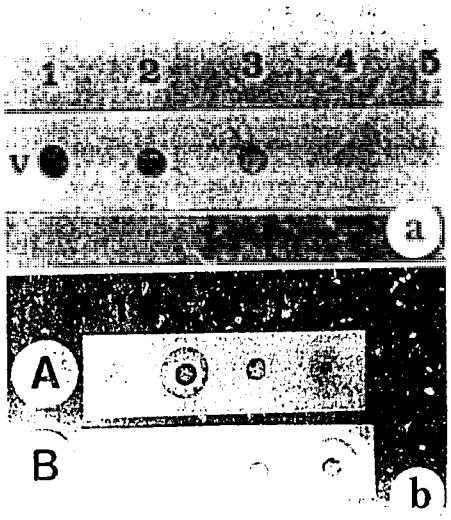


Figure 10 Sensitivity of Dot Immunobinding Assay (DIBA) for the detection of tomato yellow leaf curl virus.

a) in purified virus preparation at concentrations of 1 ng -0.1 pg (V 1-5)

b) in plant sap; A 1 = borate buffer, A 2-4 = infected plant sap at dilutions 1/2, 1/10 and 1/50 respectively. B 1 = borate buffer. B 2 = healthy tomato, B 3-4 = diseased tomato

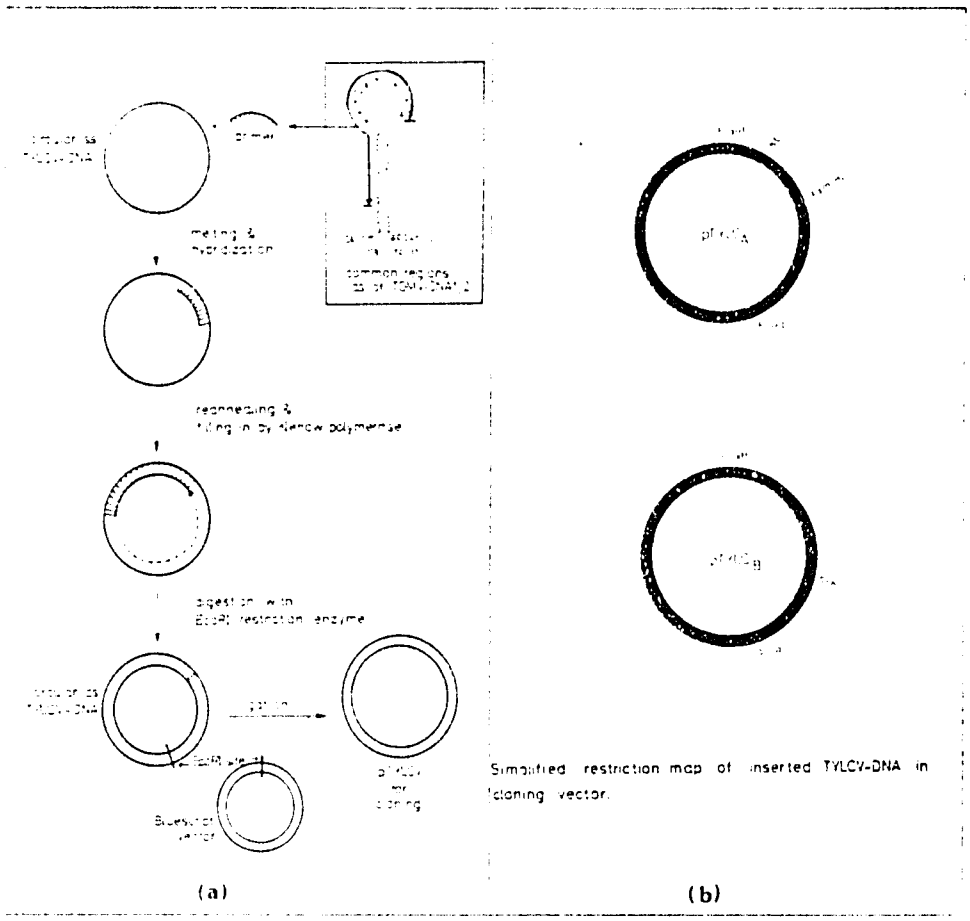


Figure 11 A scheme for synthesis of dsDNA of TYLCV (a) and gene maps of cloned DNA component A and B (b).

and determined for incorporation percentage of ^{32}P-dATP by a liquid scintillation counter, The probe was immersed in a boiling waterbath for 2 min prior to hybridization.

C. Non-radioactive labeling of DNA probe : Template DNA used was linearized dsDNA excised from plasmid vector as previously described. Purified DNA template was labeled by oligonucleotide random primed incorporation of digoxigenin-labeled dUTP according to Anonymous (1989). In a microtube, 1 µg (10 µl) of linerized DNA was denatured by heating in a waterbath for 10 min at 95 C and chilled quickly on ice/NaCl for complete denaturation. After cooling, 2 µl of hexanucleotide primers mixture and 2 µl of dNTP labeling mixture (dATP, 1 mM; dCTP, 1 mM; dGTP, 1 mM; Dig-dUTP, 0.35 mM pH 6.5) were added. The reaction volume was adjusted to 19 µl by double distilled water, and mixed with 1 µl of Klenow enzyme (2U/µl). The reaction mixture was incubated at 37 C for 16 hr and 2 µl of 0.2 M EDTA, pH 8.0 was added to stop the reaction. Labeled nucleic acid was precipitated by adding 2.5 µl of 4 M LiCl, and 75 µl of prechilled ethanol. After incubation at -80 C for 1 hr, DNA was pelleted by centrifugation to 10,000 g for 10 min followed by washing with 70% prechilled ethanol and dried in vacuum. The labeled DNA pellet was redissolved in 50 µl of tris-HCl, 1 mM EDTA pH 8.0, and kept for further hybridization test.

D. Sample preparation for virus detection : Leaf samples to be tested for TYLCV detection were prepared by three different methods as follows;

Method I. A minipreparation method described by Dellaporta *et al.*, (1983) was used. Leaf tissues of 0.5 g were ground to powder in liquid nitrogen and added with 1.5 ml of extraction buffer (100 mM tris, pH 8.0, containing 50 mM EDTA, pH 8.0, 500 mM NaCl, 10 mM mercaptoethanol). After adding 1 ml of 20% SDS, the suspension was mixed thoroughly by vigorous shaking and incubated at 65 C for 10 min. It was then added with 5 ml of 5 M potassium acetate, shaked vigorously and incubated at 0 C for 2 min. After pelleting out the precipitate, the supernatant was added with 10 ml of isopropanol and incubated at -20 C for 20 min. DNA was pelleted and dried on paper towel for 10 min. DNA pellet was redissolved with 0.7 ml of 50 mM tris buffer, 10 mM EDTA, pH 8.0. The suspension was spinned in a microcentrifuge to remove insoluble debris, and supernatant was transferred to a new Eppendorf tube. DNA was pelleted by adding 75 µl

isopropanol, and centrifuged. DNA pellet was washed with 80% ethanol, dried and redissolved in 200 µl of 100 mM tris buffer, 10 mM EDTA, pH 8.0.

Method II. This method was used to prepare DNA samples from large number of crude saps of infected tomato tissues for dot blot hybridization. Healthy and diseased tomato leaf tissues were ground each in one ml of 0.1 M tris buffer, pH 8.0 and centrifuged to remove plant debris. To each 250 µl of supernatant, an equal volume of denatured solution containing 67 µl of 3 M NaOH, 50 µl of 0.1 M EDTA and 133 µl of distilled water was added. DNA solution was boiled for 10 min in a waterbath and immediately chilled on ice. Ten fold diltutions of denatured DNA were made from 1/10 up to 1/1,000,000, and these DNA samples were used for DNA probing.

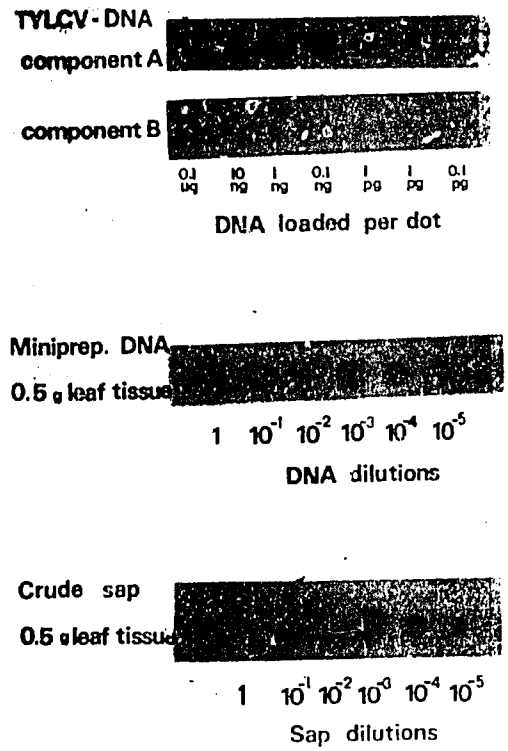
Method III. This squash blotting method (Navot *et. al.*, 1989) was performed for obtaining rapid DNA preparation either from infected leaf tissue or from whitefly vector. Tomato tip leaves were squashed onto a nylon membrane using a sterile glass rod and added with 10 µl of denatured solution (0.125 NaOH, 0.125X SSC). Whitefly adults collected from healthy and infected tomatoes were squashed either slightly or group of five onto the nylon membrane by the same procedure. The membrane was air dried before subjected to hybridization.

