Purification, Antiserum Production, Biological and Molecular Studies of Tomato Yellow Leaf Curl Virus

By

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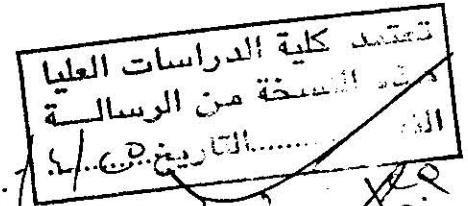

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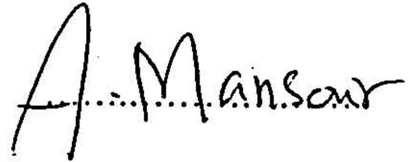

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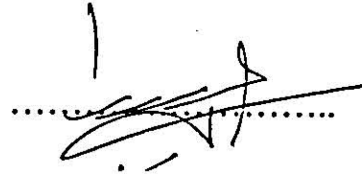

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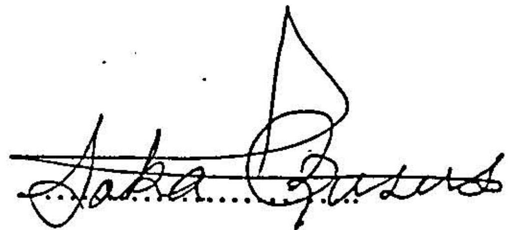

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Dedication:

To my deceased father whose sun lightens the way of every student, even it has gone away.

To my great mother, whose composure and continuos support brighten my way.

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Abstract

Purification, Antiserum Production, Biological and Molecular Studies of Tomato Yellow Leaf Curl Virus

By

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A virus isolate was isolated from tomato (*Lycopersicon esculentum* Mill.) shoots showing virus-like symptoms in the Jordan Valley. The isolate was identified as tomato yellow leaf curl virus (TYLCV) based on diagnostic assay plants, transmissibility, serology and polymerase chain reaction (PCR). The virus was purified from the tip leaves of infected tomato 30-40 days after inoculation. The modified method of purification which depends on chloroform clarification (10%v/v) and PEG (8%(w/v)) and 0.2 M NaCl precipitation gave a good virus yield for antibody production. No visible light scattering band appeared due to the virus particles after centrifugation on

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sucrose density gradients. Triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) using the polyclonal antibody produced against the local virus isolate was applicable for TYLCV detection from infected plant tissue. The test was able to detect the virus in the crude sap extracted from tip leaves of infected tomato plant to the dilution up to 1/64. Tissue blot immunosorbent assay (TBIA) detected the virus in both stem and petiole segments of infected plant tissue up to the dilution 1/2000 of monoclonal TYLCV-specific antibodies. Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and antigen-coated indirect enzyme-linked immunosorbent assay (I-ELISA) were unable to detect TYLCV from infected tissue. The genomic DNA molecules of the Jordanian TYLCV isolate was amplified from total extract of TYLCV-infected plants by the use of PCR. Synthetic oligonucleotides complementary to certain regions of the virus genome were used in a combination to amplify the full-length and the subgenomic fragments of the TYLCV DNA molecule. The test was not only able to amplify the virus genome extracted from different organs of infected tomato plants, but also was sensitive enough to amplify the virus DNA from the infective crude sap extract up to the dilution of 1/1000000. In addition, PCR was able to amplify TYLCV DNA from nucleic acid extracts of single whitefly (*Bemisia tabaci* Genn.) fed on TYLCV-infected tomato. Studying the virus fate in the whitefly vector showed that the virus DNA retained for at least 22 days that is far forth the retention of the virus antigen which persisted only for nine to ten days after 48 hr acquisition access. PCR and serological techniques succeeded to detect the virus from plants infected naturally in the field scale. PCR detected the virus in weeds of *Solanum nigrum* L., *Malva nicaensis* All. and *Datura stramonium* L. with percentages of 2, 13

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Tomato (*Lycopersicon esculentum* Mill.) is the most popular vegetable crop grown in Jordan and elsewhere depending on the availability of water irrigation. Approximately, 82000 dunums of the total arable area of Jordan is annually planted to tomato (Anonymous, 1997).

Tomato production in Jordan has, however, not yet reached its full potential due to many factors connected with climate and poor management, and above all, the high levels of pests and diseases. In regards to diseases, tomato yellow leaf curl virus (TYLCV) may be considered as the most important single factor responsible for the recurrent crop failure in the country. In the central Jordan Valley, tomato crop during the fall-growing season is affected with this virus to an extent that production has become less profitable obliging many farmers to shift their cultivation from tomato to other crops (Abu-Gharbieh *et.al.* 1978: Al-Musa, 1986). The disease is highly epidemic with incidence levels reaching 100% in fall-grown tomato. Laboratory experiment showed that TYLCV resulted in about 63% reduction in fruit yield (Al-Musa, 1982).

TYLCV is a major disease of tomato in many tropical and subtropical regions (Czosnek *et. al.* 1990; Verma *et. al.* 1975; Lana and Wilson 1976). Quantitative and qualitative yield losses in tomato crop often reach 100%

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pathogen has remained supreme in the area, defying all efforts of control and creating a sorry situation whereby no tomato plant could stay, for long, free of the disease (Makkouk, 1978: Al-Musa, 1982; Al-Musa *et. al.* 1982; Sharaf *et. al.* 1984; Al-Musa, 1986; Al-Musa *et. al.* 1987; Suwwan *et. al.* 1988; Kasrawi, 1989). Under this dramatic situation, tomato growers still take the risk and continue to grow the crop.

Epidemiologically, to predict and monitor any plant pathogen, adequate procedures for rapid and specific detection are needed. Similarly, because of the widespread occurrence of TYLCV epidemics and the potential threat to tomato production in Jordan, rapid and specific procedures for virus detection are needed in both the vector and the plant to aid in epidemiological and disease management studies. Traditionally, the diagnosis of TYLCV-infected plants has relied solely on the observation of the disease symptoms that may vary depending on the genetic background of the plant together with its physiological condition as well as on the environment. Moreover, a lot of cultivated and wild flora reacts with the virus in a complete symptomless manner, which act as a cryptic virus source that hoodwink the epidemiologists (Pico *et. al.* 1996).

Therefore, the intended project has the following aims:

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1-Purification and production of specific antibodies against TYLCV.

2-Evaluating the detection ability of different serological techniques.

3-Evaluating the potential of the PCR for amplification of TYLCV ssDNA genome from total DNA extracts of artificially infected plants and experimentally derived viruliferous whiteflies.

4-Devises methods by which the virus can simply and speedily be detected in both the plant and the vector.

5-Testing the applicability of such techniques for the virus detection from infected plants in the field.

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2.1 Tomato crop and origin

Tomato is one of the widely and commercially important vegetable crop throughout the wold for the fresh market and food industries. The crop is bred in a wide range of field climates, under plastic houses and in heated glasshouses (Atherton and Rudich, 1986).