E. **Nucleic acid hybridization and detection :** Radioactive hybridization was performed as Perbal (1988). Prehybridization was at 65 C for 3 hr followed by hybridization at 65 C for 15 hr. After washing and air dried, the filter was autoradiographed at -80 C for 24-96 hr. For non-radioactive hybridizaation the blotted filter was prehybridized in a sealed plastic bag with 20 ml hybridiztion solution (5xSSC, 1.2% w/v blocking reagent, 0.1% N-lauroylsarcosine Na-salt, 0.02% SDS) per 10 cm^2 of filter at 68 C for at least 1 hr. After that, the filter was added with 2.5 ml of fresh hybridization solution containing 25 µg of freshly denatured TYLCV-DNA labeled probe per 100 cm^2 filter. Hybridization reaction was maintained at least 6 hr at 68 C with 0.1x SSC; SDS. 0.1% (w/v). To proceed the immunological detection, the filter was washed briefly in tris buffer-I (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) and incubated in 0.5% blocking reagent in the same buffer for 30 min before washing again with tris buffer-I. The filter was then incubated with 20 ml of 1:5000 diluted antibody-conjugate Dig-AP-conjugated, 750 U/ml) in tris buffer-I at 20 C for 30 min. The unbound conjugated was removed and the filter

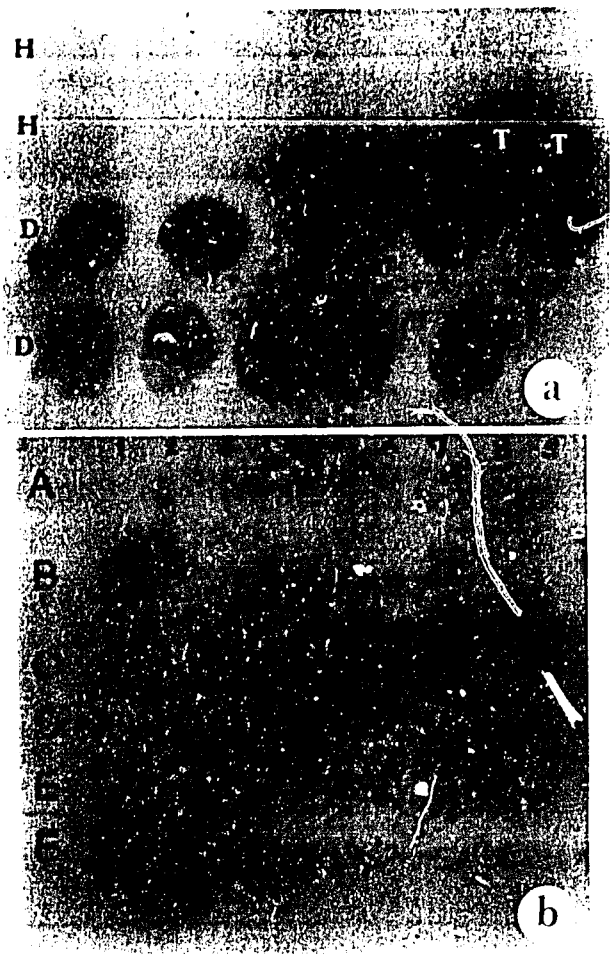
was washed twice 15 min each with 100 ml of tris buffer-I. After equilibrating in tris-buffer-II (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), the filter was incubated with 10 ml color solution in a suitable box kept in the dark. Color solution must be freshly prepared by mixing 45 µl NBT solution and 35 µl x-phosphate solution in 10 ml tris buffer-II. The reaction was then replaced with 50 ml of tris buffer-III (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) for 5 min to stop reaction and the results were recorded by photography.

Results : We have obtained that radioactive ^{32}P-oligolabeled TYLCV probe showed about 8-15% incorporation of a radioisotope after 30 min reaction. This probe could detect as low as one picogram of template DNA (Fig. 12). When ^{32}P-labeled DNA probe was applied to detect TYLCV in infected tomato leaf tissues, positive reactions were obtained in all samples resulting from the three methods of sample preparation. With minipreparation and crude sap methods, the probe could detect TYLCV up to 1/100,000 dilution. Healthy leaf tissues showed negative results for all tests. The ^{32}P-labeled DNA probe could also detect TYLCV-DNA in squashed samples from infected tomotoes (Fig. 13a) and viruliferous whiteflies which were fed on diseased tomato plants, but not from healthy ones (Fig. 13b). The developed non-radioactive Dig-labeled DNA probe showed a very high sensitivity as compared with the radioactive probe. Its sensitivity was at picogram level of homologous DNA (TYLCV-component A) and at nanogram level of heterologous DNA (TYLCV-component B) (Fig. 14a). In addition, the non-radioactive Dig-labeled DNA probe showed high sensitivity to detect TYLCV-DNA both in plant sap and viruliferous whiteflies. The probe could detect 1/10,000 dilution of infected tomato-minipreparation as well as crude sap (Fig. 14b). It could also detect TYLCV-DNA in eggs, larvae and adult whiteflies collected from diseased plant using squash blot technique (Fig. 15). This squash blot method along with the developed DNA probes served as a practical diagnostic method for TYLCV-DNA detection in a single-insect sample.

2.3 TYLCV mild strain selection : Results obtained from IEM and DIBA detection of TYLCV suggested that these two techniques were less effective when used for virus detection in crude sap. It was also shown that DNA probing was the most practical diagnostic method for TYLCV detection because of its high sensitivity and accuracy. Therefore, we have been using dot blot hybridization for virus detection and selection for natural occurring mild strain of TYLCV.



Autoradiograph [illegible] of tomato yellow leaf curl virus DNA showing [illegible] of P-labeled DNA probe [illegible]



Autoradiographs of dot blot hybridization of TYLCV-DNA with P-DNA probe

(a) Samples from healthy (H) and diseased (D) tomatoes

(b) Samples from non-viruliferous (Row A), viruliferous (Row B) Bemisia whiteflies, and diseased tissues (Row E)

C = uninfected positive control using TYLCV component A

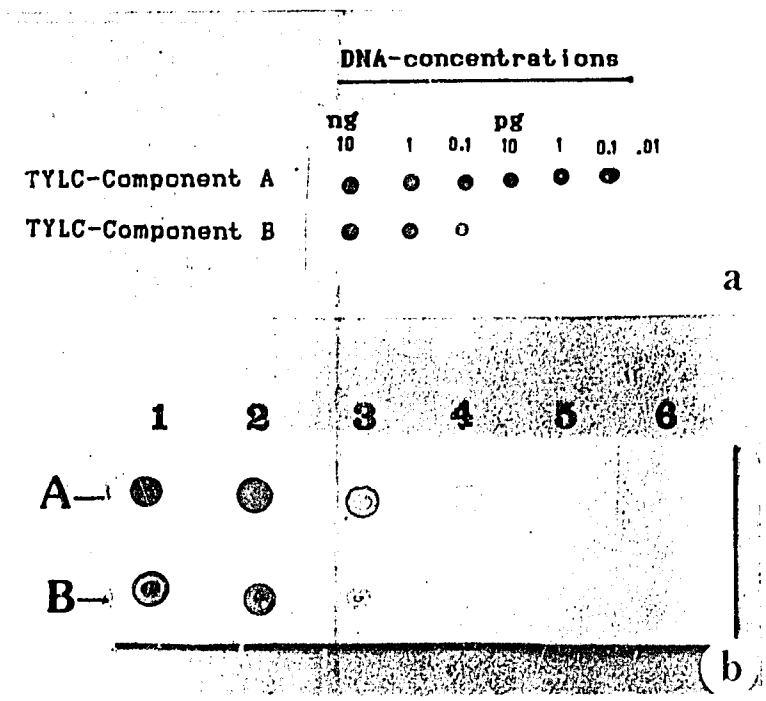


Figure 14 Dot-blot hybridization of TYLCV with probe derived from Dig-labeled component A DNA

a) sensitivity of Dig-labeled DNA probe

b) detection of TYLCV in serial dilutions of infected tomato minipreparation (A) and crude sap (B)

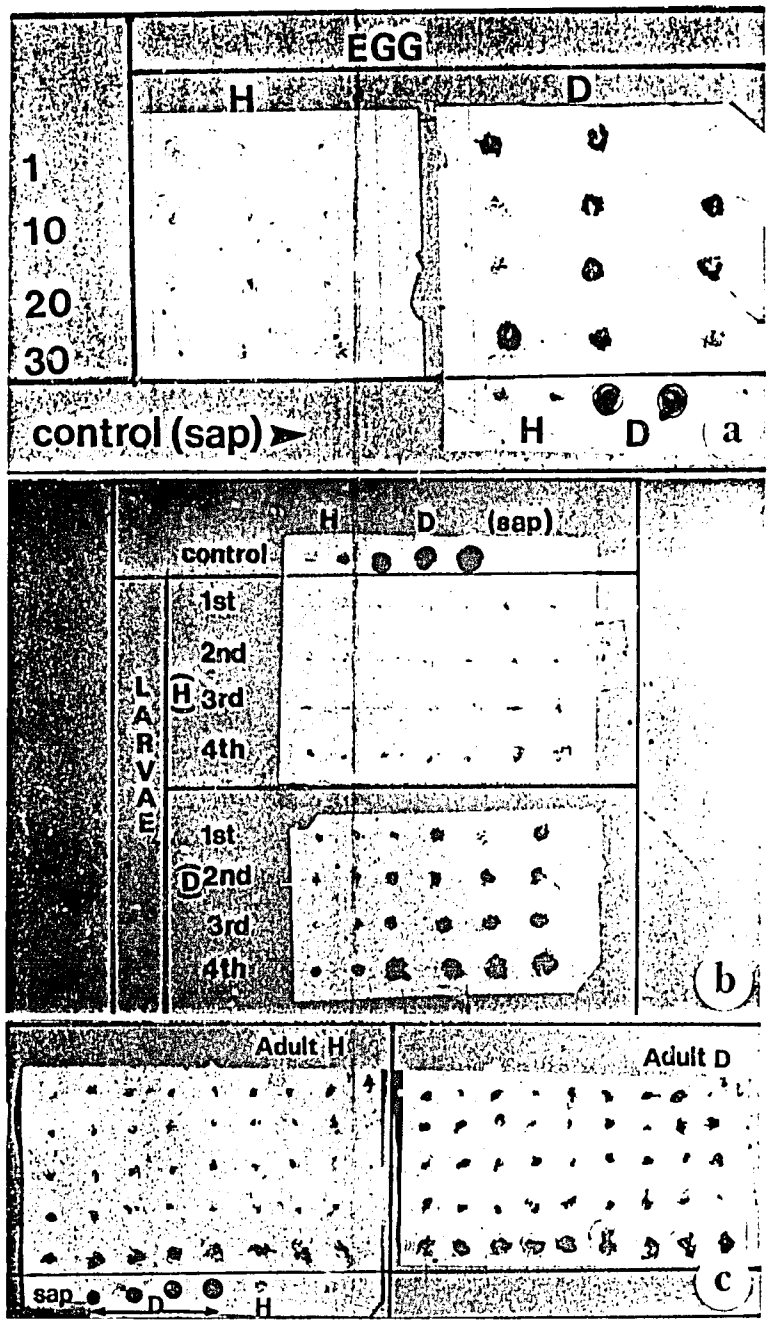


Figure 15 Detection of tomato yellow leaf curl virus in eggs (a), larvae (b), and adult (c) whiteflies using Dig-labeled DNA probe and squash blot technique. Samples were non-viruliferous (H) and viruliferous (D) whiteflies. Saps from healthy tomato (H) and TYLCV-infected tomato (D) were used as control.