The genus *Lycopersicon* is believed to originate in western South America. The genus included two subgenera, *Eulycoprsicon* and *Eriopersicon* of which the former includes two species; *Lycopersicon pimpinellifolium* (Jusl.) Mill and *Lycopersicon esculentum* Mill. *L. esculentum* contains larger-fruited types that are cultivated widely us annuals or perennials (Papudopoulos, 1991)

2.2 Tomato diseases

Tomato diseases are of two main types- parasitic, and nonparasitic. Parasitic diseases included those caused by living creatures, mainly fungi, bacteria, nematode viruses, while the nonparasitic ones are those caused by unfavorable environmental condition (Gould, 1974).

The crop is liable to infection with many viral diseases that are sometime highly infectious. The diseases include tomato mosaic cucumber

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mosaic, double virus streak, spotted with curly top, TYLCV and others (Atherton and Rudich, 1986).

2.3. TYLCV disease on tomato

2.3.1. Historical background and spread of the disease

TYLCV was first reported in Occupied Palestine since 1960s (Cohen and Nitzany, 1966). In Jordan, the virus was identified as a disease of tomato in 1978 (Makkouk, 1978). Recently, the virus has become an economically important disease worldwide (Pico *et. al.* 1996) (Table1).

2.3.2. Symptoms

Tomato plants affected by TYLCV are severely stunted. The shoots are erect and the leaflets are much reduced and abnormal in shape (Nitzany, 1975). First TYLCV symptoms on tomato plants appear 2-4 weeks after inoculation and become fully developed after a period of up to 2 months (Ioannou, 1985). The type and severity of symptoms vary according to the virus isolate, the host genetic background, the environmental conditions, and the growth stage and physiological conditions of the tomato plants at the time of infection.

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Table 1. Distribution of TYLCV along continents (Pico *et. al.* 1996)

Country	**Reference**
Africa	
Egypt	Czosnek *et. al.* (1990)
Mali	Dembele and Noussourou (1991), Czosnek *et. al.* (1990)
Nigeria	Czosnek *et. al.* (1990)
Senegal	Aminata (1991), Czosnek *et. al.* (1990)
America	
Dominican Republic	Nakhla and Maxwell (1994)
Jamaica	McGlashan *et. al.* (1994)
Mexico	Brown *et. al.* (1986)
Asia	
India	Verma *et. al.* (1975)
Iraq	Lana and Wilson (1976), Wilson *et. al.* (1981)
Jordan	Makkouk (1978), Verma *et. al.* (1975)
Lebanon	Verma *et. al.* (1975), Czosnek *et. al* (1990)
Occupied Palestine	Cohen and Nitzany 1966, Czosnek *et. al* (1990)
Taiwan	Czosnek *et. al.* (1990)
Thailand	Czosnek *et. al* (1990), Rochester *et. al.* (1990)
Turkey	Navot *et. al.* (1989), Lana and Wilson (1976)
Europe	
Cyprus	Ioannou (1985), Czosnek *et. al.* (1990)
Sicily (Italy)	Czosnek *et. al.* (1990)
Spain	Moriones *et. al.* (1993)

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Young early-infected plants are usually unfruitful due to severe flower shedding, whilst plants infected later generally produce fewer and smaller fruits than the virus-free ones (Ioannou, 1985). Leaflets that appear soon after infection are cupped downward and inwards in a hook-like shape. leaves developing later are misshapen and smaller, showing interveinal and marginal chlorosis and upward curling of the leaflet margins. Purpling can also appear on the lower surface of leaflets (Nitzany, 1975).

2.3.3. Incidence and losses in tomato crop

Tomato growing is under a constant threat of many diseases caused by whitefly-transmitted geminiviruses. TYLCV is the cause of the major disease of tomato in tomato growing sites (Al-Musa and Mansour, 1982). Weakening of the whole tomato plant and flower and fruit abscission caused by TYLCV infection result in serious yield losses (Table 2). These losses depend on the strain of the virus, the tomato cultivar and the growth stage at which plants become infected and they are particularly severe when the infection occurs before flowering (Al-Musa, 1982, Ioannou, 1985 and Nitzany, 1975). The economic effect of TYLCV on both quantity and quality of the yield makes this pathogen a serious problem in invaded areas, and a threat in regions where the tomato crop is being expanded. In

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many areas the virus has become the major disease limiting tomato production in both outdoor and protected crops (Ioannou, 1985). Disease incidence and severity have seasonal variations significantly correlated with fluctuations in the population of the vector (Ioannou and Iordanou, 1985). Under adequate conditions for the spread of the disease, it reaches epidemic proportions leading to the abandonment of the cultivated fields in many regions (Nakhla *et al.* 1994). In Mediterranean regions, the incidence of this viral disease is especially important in late summer and autumn crops (Al-Musa, 1982; Nitzany, 1975).

Ioannou (1985) reported that the average disease incidence in Cyprus varied from 20 to 100%. Late autumn and late spring plantings suffered varying levels of infection (from nil to near maximum). Similarly, Nitzany (1970) identified TYLCV on tomato in Occupied Palestine and reported that the virus is encountered in all regions and is very severe during late summer and autumn.

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Table 2. Incidence of TYLCV of two Mediterranean countries and yield loss of tomato caused by virus infection (Pico *et. al.* 1996).

Country	Growing area	Growing season	Incidenc e	Yield losses	Reference
Jordan	Jordan Valley	Autumn Spring	93-100% 0-13.2%	Under greenhouse conditions, yield losses were 63% when tomato plants inoculated 10 days after sowing	Al-Musa (1982)
Cyprus	Southern coastal zone	Summer and early autumn plantings. Winter and early spring plantings	20-100% Escaped infection	Epidemic proportions. Most important limiting factor in tomato production 50-82%	Ioannou (1985, 1987).

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2.3.4. Transmission

TYLCV is transmitted persistently in nature by the adults of *B. tabaci* Genn., its only known vector (Markham *et. al.* 1994). The virus is not transmitted congenitally to the progeny and it is not seed-borne (Pico *et. al.* 1996). The virus is retained when the vector moults but does not multiply in the vector (Nitzany, 1975). It can be transmitted experimentally by mechanical inoculation, but it is unlikely to be significant in TYLCV spread in tomato fields. The disease can be readily transmitted by grafting, but to date, no case of soil transmission has been reported (Cohen and Nitzany, 1966; Makkouk *et. al.* 1978).

2.3.5. Virus-vector relationships

The interaction between TYLCV and the vector is described as circulative and non-propagative. This means that the virus is acquired while feeding and passes through specific cell within the gut to enter the haemolymph before passing out of the insect during feeding via salivary glands (Markham *et. al.* 1994)

Cohen and Harpaz (1964) described the existing virus-vector interaction as a ``periodic acquisition`` since the insect is not able to reacquire the virus until the end of the previous infective period. The larva

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can acquire the virus and transmit it to the adult stage and they are as effective as the adults in acquiring the virus. Females transmission efficiency has been found to be higher (six-fold) than that of males (Cohen and Nitzany, 1966).