A. Detection and selection of TYLCV-mild strains in field grown tomatoes. This study was conducted after disease survey in Kamphaengsaen district, Nakorn Pathom. Shoots of symptomless tomato plants found in tomato fields with high incidence of TYLCV infection were collected. Leaf tissues were excised from shoots and subjected to hybridization. The remaining tissues were grafted to healthy tomato seedlings for virus propagation. Minipreparations of nucleic acid from leaf samples were dot blotted onto nitrocellulose membranes and hybridized with ^{32}P-labeled TYLCV-DNA probe as previously described. Tomato seedlings grafted with shoots which showed positive reactions with TYLCV-DNA probe were screened for symptom expression. Those showing no symptoms were selected for mild strains for TYLCV, and subjected to cross protectivity test.

Results : Total 241 samples of field grown tomatoes were collected from three villages in Kamphaengsaen district (Table. 1), from which 71 samples reacted with the probe. Among these, nine of them did not induce leaf curl symptoms on tomato seedlings after graft inoculation. We maintained these 9 plants as sources of TYLCV mild strains by grafting to new tomato seedlings as well as by tissue culturing.

Table 1 Field survey for selection of TYLCV mild strains.

Survey no.	Village name	No. of sample collected	No. of sample reacted with probe	No. of mild strain
1.	Ban Don Khoi (บ้านดอนข่อย)	56	19	2
2.	Ban Yang (บ้านยาง)	61	30	1
3.	Ban Nong Mai Ngam (บ้านหนองไม้งาม)	124	31	6
	TOTAL	**241**	**80**	**9**

B. Detection of TYLCV in other wild hosts. The ^{32}P-labeled TYLCV-DNA probe was used to detect the virus in several species of weeds in tomato fields. Seedlings germinated from collected seeds of several species of weed were served as healthy control. Weed plant DNAs were extracted using minipreparation melthod as mentioned above.

Results : Most of dicotyledonous weed plants collected from fields showed yellow mosaic or yellow symptoms on leaves. Some exhibited leaf curling and dwarfing (Fig. 16) Detection of TYLCV from these weed plants by dot blot hybridization revealed positive results in five weed species. Sap of some of healthy looking plants such as Seeda spp. hybridized to the probe while those of the healthy control plants obtained from seeds did not. We have also transplanted some of symptomless Seeda spp. from field into pots and maintained under insect proof condition. Most of transferred plants developed yellow mosaic symptoms similar to those seen in fields about 2-4 weeks after potting. The results indicated that weed plants in the field were commonly infected by TYLCV. Some of natural hosts of TYLCV in the fields were listed (Table. 2). It is shown that TYLCV has a very wide host range belonging to at least four plant families, e.g. Malvaceae, Compositae, Euphorbiaceae and Passifloraceae.

3. Insect Vector Studies

3.1 Rearing of insect vector

The whitefly, *Bemisia tabaci*, commonly called the tobacco or the sweet potato whitefly, is a tiny insect in the Family Aleyrodidae, Order Homoptera. It is ranked among the most noxious insects attacking field and greenhouse crops around the world. Nymphs, as well as adults, feed on the phloem sap of several plants species, thus can act as vectors for plant viruses. The most common and harmful virus disease causing severe losses for tomato production in Thailand is tomato yellow leaf curl disease which is spreaded by the winged adult whiteflies.

Adults whiteflies were collected from tomato fields around Kamphaengsaen Campus, Kasetsart University, Nakorn Pathom. Aspirator, the small collector device was used to collect the adult whiteflies from underside of the leaves. The collected whiteflies were released into the nylon screen cages containing a host plant. Sweet potato, a non-host for TYLCV, was used as a host for the whitefly. After the establishment of the whiteflies for several generations sweet patato, they were transferred to feed on healthy tomato seedlings covered with nylon screen cage (Fig. 17). Whitefly colonies that established for few generation on and caused no symptoms on tomato seedlings were selected as the healthy whitefly colonies for further experiments.

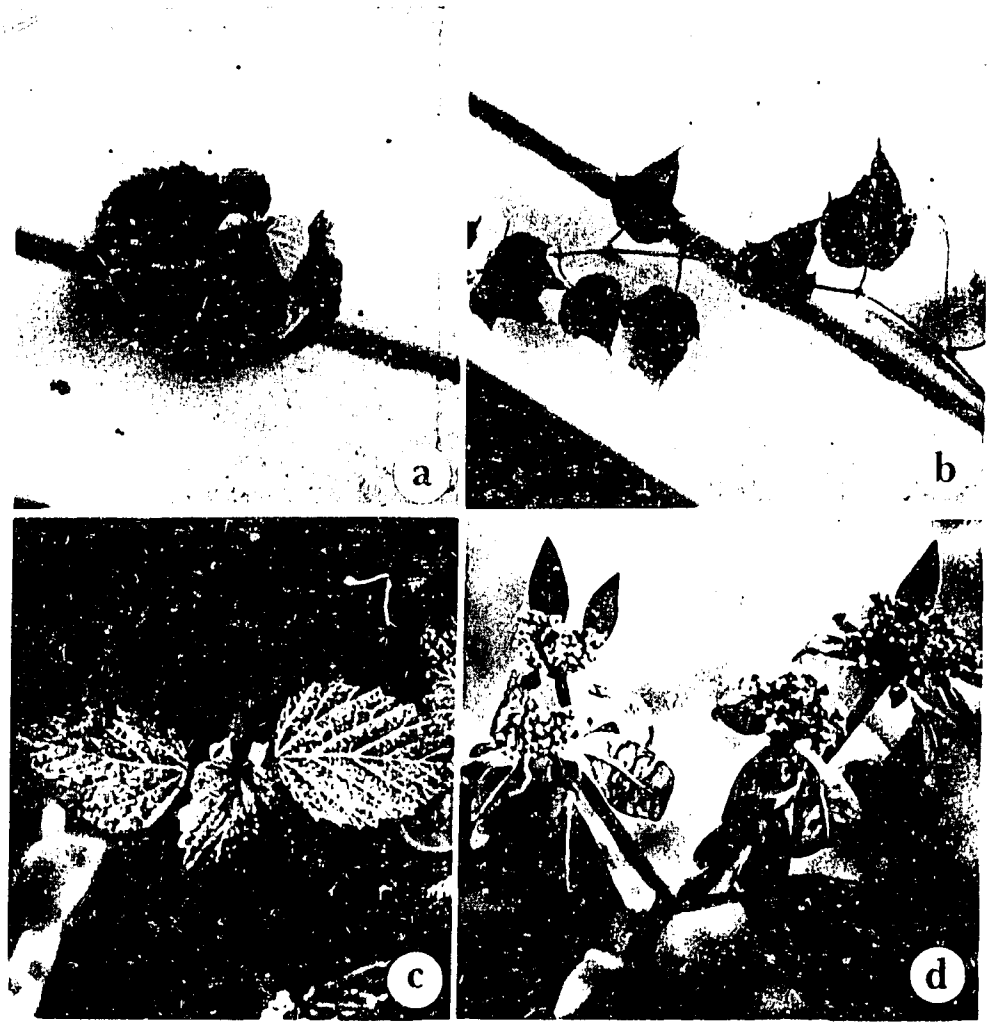


Figure 16 Natural weed host in which tomato yellow leaf curl virus was detected by using ^{32}P-labeled DNA probe.

a. *Abutilon hirtum*

b. *Passiflora foetida*

c. *Eclipta prostrata*

d. *Euphorbia heterophilla*

Table 2 Natural weed plants found in tomato fields.

Plant name (Name in Thai)	Symptom	Hybridization test with TYLCV probe
Family Malvaceae		
Abutilon hirtum (กรอบจักรวาล)	leaf curl	+
Sida spp. (ไม้กวาด)	yellow mosaic	+
Family Amaranthaceae		
Achyranthus aspera (พันธุ์งูขาว)	leaf curl	-
Family Commelinaceae		
Commelina benghalensis (ผักปราบ)	vein banding	-
Family Compositae		
Eclipta prostrata (กระน่ม)	vein yellowing	+
Eupatorium odoratum (อาทมสือ)	no symptom	-
Family Euphorbiaceae		
Euphorbia heterophilla (หญ้าดาง)	yellow leaf curl	+
Family Verbinaceae		
Lantana camara (ผักกาดอ)	no symptom	-
Family Passifloraceae		
Passiflora foetida (กระทกรก)	yellow mosaic	+
Family Solanaceae		
Physalis minima (โทมทาง)	leaf curl	-
Family Acanthaceae		
Ruellia tuberosa (ต้อยติ่ง)	no symptom	-

+ positive result, - negative result

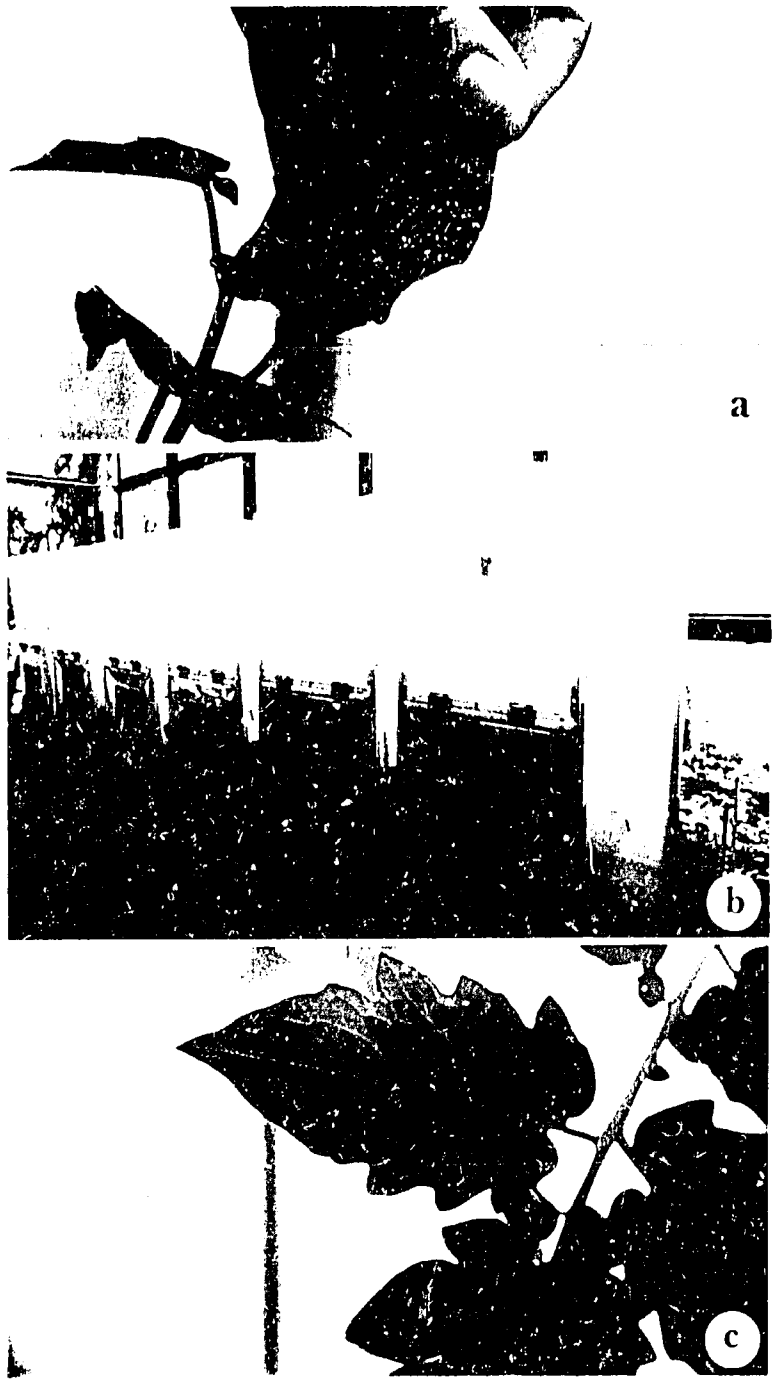


Figure 17 Mass rearing of the tobacco whitefly, *Bemisia tabaci*

a. adult whiteflies feeding on sweet potato plant

b. a set of nylon screen cages with tomato seedlings kept in screenhouse

c. adult whiteflies feeding on tomato leaf

To study the life cycle of whitefly, eggs from healthy colonies were transferred to 2 cm^2 pieces of tomato leaves which were kept in petridishes with moisted cotton (Fig. 18). The developmental cycle of the whitefly was observed daily.

Results : The whitefly, *Bemisia tabaci* has six life stages, the egg, the crawler (1st nymphal instar), two sessile nymphal instar (2nd and 3rd instar nymphs), the pupa (4th instar) and the adult or imago (Fig. 19). The morphology of different life stages can be summarized as follows;

The egg.

The egg is usully yellow in color, elongate-oval and sculptured. The apex or distal end of the egg may be broadly rounded to broadly acute. The broader basal end usually has a pedicel or stalk of varying length by which the female attaches the egg to the host. Many studies indicated that the pedicel directs the spermatozoa to the egg nucleus during fertilization and the pedicel serves as a conduit for moisture which protects the egg from dehydration. Female whitefly prefers young leaves for oviposition. Eggs are laid predominantly on the undersides of leaves and on hairy plant species, however, *B. tabaci* refrains from ovipositing on very young leaves. The egg stage usually is six days.

The crawler (1st nymphal stage)

The 1st nymphal stage is mobile but will settle within a few millimetres of the egg. The body is elongate-oval, 0.2 min. long, transpaernt or opaque, with color ranging from light green to yellow. Small amounts of powdery white wax usually are produced after the crawler settles and begins feeding. Several marginal setae and caudal setae are obviously observed. The crawler feeds for 4 days and begins to molt into the 2nd nymphal stage.

The second and third instar nymphs

These two stages of nymphal development last for 7 days. They are similar in general shape and color to the pupae. The segmentation of the legs and antennae is not distinct, may be reduced to only one segment. The body is 0.3-0.5 mm.long, more opaque with yellowish in color. Two eyespots, deep red to black in color are more conspicuous as the third instar nymph molts into the pupae. Wax secretions are common although they usually are radically different from those of the pupal stage.

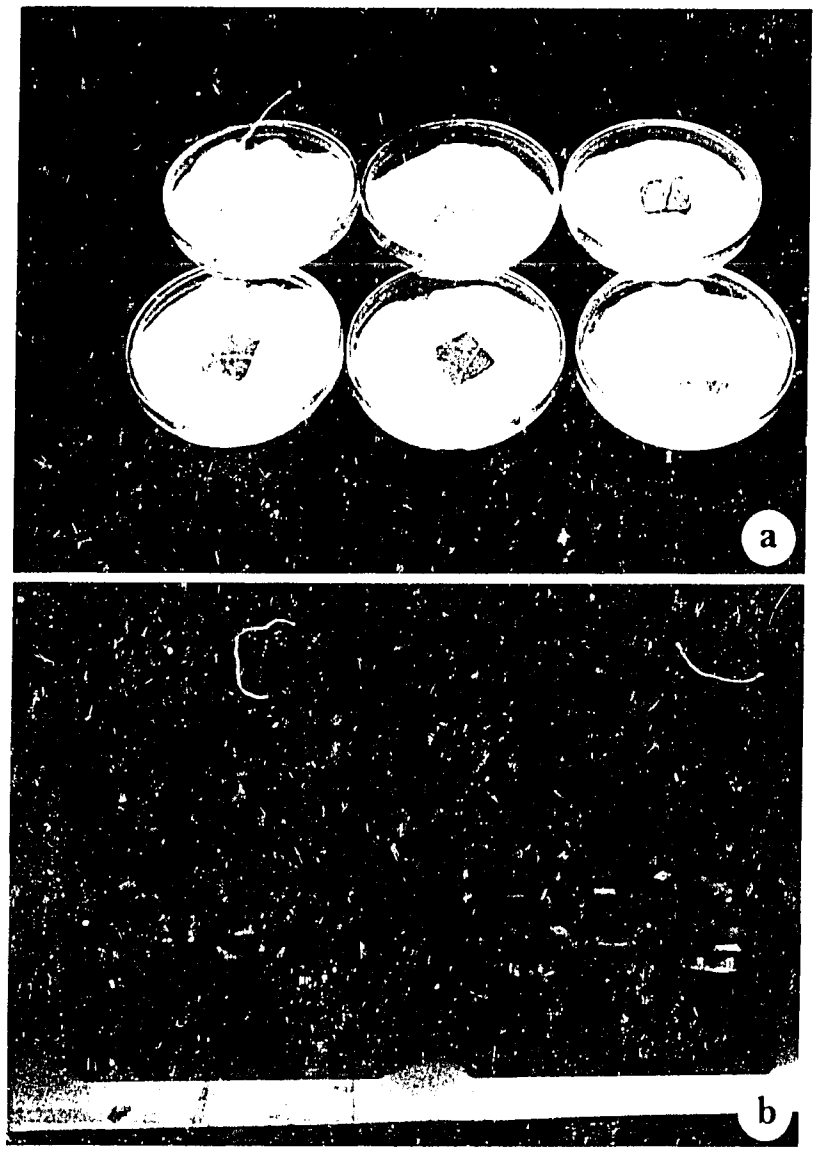


Figure 18 Laboratory rearing of the tobacco whitefly *Bemisia tabaci* for life cycle study.

a) cotton moisted petridishes containing pieces of tomato leaves with the whitefly eggs.