2.3.6. Host range

TYLCV has a narrow host range, since, it develops only in a few species of several families (Harrison, 1985).Mansour and Al-Musa (1982) reported that the host range of the Jordanian isolate is restricted largely to solanaceae.

In general, plants from different botanical families have been found to be host of TYLCV: Compositae, Leguminosae, Malvaceae, Solanaceae, and Umbelliferae (Ioannou *et. al.* 1987; Cohen and Nitzany, 1966). Some of them develop clear disease symptoms, whereas others are susceptible symptomless carriers of the virus (Cohen and Nitzany, 1966; Nitzany, 1975; Ioannou, 1985; Jorda, 1993; Mansour and Al-Musa, 1982).

In order to understand the epidemiological cycle of TYLCV in each infected area, specific studies of the flora of the infected regions have been attained, analyzing the role that different crops and weeds play as natural

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hosts of TYLCV and its vector. In many areas, TYLCV complete its entire cycle on overlapping tomato crops (Pico *et. al.* 1996) (Table 3).

Some studies spread TYLCV host range to other important crops, such as the potato, pepper, and eggplant, but the use of the results of these studies of serological diagnosic methods might imply the possibility of cross-reactions with other geminiviruses (Pico *et. al.* 1996; Macintosh *et. al.* 1992).

2.3.7. Diagnosis

Since management of TYLCV epidemics relies on rapid identification of the disease, it is imperative to be able to diagnose the disease unequivocally. The diagnosis of TYLCV-infected plants has relied solely on the observation of the disease symptoms. Since symptoms may vary according to the genetic background of the plant, its physiological conditions and its environment, diagnosis is not precise (Czosnek *et.al.* 1990).

Therefore, various techniques are currently used to detect TYLCV in infected plants or vectors.

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Table 3. Natural reservoirs of *B. tabaci* Genn. and TYLCV in two Mediterranean countries (Pico *et. al.* 1996)

Area	Reservoir	Reference
Occupied Palestine, & Jordan	**Vector Reservoirs:** Crops: *Cucumis sativus* L., *Solanum melongena* L., *Capsicum annum* L. Ornamental shrub: *Lantana camara* **TYLCV Reservoirs:** *L. esculentum* Mill., *N. tabacum* L., and *P. vulgaris* L.. Weeds: *D. stramonium* L., *Malva nicaensis* All.	Al-Musa (1986)
Cyprus	**TYLCV Reservoirs:** Crops: *L. esculentum* Mill., year-round cultivation of tomato, *N. tabacum* L.. Weeds: *D. stramonium* L. and wild *Lycopersicon* spp.	Ioannou *et. al*, (1987)

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2.3.7.1. Serology

Serological techniques can be used for TYLCV detection. A lot of difficulties concerning thee cross-reaction among related whitefly-transmitted geminiviruses due to the high homology degree of their coat protein, are the main disadvantage associated with these techniques (Pico *et. al.* 1996; Macintosh *et. al.* 1992; Muniyappa *et. al.* 1991).

2.3.7.2. PCR technique

The polymerase chain reaction (PCR) is an extremely sensitive and specific technique for the detection and identification of plant viruses, and it can be used to investigate precise question about the genetic diversity of the viruses (Mehta *et. al.* 1994; Rojas *et. al.* 1993).

The sensitivity of PCR is based on the use of oligonucleotide primers that are complementary to regions flanking the DNA sequence to be amplified. Because PCR amplifies nucleic acid, the technique could be useful in overcoming many of the present difficulties associated with serological detection method, e.g. Low titer of antigen and developmental or environmental regulation of the antigen production and the problem of the cross reaction (Rojas *et. al.* 1993; Pico *et. al.* 1996)

Navot *et. al.* (1992) used PCR to amplify the full-length as well as subgenomic fragment of TYLCV DNA molecules extracted from

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infected plant tissue. In addition, the author amplified the DNA molecules from individual *B. tabaci* Genn. fed on TYLCV-infected plant and from whiteflies collected from naturally infected bean plants in the field. Similarly, Mehta *et. al.* (1994) used PCR for specific amplification and detection of TYLCV from viruliferous *B. tabaci* Genn.. The test was reported to be sensitive to detect the virus in individual whitefly in mixed samples of up to 25 (1 viruliferous: 24 nonviruliferous) individuals.

Furthermore, Rojas *et. al.* 1993 and Deng *et. al.* 1994 used PCR with degenerated primers for detection and differentiation of whitefly-transmitted geminiviruses in plants and vector insects.

2.4. Virus biology and structure

TYLCV has characteristic geminate particle morphology (20x30 nm in size) consisting of two incomplete icosahedra. The virus posses a unique 2787 nucleotide, circular and single-stranded DNA genomic component, unlike other whitefly-transmitted geminiviruses with a bipartite genome (Lazarowitz, 1987). The particle structure and the cytopathological changes found in infected tissues clearly show

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that the causal agent of this disease is a geminivirus (Russo *et al.* 1980).

2.5. Purification of TYLCV

Since TYLCV is a geminivirus with low concentration inside the infected plants, the method of purification should give a good yield of viron. Therefore, Czosnek *et. al.* (1988) purified TYLCV from infected tomato and *D. stramonium* L. plants, 1-2 weeks after the symptom development. Ethylene diaminetetraacitic acid (EDTA), sodium sulfite, 2-mercaptoethanol and Triton X-100 were used in extraction buffers. The virus was clarified either by chloroform (10%) or gravitational force of 8,000 *g* for 10 min. Ultra-speed centrifugation was used to precipitate the partially purified virus from the virus suspension. The virus was finally purified on rate zonal sucrose gradient after exposure to gravitational force of 90,000 *g*.

Similarly, Rochester *et. al.* (1990) modified and used the purification method of cassava latent virus (CLV) to purify TYLCV (Sequeira and Harrison, 1982). Accordingly, the virus was purified from infected leaves of tomato plants 3wks after transmission by whiteflies. 0.1 M tris-HCl buffer, pH 8.4, containing 1% thioglycerol was used to extract

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the virus. Chloroform was used in clarification treatment and PEG (4% w/v) and 0.2 M NaCl were used to precipitate the virus. After resuspension, the virus was concentrated by the gravitatinal force of ultra-speed centrifugation. Cesium sulfate was used during isopycnic centrifugation to give a yield of 1-2 mg per kilogram starting material.

2.6. Position of TYLCV in Jordan

2.6.1. Tomato crop; its importance and cultivation habits

Tomatoes are among the major vegetable crops grown in Jordan. About 82000 dunums of the total arable area are planted to tomato (Anonymous, 1997). Tomatoes are planted in the Jordan Valley during two main planting seasons, namely: the fall and spring seasons. For the fall season, transplants must be available in September and October. Thus tomato seedlings must be raised in August and part of September (Al-Musa *et. al.* 1982).