b) incubation of petridishes for the development of the whitefly

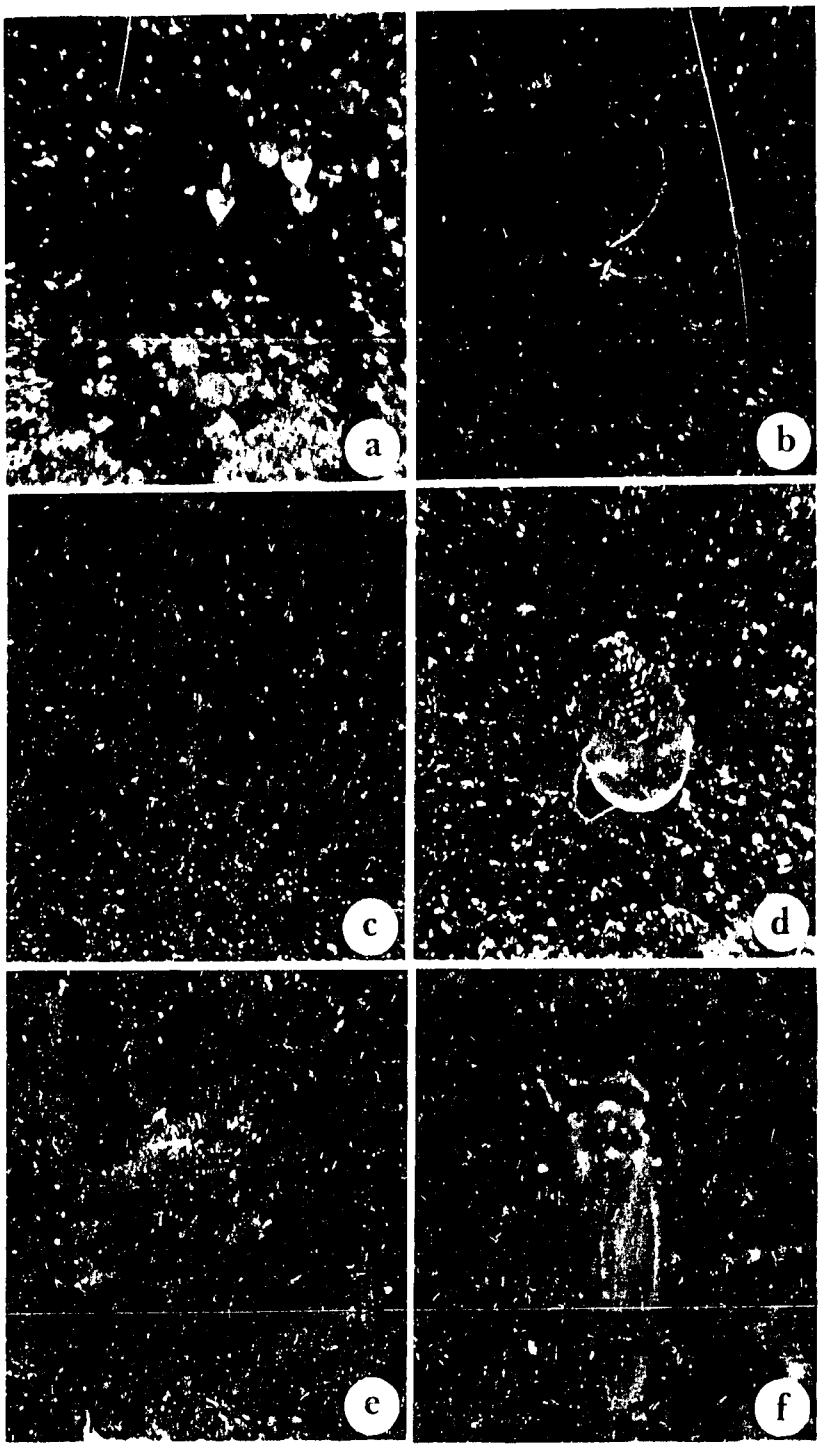


Figure 19 Life cycle of the tobacco whitefly *Bemisia tabaci*
a) the eggs, b) the crawler (1st nymphal instar),
c) the 2nd nymphal instar, d) the 3th nymphal instar,
e) the pupa, and f) the adult or imago

The pupa

The pupa shape is oval or nearly circular of approximately 0.8 mm long with the widest area of the body just posterior to the transverse molting suture. Most pupae are nearly flat in profile. As development continues, particularly with the development of the adult internally, the dorsum of the pupa becomes more convex or elevated. Whitefly pupae often reflect the color of the host, usually some shade of green. They may exhibit iridescent colors, usually blues, violets and greens which is probably due to fine striations in the cuticle which refract light. The internal organs show yellow or orange coloration, and the compound eyes of developing adults show as red spots through the papal case. The pupae produce a thin layer of transparent wax over most or all of the body surfaces and often is not readily apparent to the unaided eye. Whitefly pupa in subfamily Aleyrodinae lacks compound wax pores and leg claws, have only one pair of lingular setae and has adhesive or circular discs at the apices of the legs. Antennae and legs appear as single unsegmented organs. Certain setae include anterior and posterior marginal, cephalic, mesothoracic, metathoracic, caudal and ventral abdominal setae are normally found on whitefly pupae. It has been shown that the number, size and placement of dorsal setae in *Bemisia tabaci* may be correlated with the amount, distribution and construction of leaf hairs on the host surface. The pupal stage is approximately 6 days.

The adults

Emergence of adult *Bemisia tabaci* takes 5-15 min and is followed by wing expansion, which occurs on or near the pupal case and takes 40-50 min. Emergence generally occurs during the day. Males emerge on average a little earlier than females. Adult whiteflies appear white owing to the white powdery wax secretions produced shortly after emergence from the pupa. The actual colour of the adult integument is usually yellow. The body length is usually shorter than 2.0 mm; total length from vertex to wing tip no greater than 2.0 mm, and the wing span is no greater than 3.5-4.0 mm. The wing venetion is reduced primarily to the costal-subcostal veins, radius and occasionally the cubitus vein. There are four ventral abdominal wax plates in males and two ventral abdominal wax plates in females. Light microscopic observation reviewed that males have a very short simple aedeagus and have extremely a long pairs of clasper.

3.2 Insect Population Studies :

There is certainly a need for monitoring whitefly population levels to determine the success and continued reliability of a control program. To access the fluctuation of whitefly population around the year

in Kamphaengsaen area, two experimental plots were used as sampling sites, the tomato experimental plots of Asian Vegetable Research and Development Center and tomato plots of Department of Entomology, Faculty of Agriculture, Kasetsart University. In both plots, watering and fertilizing were practised according to the regular schedule to ensure normal development of the plants. However, no insecticide was used throughout the experiment.

Several studies have demonstrated that the most attractive color for adult whitefly is yellow. The attraction of the yellow color can be explained by the importance of the whitefly laying its eggs on young leaves, which are more yellowish than older leaves. In this study we used flat yellow plastic plate of 15 cm x 20 cm as traps. The traps were placed horizontally on the wood stick, of approximately 75 cm in height above the ground level. The plastic plate was covered with clear plastic bag in which the outer layer was pasted with vaseline as sticky material to trap the adult whitefly. Seven days after transplanting tomato plant, these complete set of sticky traps were placed in every one of the other row of plant, 3 meters apart. Ten sticky traps were used per row of tomato plant of 30 meters long. In every three days, the sticky plastic bags were collected in the early morning, brought back to the laboratory and the number of adult whitefly sticked on the vaseline trap were counted with the aid of the stereomicroscope. This study was performed during the period of June 1991 to May 1992 which covered the wet and dry season of the year. The collected data were the average of 3 plantings of the tomato crop. Daily minimum and maximum temperature and level of rainfall were also recorded throughout the study.

Result : The results indicated that whitefly *Bemisia tabaci* population fluctuated throughout the year (Fig. 20). The number of adult whitefly caught on the trap could vary from 0 to 7 per trap. *B. tabaci* population increased rapidly from 0.5 whiteflies per trap in June 1991 to the peak of 5 whiteflies per trap in July and August 1991. During September 1991 to March 1992, there was no abrupt fluctuation of the whitefly population. The population density remained in the average of 0.5-2 whiteflies per trap. However in April 1992 and May 1992 the population started to increase to the peak of 7 whiteflies per trap. The result suggested that population density of adult whitefly throughout the year fluctuated into 3 periods (Fig. 21). From June to September, the average no. per trap was 3.5, from November to December, the population droped down to the average no. of 0.5 whitefly per trap and from January to May, the population increased to the average of 2 whiteflies per trap.

The number of *B. tabaci* per trap during wet season (June-September, 1991) was significantly higher than those of dry season (November-December 1991 and January-May 1992).

Correlation between population density of adult whitefly and daily temperature was determined by recording the temperature in the tomato field daily around the year. The result shown in Fig. 22 which indicated that the daily minimum temperature during the period of June, 1991 to May, 1992 in Kamphaengsaen district was in the range of 18-20 C.Number of adult whitefly per trap was in direct correlation with daily minimum temperature. During July-August 1991 when the daily minimum temperature went up to 23-25 C, population of adult whitefly could raise up to 5 individual per trap. The same phenomenon occurred during the period of April to May 1992 in which population of adult whitefly started to increase in accordance to the increase of the daily minimum temperature. During the dry and cool period of the year that is in December 1991 to Juanuary 1992 in which the daily minimum temperature droped down to 18 C, no adult whiteflies were caught in the traps. The average number of whiteflies per trap in correlation with the daily minimum temperature (Fig. 22) was in an agreement with the result shown in Fig. 23.

Study on the population density of tobacco whitefly, *Bemisia tabaci* in tomato field in Kamphaengsaen district during June 1991 to May 1992 by using yellow sticky traps can be concluded as follows; the number of whitefly catched was in correlating with weather data. Several parameters and models were tested by using regression analysis. The results showed that the number of whitefly per trap in a given month was moderately correlated with the average daily minimum temperature ($r = 0.06057$, $p = 0.0483$). The model can be expressed as $Y = -9.0603 + 0.4872 X$), where Y is the number of whiteflies per trap and X is the average daily minimum temperature. Other variables such as daily maximum temperature and rainfall produced low correlation coefficient value ($r = 0.4409$ and 0.1369, respectively). The number of whitefly per trap during wet season (June-September 1991) was significantly higher than those of dry season (November 1991 - January 1992).