2.6.2. Economic importance and disease incidence

Tomato crop is liable to infection with much disease among which, viruses occupy the maximum menaces. TYLCV is one of the most important factors limiting production of fall grown tomatoes in Jordan (Al-

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Musa and Mansour, 1982). The first record of virus identification was made by Makkouk (1978). Infection with TYLCV caused drastic yields reduction, that many farmers were forced to shift from tomato to cucumber cultivation (Al-Musa, 1986). Under glasshouse conditions, yield losses approach 63% when tomato plants were inoculated with the Jordanian isolate of TYLCV 10 weeks after sowing. The disease incidence at the end of the tomato growing seasons ranged from 0 to 13.2% in spring grown tomatoes and from 93 to 100% in fall-grown tomatoes (Al-Musa, 1982).

Makkouk (1978) identified TYLCV in nine locations throughout the Jordan Valley as the most serious and wide spread virus in tomato fields inflicting severe losses. Subsequently, Abu-Gharbieh (1979) reported that over 100 tomato cultivars used by Jordanian farmers were susceptible to TYLCV. Some cultivars showed less severe symptoms, however, and their fruit setting was better than the susceptible standards.

Al-Musa and Mansour (1982) reported that TYLCV occupies a predominant occurrence of TYLCV in Jordan over the tomato viruses including tomato mosaic virus (TMV), cucumber mosaic virus (CMV) and potato virus Y (PVY).

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3.1 Virus identification

3.1.1. Sample collection

Tomato fields in several locations of Jordan were visited and young shoots and leaves with symptoms suggestive to tomato yellow leaf curl virus (TYLCV) were collected.

3.1.2. Virus inoculation

Scions (about 1-1.5 cm long) obtained from young shoots of field-grown plants with prominent symptoms were grafted onto virus-free young tomato seedlings. The stock and scion were firmly bound with parafilm tapes and the pots containing the graft were covered with bell jars or perforated transparent plastic sacs or placed in the shade for 2-3 days after grafting. In very hot weather, the grafted plants were kept under mist condition (25-30 °C and 100% relative humidity).

Inoculated plants were kept in insect-proof cages under glasshouse conditions (25-35 °C) and observed for appearance of symptoms.

3.1.3.Virus culture

For pure culture, the virus was passed through single adult whitefly (*Bemisia tabaci* Genn.) transmission and maintained in both jimsonweed (*Datura stramonium* L.) and tomato (*Lycopersicon esculentum* Mill.). Inoculation was done either by grafting or by whitefly transmission.

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Jimsonweed and/or tomato plants were inoculated at the two-leaf stage and then sprayed with insecticide (Confidor). All plants were grown in an insect-proof glasshouse and sprayed biweekly with Confidor.

3.1.4. Whitefly culture

Whiteflies were cultured on cucumber (*Cucumis sativus* L.) grown in muslin-covered cages held in an insectary glasshouse at temperatures of 26-32 C^0. *B. tabaci* Genn. was differentiated from the glasshouse whitefly, *Trialeurodes vaporariorum* (Westwood) depending on pupal stage (Gerling 1990; Avidov and Harpaz 1969).

3.1.5. Diagnostic assay hosts

Diagnostic assay plants of TYLCV used for the virus identification were described by Mansour and Al- Musa (1992)

Recovery of the virus by successful back-transmission to tomato indicated that the species concerned was a host of TYLCV. Species from which the virus was not recovered were considered as apparently immune to TYLCV.

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3.1.6. Transmissibility

Transmission experiments of TYLCV with *B. tabaci* Genn. were conducted in laboratory as described by Mansour and Al-Musa (1992). The whiteflies were given 48 hrs acquisition access period on TYLCV-infected tomato and 48 hrs inoculation access period on healthy tomato plants.

In the above-mentioned experiment, the inoculated plants were kept in insect-proof cages and watched daily for symptom development.

3.1.7. Serology

Triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) was employed in the identification task of TYLCV isolates. The monoclonal and polyclonal antibodies specific for TYLCV were purchased from ADGEN Ltd. (Scotland, U.K.). The complete procedure was mentioned in section 3.3. Tissue blot immunosorbent assay (TBIA) was also used in the identification process of TYLCV isolates (Section 3.3).

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3.1.8. Polymerase chain reaction (PCR)

PCR was also employed in the identification stream of TYLCV isolate using one primer corresponds to the viron positive strand and three primers complementary to the viron strands. Details are mentioned in section 3.4.

3.2. Purification of TYLCV

To study the basic properties of any virus, it is essential to be able to obtain purified preparations that still retain infectivity. Similarly, to produce a specific antiserum for TYLCV, it is essential to be able to isolate preparations that are more or less free of host materials and are infectious. The following work describes the procedures that were selected to isolate and purify the Jordanian isolate of TYLCV.

3.2.1. Determining of TYLCV propagative host plants

Plants of jimsonweed and tomato were selected for assaying the virus concentrations in their tissues (Czosnek *et. al*. 1988). All plants were graft-inoculated using TYLCV-infected scions from tomato source. One month later, all plants were tested by TAS-ELISA (Section 3.3.) and the virus content was quantified at 405 nm using ELISA-Reader one hr after the substrate incubation.

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3.2.2. Determination the proper time of harvesting infected tissue

Ten plants of tomato were mechanically grafted with TYLCV-infected scions during the 2nd and 3rd true leaf stages. Back indexing using quantitative TAS-ELISA (Section 3.3.) was performed at different periods including one, two, three, four, five and six wks post inoculation. The assayed tissue was always obtained from the uppermost leaves of the main stem. After well's charging with *p*-nitrophenol phosphate, the absorbency at 405 nm was measured 60 min thereafter.

3.2.3. Determination of virus concentration in different parts of tomato plant

Different parts of tomato evincing typical symptoms of TYLCV were tested for their virus concentration using serological assays. These parts included: tip leaves, petioles of the tip leaves, the middle-age leaves, petioles of middle-age leaves, the older leaves, petioles of the older leaves, upper part of the stem, lower part of the stem, roots and flowers. Each part was macerated and tested by a quantitative TAS-ELISA (Section 3.3.). Parts from healthy plants were processed similarly to represent the negative control.

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3.2.4. Virus source for purification

Based on the results obtained by studying the proper time of harvesting and plant parts with maximum virus concentration, tip leaves of tomato showing typical symptoms of TYLCV were harvested 30-40 days after inoculation, The tissue was used as a virus source for all steps of purification.

3.2.5. Viron purification

3.2.5.1. Comparison of extraction media

Different media were evaluated for their ability to extract TYLCV from tomato source plants. These were:

Medium 1: 0.2 M phosphate buffer containing 0.004 M EDTA and 1% 2- mercaptoethanol, pH 7.8.

Medium 2: 0.1 M phosphate buffer containing 0.3% ascorbic acid, pH 7.2.

Medium 3: 0.01 M phosphate buffer containing 0.1% thioglycolic acid (TGA), pH 3.9.

Medium 4: 0.1 M phosphate buffer, pH 7.2 containing 0.002 M EDTA and 0.01 M Na2SO3. The homogenate was made 1% (v/v) with Triton X-100.

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Medium 5: 0.1 M sodium citrate buffer, pH 6.0 and 5 ml/liter thioglycerol

Medium 6: 100mM trisodium citrate, 18.5 mM ascorbic acid, 60mM sodium sulfate, 5mM EDTA, pH 8.0 and 1% (v/v) 2-mercaptoethanol. Triton X-100 was incorporated to the homogenate by 2.5% (v/v).