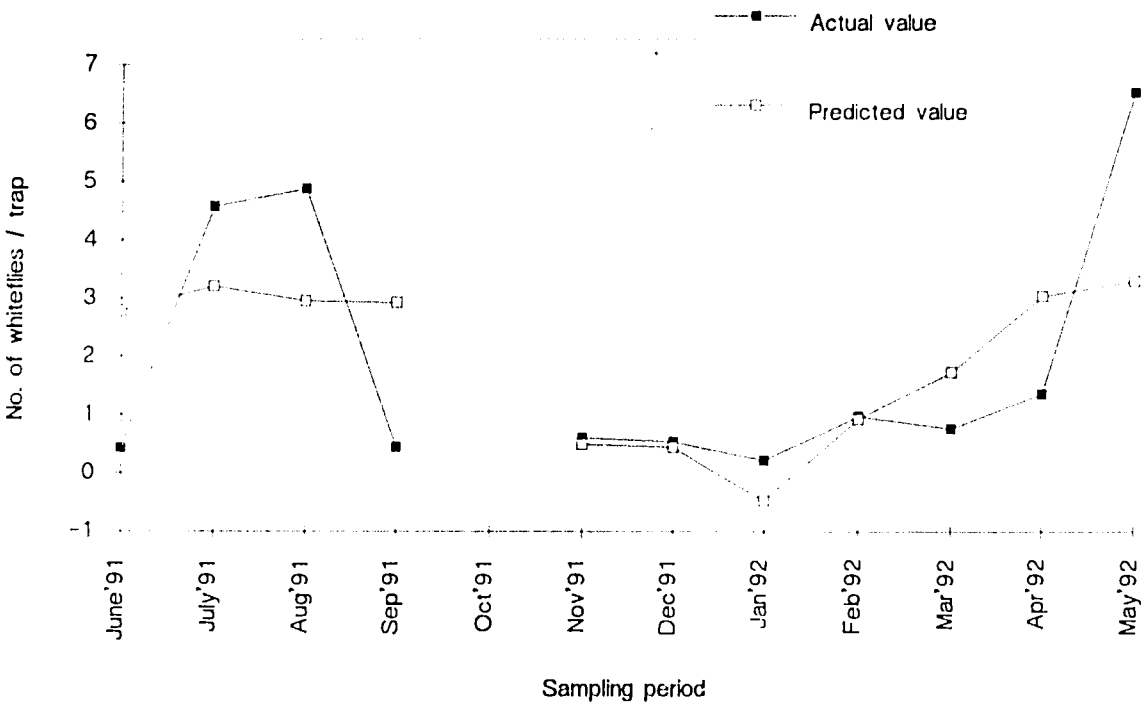


Figure 20 Number of whiteflies per trap during June 1991 to May 1992

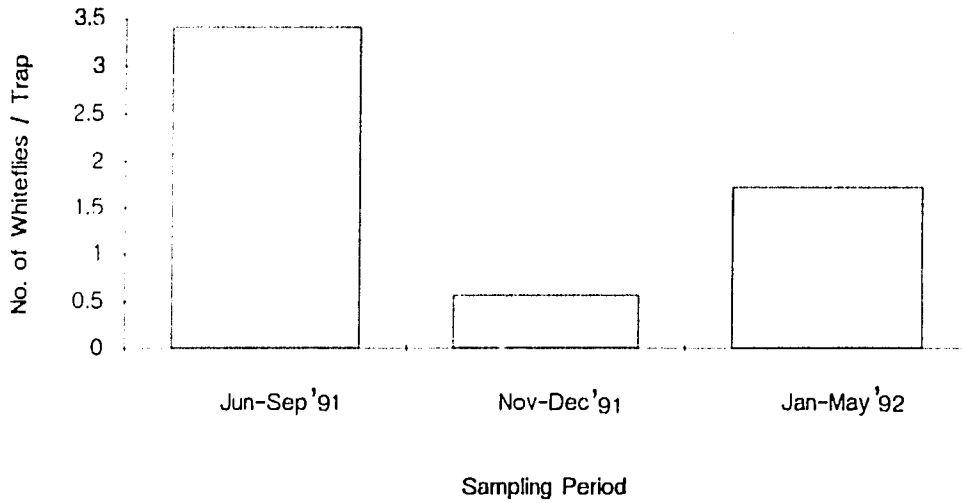


Figure 21 Average number of whiteflies per trap

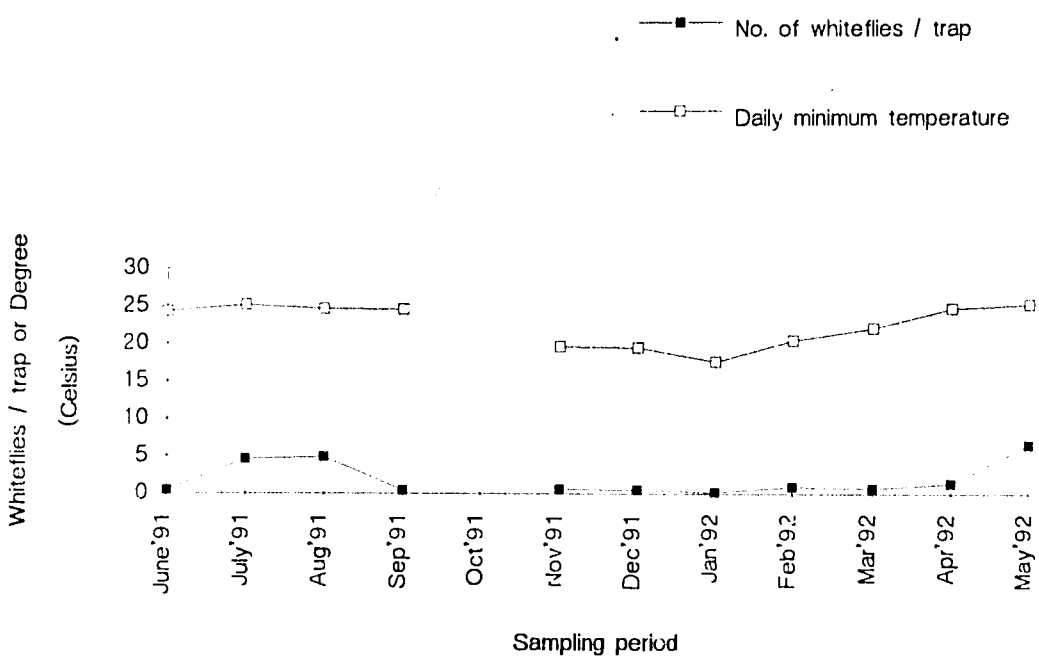


Figure 22 Number of whiteflies per trap in relation to daily minimum temperature during June.

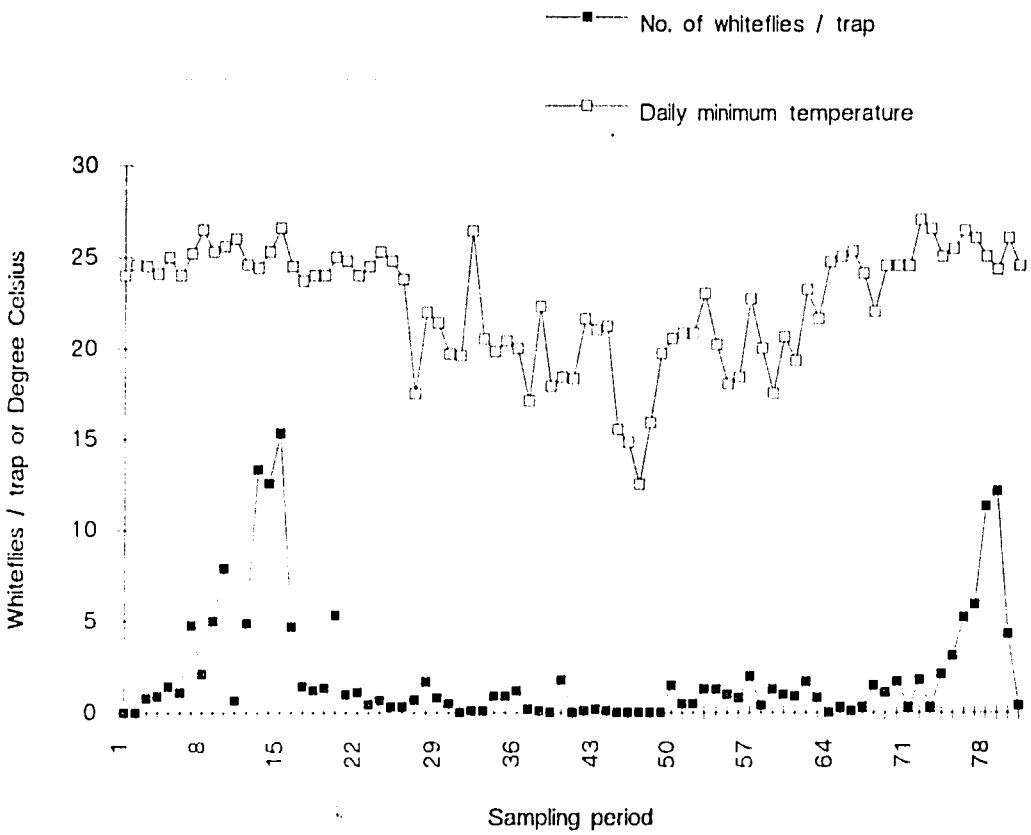


Figure 23 Average number of whiteflies per trap and daily minimum temperature.

3.3 Insect (membrane-feeding) Transmission : A glasstube, 2.5 x 10 cm in size, was designed as an insect container for membrane feeding test. One end of the tube was covered with a fine nylon screen. Ten, twenty and thirty whiteflies were placed in each glasstube and sealed the other end with a thin parafilm. A drop of 200 µl component A TYLCV was spot on the surface and covered with another layers of parafilm to spread the sample between the double layers of parafilm sheet. These insect containing glasstube were kept in the box illuminated only on top to stimulate insect feeding (Fig. 24). After 5 hrs feeding time, the whiteflies were squash blotted onto the nitrocellulose membranes to assay for the presence of component A by using non-radioactive probe. For the acquisition of virus particles through membrane feeding, a drop of crude sap from infected plant was used. After membrane feeding, a glasstube with the whiteflies was placed underneath tomato seedling and covered with plastic beaker. A fine screen was used to replace the bottom part of the beaker to allow aeration and plant growth. The whiteflies in each glasstube were released by removing the parafilm sheets from the glasstube. After one week, a glasstube and whiteflies were removed and leaf curl symptoms on seedlings were observed weekly (Fig. 25).

Result : We have detected component A of TYLCV and TYLCV from crude sap from the whiteflies after membrane feeding (Fig. 26). It indicated that acquisition of the virus or viral components by the whitefly vector is possible. However, we have not applied much of this technique as a mean for virus inoculation because mild strains being used are selected from natural infection, not from *in vitro* mutagenesis. Direct feeding on infected tomato plants is the most efficient mean of acquisition and transmission of the still considered by the whitefly vector.

4. Production of Pre-immunized Seedlings

The project was aimed at searching for the possible methods to cost-effectively produce pre-immunized tomato seedlings. There were two approaches to be investigated, first, to produce pre-immunized seedlings *in vitro* and second, to pre-immunize the seedlings by insect vector. Experiments were designed to study the potential and comparative advantages of these two systems to suggest which one was practical and economical for the production of resistant tomato seedlings.