Medium 7: 0.1 M sodium phosphate, pH 7.0, 2.5 mM EDTA, 10 mM sodium sulfite, 0.1% (v/v) 2-mercaptoethanol and 1% (v/v) Triton X-100.

Subsequently, tip leaves of infected plants dried by liquid nitrogen were crushed to a fine powder and homogenized in different media (1 gm of fresh weight/3 ml of medium). The expressed juices were emulsified with 10% ice-cold chloroform and subjected to one cycle of different centrifugation (30 min at 8000 *g*; 5 hrs at 120,000 *g*). The final pellets were resuspended by 1ml buffer containing 6.056 gm of tris (hydroxymethyl) aminoethane and 7.56 gm of sodium sulfate in one liter of distilled water. Preparations were assayed for TYLCV using the quantitative TAS-ELISA (Section 3.3.).

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the suspensions were removed by further low speed centrifugation at 8000 *g* for 5 min.

100 *ul* aliquots from the supernatants were incorporated into ELISA plate together with aliquots obtained from healthy tissues processed in the same way, and the test was done typically. The absorbency values were measured 60 min at 405 nm using ELISA-Reader.

3.2.5.3. Precipitation of TYLCV by different agents

Several methods of virus precipitation were tested for their applicability towards TYLCV. Accordingly, the virus was precipitated from the chloroform clarified aqueous sap of tomato by applying the following precipitation methods:

Method 1: Ammonium sulfate 20% (w/v).

Method 2: Ammonium sulfate 40% (w/v).

Method 3: 0.1 M NaCl.

Method 4: 0.8 M NaCl.

Method 5: Polyethylene glycol (PEG) 4% (w/v).

Method 6: PEG 8% (w/v) and 0.2 M NaCl.

Method 7: Ammonium sulfates 30% (w/v) and 0.1 m NaCl..

Method 8: Reducing the pH to 4.5.

Method 9: Ultra-speed centrifugation at 140,000 *g* for 7 hrs.

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The treatments from 1 to 8 were stirred for 60 min and then submitted to high-speed centrifugation at 65,000 *g* for 3 hrs. The pellets were resuspended in buffer containing 6.056 gm of Tris (hydroxymethyl) aminoethane and 7.50 gm of sodium sulfate in one liter of distilled water as a final volume (1ml/pellet). The virus content in the pellets was quantified by TAS-ELISA (Section 3.3.).

3.2.5.4. Purification method

According to results of the propagative host plant of TYLCV, organs with maximum virus content and the proper time of harvesting together with the results obtained from studying the best methods of virus extraction, clarification, precipitation together with the method of Czosnek *et. al.* (1988), a modified purification method was used as follows:

One hundred gram of tip leaves of tomato plants were harvested 30-40 days after graft inoculation with TYLCV and used as a virus source. After crushing in liquid nitrogen and homogenization in the medium containing 0.1 M sodium phosphate, pH 7.0, 2.5 m M EDTA, 10 m M sodium sulfite, 0.1% (v/v) 2-mercaptoethanol and 1% (v/v) Triton X-100 (medium 7; 3 ml/gm tissue), the homogenate was stirred overnight at 4 C^0, and then squeezed 2 times through double layers of cheesecloth. The sap

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was emulsified with ice-cold chloroform (10%) (v/v) for 30 min at 4 °C, then submitted to refrigerated low speed centrifugation at 8000 *g* for 20 min. Solid PEG 8%(w/v) and 0.2 M NaCl were added to the aqueous phase and the mixture was stirred for 2 hrs at 4 °C, then submitted to high speed centrifugation at 65,000 *g* for 3 hrs. The pellets were resuspended in 0.1-M phosphate buffer, pH 7.0 containing 2mM EDTA (resuspension buffer), held overnight at 4 °C and then submitted to two cycles of low speed centrifugation at 8,000 *g*. The supernatants were collected, layered above 20% sucrose cushion (3ml/tube) and then submitted to ultra-speed centrifugation at 120,000 *g* for 7 hrs. The pellets were resuspended in the resuspension buffer and stored at –20°C.

Sucrose density gradient centrifugations were made by layering of 1-2 ml of the partially purified virus preparation on the top of the density gradients (100 g tissue/gradient). The density gradients were 15-ml, (10-35%) and 32-ml, (10-50%). The gradients were fractionated (1.5 ml/fraction for the 15-ml gradient and 2ml/fraction for the 32 ml gradient) after being subjected to ultra-speed centrifugation at 90,000 *g*. The times of centrifugation were 4 and 15 hrs for the 15-ml and the 32-ml gradients, respectively.

Spectrophotometry at (260 and 280 nm), PCR (Section 3.4) and TAS-ELISA (Section 3.3.) were used to check the virus presence in each

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fraction. Also, a quantitative TAS-ELISA was employed to assess the virus content in the virus fraction.

In all experiments mock preparation of saps obtained from TYLCV-free tomato plants were processed and assayed similarly.

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3.3. Antiserum production and serological studies

3.3.1. Antiserum production

Antiserum was produced by immunization of rabbit intramuscularly with 1 ml of emulsified antigen (purified preparation of TYLCV obtained from both purification methods) in Incomplete Freund's Adjuvant. Subsequent three injections were done at weekly intervals using 0.5 ml antigen. The animal was bled from the marginal ear vein after one week of the last injection and repeated three times at weekly intervals. Booster injection with the same dose was done after the third bleeding.

Serum was separated after allowing the blood to clot at room temperature and then centrifuged at 8000 *g* for 15 min. Serum was carefully separated and aliquoted after addition of sodium azide as a percentage to a final concentration of 0.02 g/ml. (Walkey, 1985)

3.3.2. Determination of the antiserum titer

The titer of the antiserum; the highest dilution of the antiserum that react with its own homologous virus, was determined using (TAS-ELISA) (Section 3.3.). Tenfold dilutions of antiserum were tested against twofold dilutions of crude sap using chase board method. The cross point that developed the strongest reaction was determined and the donor opposite

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dilutions of both antiserum and antigen were chosen for the subsequent work. (Hill, 1984)

3.3.3. Antibodies' sources and use

Goat-anti-mouse conjugate together with polyclonal (0.2 ml) and monoclonal (0.2 ml) TYLCV-specific antibodies was purchased from ADGEN Ltd (Scotland, U.K.). The above mentioned purchased materials were used in all serological tests devoted for virus identification and purification works that are discussed in sections 3.1 and 3.2.

The produced polyclonal antibodies against the local TYLCV isolate was used in the rest of the work that included evaluation of serological tests for TYLCV detection (Section 3.3), PCR (immunocapture PCR) (Section 3.4.) and studying TYLCV in the field (Section 3.5).