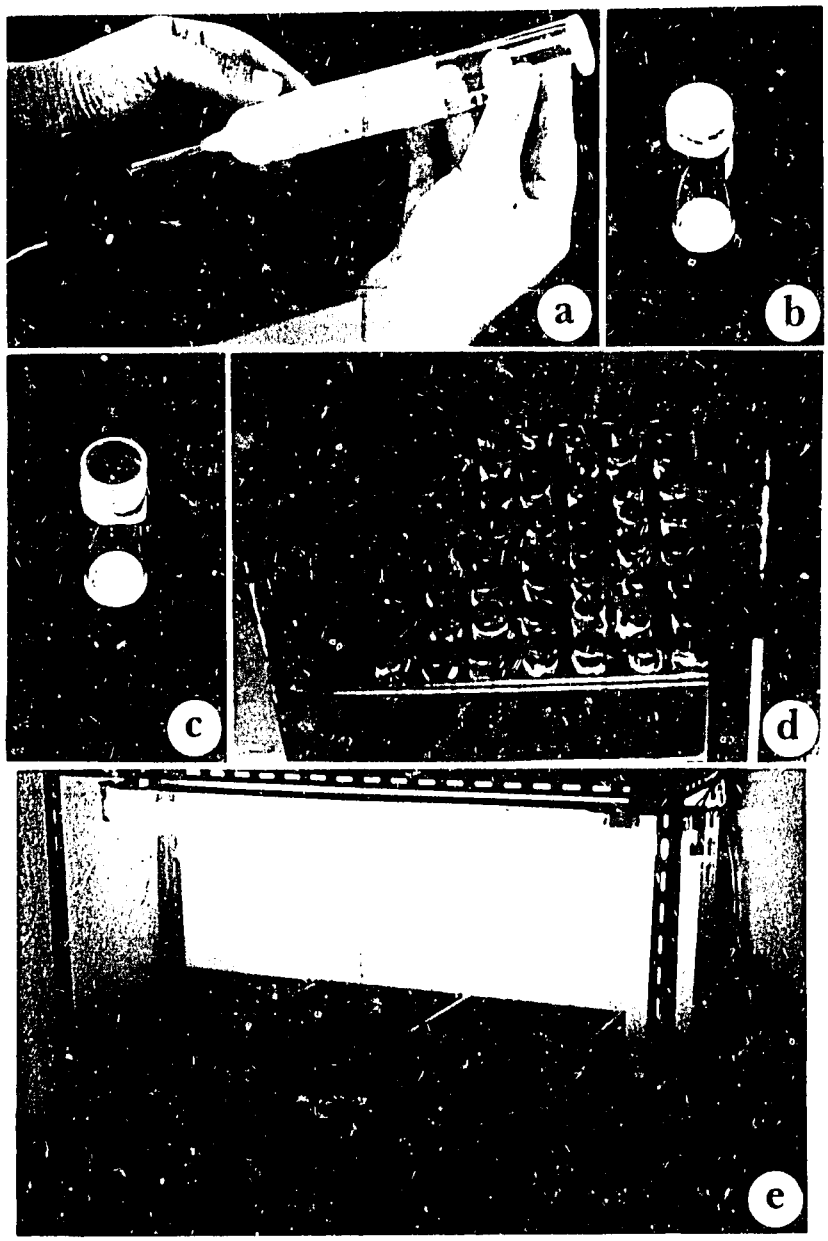


Figure 24 Acquistion of tomato yellow leaf curl virus through the membrane-feeding of the whitefly

a) transferring the whitefly adults from an aspirator into a glasstube
b) the whiteflies in parafilm sealed glasstube
c) a top view of the glasstube showing a layer of sap sample covered by prrafilm sheets
d) a set of glasstubes in the box
e) box illumination to stimulate insect feeding.

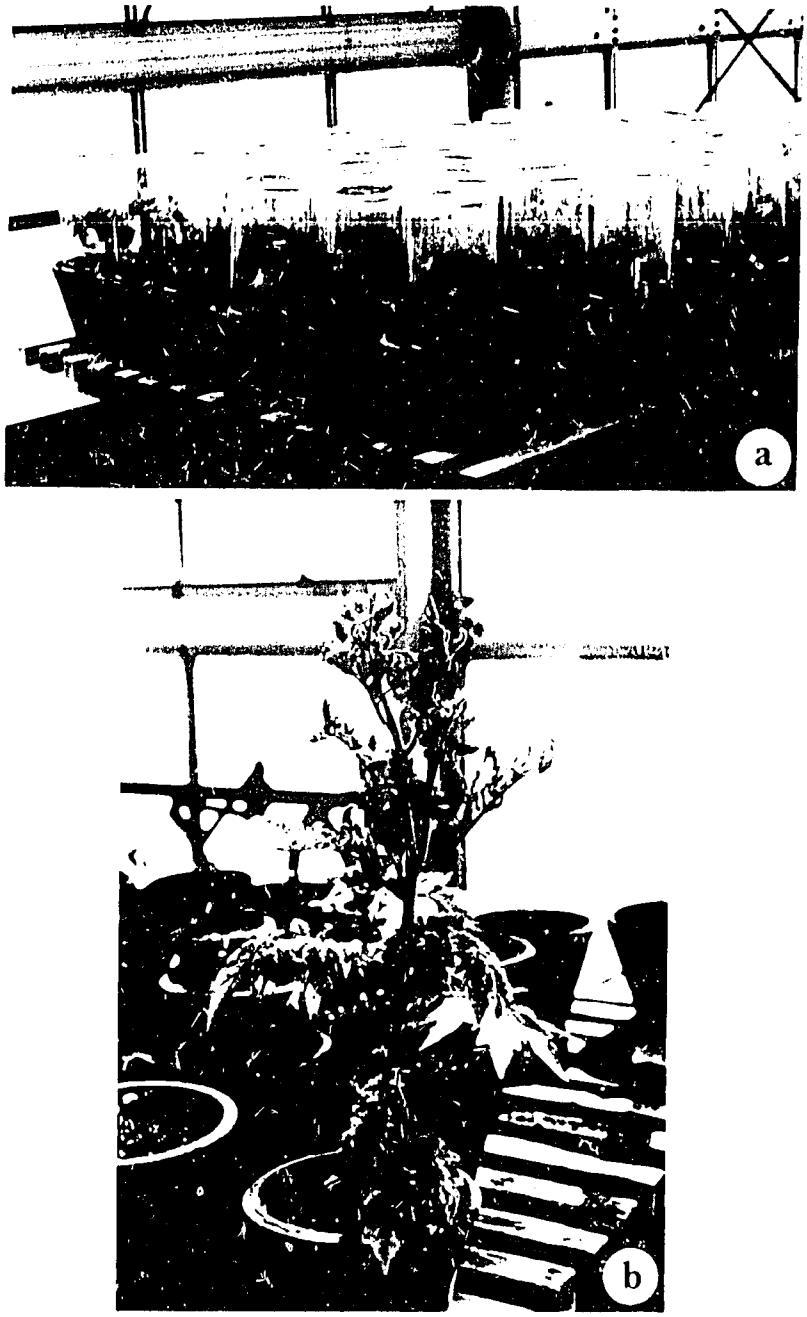


Figure 25 Transmission of tomato yellow leaf curl virus by the whitefly which acquired the virus through membrane feeding.

a) release of viruliferous whiteflies to tomato seedlings after membrane feeding

b) a tomato plant showing tomato yellow leaf curl symptom after whitefly transmission

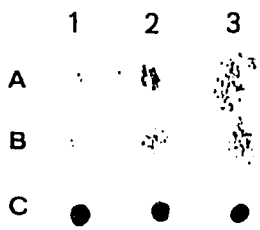


Figure 26 Acquisition of component A of TYLCV by the whitefly through membrane-feeding
Row A : component A + healthy sap, Row B :component A, Row C : component A (control without feeding) 1, 2 and 3 indicated number of 1, 10 and 20 whiteflies/dot, respectively.

4.1 *in vitro* pre-immunization : One-month old seedlings graft inoculated with TYLCV were used as virus source. After two weeks, stem pieces with lateral buds were excised, rinsed with water, and surface sterilized with 20% chlorox solution for 10 min . They were rinsed with three changes of sterile distilled water and cultured on MS medium (Murashige and Skoog, 1962). Cultures were kept illuminated at 25 C with 2,000 lux, 16 hr/day. Tissues from healthy seedlings were also subjected to the same treatment as control.

Germinating buds were used as samples for sub-culturing onto the same medium. Regenerated shoots, 3-4 cm in height were excised and transferred to rooting medium consisting of MS medium and 2% sucrose. Rooted plants were transferred to 1:1 vermiculite and burn husk mix and maintained in a growth chamber at 22-26°C for 1 week prior to field planting.

To detect TYLCV in cultured tissues and regenerated plants, healthy and inoculated tissues were homogenized in denaturing solution (1 µl/mg of tissue) containing 0.125 N NaOH and 0.125 x SSC (20 x SSC : 3 M sodium chloride, 0.03 M sodium citrate, pH 7.0). The extracts were centrifuged at low speed (5,000 g) for 5 min. The supernatant of extracted tissues were serially diluted in the same buffer. One µl of sample was dotted on nitrocellulose membrane which has been pretreated by presoaking in distilled water, soaking in 20 x SSC for 30min and air drying. After dot-blotting, the nitrocellulose membrane was baked at 80 C under vacuum for 2 hrs. TYLCV in samples was detected with DNA probe by using a DIG labeling kit (Boeringer Mannheim) as previously described.

Results : Lateral buds grew well on MS medium without modification of plant hormones. Quantitative analysis of TYLCV-DNA in lateral buds derived from cultured stems at 5 days intervals indicated that the viral DNA varied in the range of 0.35-3.20 µg/mg tissue, depending on period of infection and tomato genotypes . Most of genotypes had higher viral DNA content during the first 10 days an then tailed 30 days after inoculation (Table 3). The persistance of TYLCV in cultured tissues was demonstrated at least, up to one year. It is concluded that TYLCV, a phloem limitted virus could pass through the somatic embryogenesis and persist after sub-culturing. Seedlings derived from TYLCV-infected tissues should contain the same virus or the one to be used for pre-immunization.

Table 3 Quantitative analysis of TYLCV-DNA from different cultivars of tomato in tissue culture at 5 days interval after inoculation.