3.3.4. Serological tests

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) (Clark and Adams, 1976), antigen coated indirect enzyme-linked immunosorbent assay (I-ELISA) (Clark, *et. al.* 1986), triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) (Macintosh *et. al.* 1992; Muniyappa *et. al.* 1991) and tissue blot

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immunosorbent assay (TBIA) (Torrance, 1998) were tested for their practicality in TYLCV detection from infected plant tissue.

3.3.4.1.Triple-antibody sandwich immunosorbent assay (TAS-ELISA)

Upon the recommendation of the manufacturer (Adgen Ltd. Scotland, U.K.) for the triple antibody sandwich immunosorbent assay (TAS-ELISA), the following buffers and gradients were used:

1), Coating buffer (1.59 gm of sodium carbonate and 2.93 gm of sodium hydrogen carbonate in one liter of distilled water as a final volume, pH 9.6)

2) Phosphate buffered Saline (PBSx10) (80 gm sodium chloride, 2 gm potassium dihydrogen orthophosphate, 11.5 gm of disodium hydrogen orthophosphate, 2 gm of potassium chloride in one liter of distilled water as a final volume, pH 7.2)

3) Wash buffer (PBS+Tween 20) (1 liter of phosphate buffered saline and 0.5 ml of Tween 20).

4) Extraction buffer (6.056 gm of tris(hydroxymethyl) aminomethane and 7.56 gm of sodium sulfate in one liter of distilled water as a final volume, pH 8.5)

5) Conjugate buffer (Mab buffer) (0.2 gm of bovine serum albumin and 100ml of PBST).

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6) Substrate Buffer (90.39 gm of diethanolamine, 19.82 gm of diethanolamine-HCl and 0.1 gm magnesium chloride in one liter distilled water as a final volume, pH 9.8).

TAS-ELISA was done essentially as described by Macintosh *et. al.* (1992) and Muniyappa *et. al.* (1991) except that a range of leaf extraction media and buffers were used as recommended by Adgen Ltd. (Scotland, U.K.).

Accordingly, 100 *ul* aliquots of polyclonal TYLCV-specific antibodies diluted at 1/1000 in coating buffer were added to each well of the microtiter ELISA plate then incubated for 4 hrs at 37 °C. After 3 successive washing with PBS-T, 100 *ul* aliquots of the plant sap extracted by grinding 1 gm of tissue in 10 ml of TYLCV-extraction buffer were added prior to overnight incubation at 4 °C. After similar washing and removing of the excess sap, aliquots of similar volumes of monoclonal TYLCV-specific antibodies were added and the plate was incubated for 2 hrs at 37 °C. Subsequently, 100 *ul* aliquots of goat anti-mouse IgG diluted to 1/1000 with conjugate buffer were added similarly to the wells after 3 successive washings and the plate was warped and incubated for 3 hrs at 37 °C followed by 3 successive washings. 100 *ul* aliquots of *p*-nitrophenyl phosphate diluted in the substrate buffer at 1m gm/ml were added and the plate was immediately incubated in dark at room temperature for 1 hr.

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3.3.4.2. Sensitivity evaluation of TAS-ELISA

Sensitivity of TAS-ELISA was evaluated through its ability to detect the virus at different dilution of infective sap extract. Accordingly, crude sap was extracted from TYLCV infected tomato plants and exposed to 2-folds dilution until 1/256.

All aliquot dilutions were incorporated into ELISA wells and the test was run typically. Absorbency at 405 nm was measured 1 hr after incubation with the substrate. Crude sap extracted from healthy tomato was processed similarly.

3.3.4.3. Double antibody sandwich imunosorbent asay (DAS-ELISA)

DAS-ELISA was performed as previously described by Clark & Adam (1976). Accordingly, 100 *ul* aliquots of purified immunoglobulin, appropriately diluted in coating buffer, were added to each well of the microtiter plates, then incubated at 37 °C for 3 hrs. Thereafter, the plate was washed 3 times by flooding wells with PBS-Tween and left to soak for 3 min in each wash. 100 *ul* aliquots of test samples extracted in TYLCV grinding buffer, were added to the wells and then the plate was covered and incubated at 4°C for overnight. After 3 subsequent washings, the wells were charged with 100 *ul* aliquots of specific conjugate (goat anti-

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rabbit conjugate) appropriately diluted in Mab buffer, covered and incubated at 37°C for 3 hrs and then washed.

100 *ul* aliquots of enzyme substrate (*p*-nitrophenyl phosphate) were added to wells and incubated for 1 hr at room temperature.

For DAS-ELISA using monoclonal antibodies, the same procedure was repeated and the polyclonal antibodies were replaced with monoclonal ones and the goat-anti-rabbit IgG was replaced with goat-anti-mouse IgG.

3.3.4.4. Antigen-coated indirect enzyme-linkage immunosorbent assay (I-ELISA)

I-ELISA described by Clark *et. al*. (1986) was tested for its practicability for TYLCV detection. Accordingly, tip leaves of tomato plants showing typical symptoms of TYLCV were macerated in TYLCV-extraction buffer (1/10) (w/v) and then divided into 0.1 ml aliquots which were separately added to the wells of the microtiter ELISA plate. The plate was refrigerated overnight at 4 °C. After the sap extract was discarded, the plate was washed three times with PBS-Tween buffer. The wells were charged with 0.1 ml of TYLCV-specific polyclonal antibodies diluted in Mab buffer. After 3 hrs incubation at 37 °C, the antibodies were discarded and the plate was washed 3 times with PBS-T. Goat-anti-rabbit conjugate diluted at 1/3000 (v/v) in conjugate buffer was added to the wells (0.1

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ml/well) and the plate was incubated for 3 hrs at 37 °C. After conjugate discarding and plate washing, 0.1 ml of the substrate was added.

In all the above mentioned ELISA tests, dilutions of TYLCV-specific antibodies and goat anti-mouse conjugate were selected depending on comparative preliminary ELISA tests using sets of positive and negative controls. Concerning the TAS-ELISA, the recommendation of Adgen Ltd. (Scotland. U.K.) was also considered. The results of ELISA tests were recorded at the end of incubation period (1 hr after the substrate incubation. Therefore, conversion of the chromogenic substrate *p*-phenyl phosphate (colorless) to *p*-nitrophenol (yellow in alkaline solution) was monitored by subjecting the plates to automatic ELISA Reader, and the absorbency was measured at a wave length of 405 nm.

Incubation time was kept constant within the experiment and the tests were done in duplicate for each specimen including positive and negative controls. Test values were considered positive when they exceed the value of the negative control as recommended by Adgen Ltd. (Scotland, U.K.).

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3.3.4.5. Tissue blot immunosorbent assay (TBIA)

Leaf petioles and stems of tomato were printed onto a dry nitrocellulose membrane and the residues were removed by a brush. The tissue blots were washed 3 times (5 min each) with phosphate buffer saline containing 0.05% Tween 20 (PBS-T) pH 7.4. The tissue blots were then immersed for 2-3 min in 1 *u*g/ml polyvinyl alcohol (PVA) in PBS-T, then washed 3 times as pointed above. The tissue blots were incubated for 2 hr at room temperature with TYLCV-specific monoclonal antibody diluted up to 1:2000 in Mab, pH 7.4. After 3 successive washings, the tissue blots were incubated for 2 hrs at room temperature with alkaline phosphatase-labeled goat anti-mouse immunoglobulins diluted to 1:1000 in conjugate buffer. Thereafter, the tissue blots were incubated in substrate solution containing 14 mg of nitroblue tetrazolium and 7 mg of 5-bromo-4- chloro-3-indolyl phosphate in 40 ml of substrate buffer (0.1 M Tris-HCl, containing 0.1 M NaCl and 5 mM MgCl2), pH 9.5.

A positive result was considered by achieving a purple color on the tissue blot. No coloration indicates the negative reaction.

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gloves and putting on a special mouth mask, and sheilder was put when using ultraviolet light. These precautions, were done to prevent contamination of the isolated DNA and also to protect from the materials that are potentially hazardous to human kind (e.g. ethedium bromide).

3.4.2. PCR reagents

The PCR reagents were conditioned for TYLCV as follows:

3.4.2.1. Enzyme

The thermostable DNA polymerase extracted from thermophilic bacterial species *Thermus aquaticus* (*Taq*) was provided by the Sigma Company (Saiki *et. al.* 1988). The enzyme was stored at –20 °C and 0.2 *u*l was used directly in each PCR (Michael *et. al.* 1990).

3.4.2.2. Deoxynucleotide triphosphates

A set of the four deoxynucleotide triphosphate (dNTPs) including d-ATP, d-CTP, d-GTP and d-TTP was purchased from Allied Technological Group (Alltech), Paisley, U.K. Primary stocks were diluted in demonized distilled water to 2 mM each, aliquoted, and stored at –20 °C. Volume of 2.5 *u*l of dNTPs was used for each 25 *u*l of PCR (Michael *et. al.* 1990; Mehta *et.al.* 1994; Navot *et al.* 1992).

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3.4.2.3. Oligonucleotide primers

TYLCV-specific primers were purchased from the Alltech Company, Paisley, U.K.. The primer sequences were (from `5 to`3) P1V, ATACTTGGACACCTAATGGC, nt 61-80, P2C, AAGTAAGACACCG ATACACC, nt 41-60, P4C, TGGACATCTAGACCTAAG, nt 2054-2071, P5C, AGTCACGGGCCCTTACAA, nucleotides 456-473. The sequence of primer PIV corresponds to the viron (+) strand, whereas, primer P4C is complementary to the viron strand (Fig 1) (Navot *et.al.* 1992).

Working stocks (500 *u*l) of 2 *u*M concentration were prepared from each primer according to their concentrations

3.4.2.4. Template DNA

The ssDNA templates of TYLCV suspended in Tris-EDTA (TE) buffer (appendix 1) buffer were stored frozen. The optical density of each template was measured spectrophotometrically at 260 nm, and thereby, the concentration in ug/ml was determined. Volume of 10 *u*l was incorporated to each PCR (Michael *et. al.* 1990).

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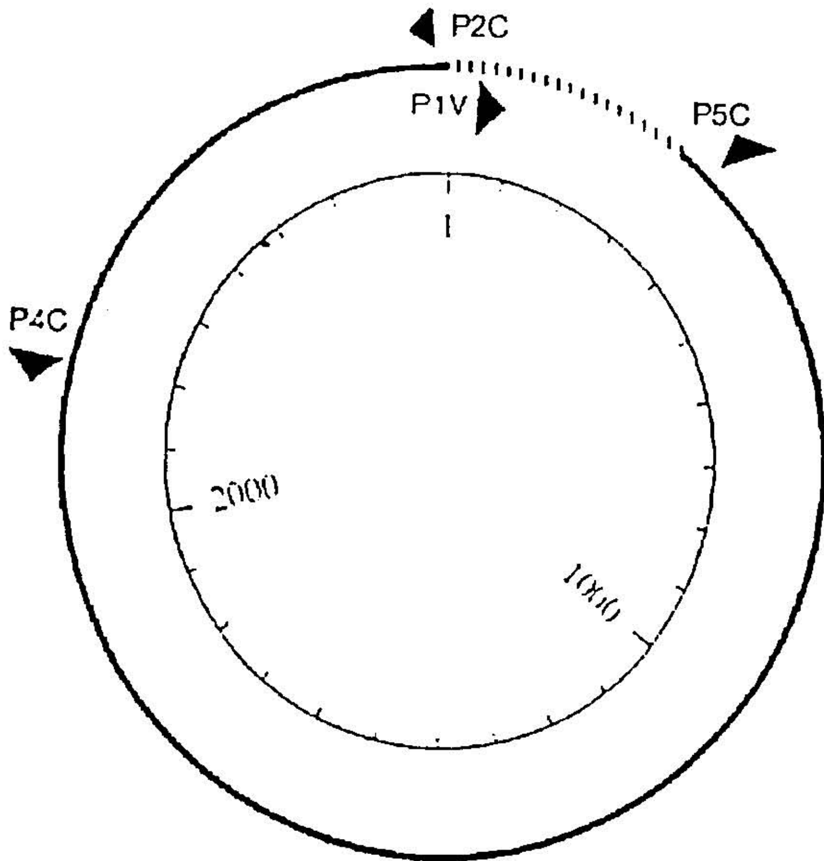

Fig 1. Location of the primers used for amplification of the complete TYLCV genome (P1V and P2C) and of subgenomic fragments (P1V and P4C, and P1V and P5C). The cross-hatched region of the outer circle (nucleotides 1-314) denotes the intergenic region of the viral genome. Arrows indicate the direction of DNA synthesis from each primer.

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3.4.3. PCR cocktail

The PCR cocktail was constituted aseptically in 500-ul Eppendorf tubes to achieve a final volume of 25ul (Navot *et. al.* 1992). Each cocktail included the following:

2.5 *u*l of 10X *Taq* buffer (TBE)

2.5 *u*l of 2 mM dNTPs

2.5 *u*l of 2 *u*M P1V primer

2.5 *u*l of 2 *u*M P4C primer

4.8 *u*l Water

0.2 *u*l *Taq* polymerase

10 *u*l DNA template

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3.4.4. DNA extraction protocols

3.4.4.1.DNA extraction from viruliferous whiteflies

Protocol 1:

This protocol was done according to Mehta et. al. (1994). Accordingly, 100 *ul* of homogenization buffer (2) (appendix 1) was added to the individual whiteflies in the Eppendorf tubes and ground thoroughly with a sterilized needle. Thereafter, 1 *ul* of proteinase K (50 ug/ml) was added, and the sample was incubated at 65 °C for 30 min. 3.5 *ul* of potassium acetate was then added and the tube was kept in ice for 30 min before centrifugation for 15 min at 10,000 *g*. The supernatant was separated and exposed to a further centrifugation for 5 min at similar revolution speed, then collected and extracted twice with an equal volume of TE-saturated phenol: chloroform (1:1). The aqueous phase was transferred to a new tube and extracted with an equal volume of chloroform. After transferring the resultant aqueous phase to a new tube, 2.5 volume of 100% ethanol was added, vortexed well, and incubated for 15 min at –80 °C. After incubation, the tube was submitted to 15 min centrifugation at 10,000 g and the resultant supernatant was discarded. The pellet was washed with 500ul of 70% ethanol, dried, and then resuspended in 25 *ul* of sterile distilled water.