Cultivars	Amount of viral DNA (ng/mg tissue)					
	Day after inoculation					
	5	10	15	20	25	30
Seeda	1.89	2.16	2.12	1.57	1.18	1.15
VF 134-1-2	2.30	3.20	2.93	2.40	2.24	1.09
Seedathip I	3.01	1.22	1.36	1.18	1.81	0.35
Seedathip II	1.30	1.47	1.06	1.30	1.31	1.33
TN #3	2.21	1.41	1.47	1.54	1.18	1.66
B-200	1.12	0.96	0.67	1.15	1.26	2.02

4.2 **Pre-immunization by Insect Vector :** Attempts were made to mass inoculate seedlings with TYLCV by using the whitefly vector. Viruliferous whiteflies feeding on TYLCV infected Seeda tomato were transferred to feed on 2 week-old tomato seedlings grown in 1 ft^2 plastic container. Inoculation feeding times were 1, 7 and 15 hrs after which all the whiteflies were eradicated. Infected seedlings were determined by dot-blot hybridization using DIG labeling probe as previously described.

Result : The viruliferous whitefly could readily transmit the virus after 1 hr inoculation feeding time. Transmission percentages increased with an increase of inoculation feeding time (Table 4).

For practical use, we have studied the effects of number of plant on transmission by the whiteflies. In this case, number of seedling per plastic container was increasedup to 100 seedlings/container. We obtained transmission percentages ranging from 65-80% (Data not shown). It is therefore concluded that pre-immunization of tomato seedlings by the whitefly vector is more practical and better cost effective than *in vitro* pre-immunization system.

Table 4 **Transmission percentages of tomato yellow leaf curl virus (TYLCV) by the whitefly, *Bemisia tabaci* at different inoculation feeding times. Fifty insects were used for each treatment.**

Inoculation feeding time (hr)	Number of seedling Infected/total	% Transmission
1	21/50	42
7	33/50	66
15	34/50	74

5. Field Trials

5.1 Effects of mild and severe strains of TYLCV on symptom development

Seven out of 9 mild strains of TYLCV, designated as, M1-M7 were tested for symptom expression on field grown tomatoes. Each strain was graft-inocutated to 15-35 tomato seedlings and kept in greenhouse for 3 weeks (Fig. 27). They were then transplanted in the field and symptom expression was recorded 20 and 40 days after planting. Seedlings graft-incoulated with severe strain and healthy seedlings were also planted as control.

Result : Field testings for symptom expression of selected mild strains were performed during May-July, 1987 and February-April, 1988 (Table 5). Seedlings infected with mild strains showed mild symptoms 20-30 days after field planting while those with severe strain exibited only severe symptom. Healthy seedlings also showed high degrees of severe symptom expression due to field infection. However, symptoms on mild strain infected plants developed severe symptoms 40 days after field planting. There were few plants infected by mild strains still expressed mild symptoms, suggesting the possibility of symptom suppression. Strains M4, M5 and M7 were therefor selected as potential mild strains for further uses.

Figure 27 Seedlings of Seeda tomato graft-inoculated with mild strain (Left), severe strain (Middle) of TYLCV and healthy (Right) before field planting.

Table 5 Effects of mild strains of TYLCV and disease development in field grown tomatoes.

Strains	Inoculated plants	Symptom (20 days)		Symptom (40 days)	
		severe	mild	severe	mild
A. May - July 1987					
M1	15	13	2	15	0
M2	15	13	2	15	0
M3	15	13	1 (1)*	14	0
M4	15	6	5 (4)*	8	3
Severe	15	15	0	15	0
Healthy	35	29	2 (4)*	29	2
Total	**110**	**89**	**12**	**96**	**5**
B. February - April 1988					
M5	18	9	5 (4)*	12	2
M6	25	25	0	25	0
M7	28	23	4 (1)*	26	1
Severe	18	16	0 (2)*	16	0
Healthy	30	15	15	27	3
Total	**119**	**88**	**24**	**106**	**6**

(*) = number of dead plant

5.2 Field Performance of mild strain-inoculated tomato : One month old seedlings of Seeda tomato were graft-inoculated with infected tissues of mild strain (M7). Tissues from healthy and severe strain-infected plants were also grafted into young seedlings to severe as control. Inoculated plants were kept in the greenhouse for 3 weeks and then planted in the field. Plants were frequently checked for TYLCV, especially in those showing no external symptoms. Severity of symptom, plant height and fruit setting were recorded for 2 months after planting.

Result : Although this field trial did not illustrate the effects of severe and mild strains on the yields of tomato, partly due to the unfavorable condition (March-April), the experiment clearly showed that severe strain had significant impact on plant growth and fruit setting (Table 6). It has been shown that if young seedlings were infected before flowering stage, infected seedlings were no longer productive. Plants infected with mild strain exhibited field performance comparable to healthy plants. Severe symptoms were observed among mild strain infected plants. It is not clear whether it was due to the lack of cross protection or the over expressions of severe strain in mixed populations of mild strain being used in this study.

Table 6 Field performance of Seeda tomato plants infected by mild and severe strains of TYLCV

Strain	No. of Plant	Disease Rating*	Average Growth (cm)	Fruit setting %
Severe	21	3	26.75	0
Mild	93	1-3	41.38	73.85
Healthy	50	0-3	49.95	80.95

*Disease rating
0 = no symptom
1 = mild yellowing
2 = curling of new leaves and shoot
3 = leaf curling, severe stunting, plant died

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

This project was carried out by a group of Thai scientists specializing in the areas of plant virology and insect virology. They have been trained in US. and Japan for their advance degrees. In Thailand, they have committed themselves to research and development in Plant Protection Virology, and have teaching responsibilities at Kasetsart University, the country leading university in agriculture. The support provided by AID/SCI PSTC research project has tremendous impact on research individuals to perform their duties, research laboratories to strengthen their facilities and Kasetsart University to her graduate program. It should be mentioned that Virus and Mycoplasma Lab., Plant Pathology Department, Insect Virology Lab., Entomology Department, College of Agriculture, and Plant Genetic Engineering Unit, the Central laboratory and Greenhouse Complex of Kasetsart University were significantly improved to be able to conduct research in molecular plant pathology and molecular biology. Research collaborations with leading researchers and laboratories in developed countries like Dr. Roger N. Beachy, The Scripps, USA; Dr. Peter Waterhouse, CSIRO, Australia; Dr. James Dale, Queensland University of Technology, Australia; Dr. M. Ikegami, Tokyo University of Agriculture, Japan; Dr. Douglas P. Maxwell, University of Wisconsin, USA and Dr. H. J. J. Nijkamp, Free University, The Netherlands are examples of scientific capability improvement of the group.

Research results have elucidated several aspects of plant-virus-vector relationships of tomato yellow leaf curl disease. Among these findings were the detection of TYLCV by the modern technology. We have identified mild strains of TYLCV and confirmed their presence by this technique. It is very useful for breeders working in the field to select the potential tomato lines which are resistant to virus infection but not due to disease escape. We have demonstrated the sole vector of this virus, the whitefly, *B. tabaci* and its population dynamics. We also believe that in order to have the successful disease control program, insect population will have to be carefully monitored.

PROJECT ACTIVITIES/OUTPUTS

1. Publication :

THONGRIT, D., S. ATTATHOM AND T. SUTABUTRA. 1986. Tomato yellow leaf curl virus in Thailand. FFTC Book Series No. 33. Taiwan. Republic of China.

ATTATHOM. S., P. CHIEMSOMBAT, T. SUTABUTRA AND R PONGPANITANOND. 1990. Characterization of Nucleic Acid of Tomato Yellow Leaf Curl Virus. Kasetsart J. (Nat. Sci.) 24 (7) : 1-5.

KALAYAWUDHIPONG, P., S. ATTATHOM AND R. VISESSUWAN. 1990. Regeneration of Seeda Tomato Plants from Isolated Protoplasts. Kasetsart J. (Nat. Sci.) 24 (5) : 6-11.

CHIEMSOMBAT, P., W. KOSITRATANA, S. ATTATHOM, T. SUTABUTRA AND N. SAE-AUNG. 1990. DNA Probe and Nucleic Acid Hybridization for Plant Virus Detection. Kasetsart J. (Nat. Sci.) 24 (5) : 12-16.

SUTABUTRA, T., S. ATTATHOM, K. KOSITRATANA, T. ATTATHOM AND P. CHIEMSOMBAT. 1991. Detection of tomato yellow leaf curl virus in whitefly with a non-radioactive probe. Workshop on AID/SCI Funded Research in Agricultural Biotechnology. Kasetsart University, Bangkok. p 110-113.

2. Training

2.1 Non-radioisotope Probe for Plant Virus Detection. 1991.
(Co-organizer : Dr. I. Uyeda, Hokkaido University, Japan)
A two-week training course for Thai Virologists and Plant Virologists to apply molecular biology technique to detect plant viruses including TYLCV.

2.2 Plant Virus Gene Amplification by PCR. 1991.
(Co-organizer : Dr. M. Ikegami, Tokyo Nodai, Japan)
A week training course designed for Thai researchers how to utilize PCR technology as a tool for plant virus diagnosis. TYLCV-CP gene was used as a model.

2.3 Master Class in Plant Virology. 1992.
(Co-organizers : Australia Center for International Agricultural Research and Queensland University of Technology)
A two-week course designed for 20 plant virologists in Thailand and neighbouring countries to introduce the basic concepts in molecular plant virology. TYLCV was one of the subjects for discussion.

PROJECT PRODUCTIVITY.

We have accomplished most of the project objectives. However, field performance of the selected mild strains to protect against severe strain was not up to the level of our anticipation. It is not quite clear whether it was due to the "mixed" population of mild strain or the instability of mild strain in natural condition.

FUTURE WORK

Research results of this project have marked the way to basic and applied research in plant virology that could contribute to the better and more efficient disease control program. Here are some examples of future work:

1. Molecular biology of TYLCV.

It is obvious that this small ss-DNA virus has potential risk to crop production in tropical region. We have to learn more on genetics and biology of this virus, especially, the viral genomes and their functions. Molecular biology of this virus would help us understand the mechanism of cross-protection and design better control strategy.

2. The biology of the whitefly, *B. tabaci*

It has been clearly demonstrated that if one could prevent whitefly transmission of TYLCV, there will be no infected tomato plant in the field. We have tried very hard to control the virus and manipulate the plant to be more resistant to infection. Little has been done to control the whitefly at large scale. Chemical spray is proven not to be an effective way to control virus transmission. Biological control of whitefly population through natural parasites, predators and pathogens should be fully investigated. Tropical environment provides a good opportunity for biocontrol agents to control the target pest.

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