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Protocol 2:

Individual adult whiteflies were placed in Eppendorf tube and ground thoroughly in 50 μl of salt Tris-EDTA (STE) buffer (appendix 1) and were ground using a sterilized needle. Then, the tube was centrifuged for 10 min at 10,000 *g*. The supernatant was collected and used directly for PCR (Mehta *et. al*. 1994).

3.4.4.2. DNA extraction from TYLCV-infected plant tissue

Protocol 1:

Nucleic acid of TYLCV was extracted from infected plant by standard method described by Dellaporta *et. al*. (1983). Tip leaf samples weighing approximately 0.3 gm, in 1.5 ml Eppendorf tubes were dried in liquid nitrogen and then ground to a fine powder with a glass rod. Then, 1 ml of extraction buffer (1) (appendix 1) was added and the tubes were kept for 5 min at 65 °C 300 μl of 5 M potassium acetate per 0.3 gm tissue were added to each tube and incubated on ice for 10 min, then centrifuged for 10 min at 10,000 *g*.

The supernatants were transferred to new tubes and the nucleic acids precipitated with 700 μl isopropanol. The pellets were subsequently

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washed with 70% and 100% ethanol, dried and resuspended in 100 ul TE (Crespi *et. al.* 1991).

Protocol 2:

The method of Dellaporta *et.al.* (1983) was modified in the step concerning the nucleic acid separation from the protein debris. The same weight of plant tissue was extracted in the same volume of the extraction buffer and incubated similarly at 65 °C. After the addition of 300 ul of 5 M potassium acetate, incubation for 10 min on ice, and low speed centrifugation for 10 min, the supernatants were mixed thoroughly with buffer (TE)-saturated phenol and submitted for centrifugation at 10,000 *g* for15 min.

The supernatants were obtained, treated with 700 ul isopropanol and exposed for 10 min low speed centrifugation. The pellets were washed similarly and resuspended in 100 ul TE buffer each.

3.4.5. Immunocapture-PCR (IC-PCR)

3.4.5.1. Immunocapture with polyclonal antibodies (IC-PAB)

1.5 ml Eppendorf tubes were coated with 200 ul of TYLCV-specific polyclonal IgG (1-2 ug/ml in coating buffer, pH 9.6 (appendix 1)) for overnight at 4C. After 3 successive washings with PBS-T, 200 ul

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of the sap extract prepared in 0.2 M KPO4, pH 6.0 or PBS buffer (0.1 gm/200 ul) was added to each tube and incubated for overnight at 4 °C and then washed similarly. The viral nucleic acid was released by the subsequent addition of 175 ul TE buffer, 5 ul proteinase K (20 mg/ml) and 20 ul of 10% SDS followed by incubation for 2 hrs at 37 °C.

200 ul of Tris-saturated phenol (pH 7.6) was added to the mixture, vortexed and then centrifuged for 5 min at 10,000 *g*. The aqueous phase was transferred to a cleaned Eppendorf tube, extracted twice with chloroform, vortexed and then centrifuged at 10,000 *g* for 5 min. The aqueous phase was transferred to a cleaned Eppendorf tube, 2.5 volume of cold absolute isopropanol and 0.1 volume of 3 M sodium acetate, pH 5.5, then incubated at –70 °C for 15 min or –20 °C for 2 hrs or overnight. After centrifugation at 10,000 *g* for 10 min, the aqueous phase was discarded and the pellet was washed subsequently with 70% and 100% ethanol, dried and resuspended in 50ul of TE buffer (Anonymous, 1995).

3.4.5.2. Immunocapture with monoclonal antibodies (IC-MAB)

The same procedure was repeated as pointed above for the Immunocapture PCR except that polyclonal antibody was replaced with monoclonal one (Anonymous, 1995).

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3.4.6. Amplification of TYLCV DNA

The amplification protocol was used as described by Sambrook *et. al.* (1989). Briefly, the cycling protocol was initiated in tubes containing 25 *u*l of PCR cocktail using the following cycling program:

Initial cycle	
Annealing temperature	65 °C for 5 min
Extension temperature	72 °C for 5 min
Denaturation temperature	92 °C for 1 min
Subsequent cycles	55 °C for 2 min
	72 °C for 4 min
	92 °C for 1 min

The reaction was performed 35 cycles using Perkin Elmer automated thermocyclers. The amplified samples were subjected to electrophoresis in 1.2 % agarose gel, stained with 0.5 *u*g/ml of ethedium bromide, and photographed (Navot *et. al.* 1992).

3.4.7. Gel electrophoreses

Electrophoreses was employed for amplified DNA product of PCRs. The process was done in a gel consisted of 1.5 gm agarose and 100 ml of 1X TBE buffer (appendix 1). After boiling, the agarose gel was poured

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into a casting tray, allowed to solidify for around 30 min. Running buffer of 1X TBE was added to sink the gel. PCR products were mixed with bromophenol blue (3.5 ul/PCR cocktail) and then loaded on the gel using micropipite. The gel chamber was connected with a power supply to permit DNA migration toward the anode (+ve pole). Electrophoreses was started at 70 volts for 5 min then at 90 volts for 1-2 hrs. The gel was removed from the tray and stained for 30 min with 0.5ul/ml ethedium bromide. After destaining for 30 min with tap water, the amplified DNA products were visualized by ultraviolet light and photographed by Polaroid films (Saifan, 1999).

3.4.8. Fragment sizes estimation of PCR products

Fragment sizes of DNA products were estimated depending on the standard curves that represent the typical relationship between the fragment sizes in (bp) and the mobility of DNA bands in Lambda *Hind III EcoR1*, 500-base and PBR DNA size markers (Saifan, 1999).

3.4.9. Band scoring

DNA bands in each amplification product were scored to be present or absent by visual testing of the gel photograph. The positive bands were

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compared with those developed by the standard PCR products that were amplified from TYLCV-infected plant tissue.

3.4.10. Threshold of TYLCV detection in plant sap by PCR

The cut point of PCR detection of TYLCV from infective plant sap was determined by DNA amplification obtained from crude sap dilutions. Therefore, the DNA was extracted from the infective tomato sap after being exposed to10-fold dilutions using the methods of Dellaporta *et. al.* 1983 (modified protocol/protocol 2). The PCR products were loaded in gel electrophoresis and the resultant bands were noticed.

The same procedure was repeated for IC-PCR using the polyclonal antibody produced against the local isolate of TYLCV.

3.4.11. PCR detection of TYLCV genome from different plant parts

Two tomato plants were grafted with TYLCV-infected scions and kept under glasshouse conditions. Thirty to forty days after inoculation, parts of root, tip leaf, middle-aged leaf, older leaf, upper, middle and lower stem portions were dissected. Each part was ground in buffer containing 6.056 gm of tris (hydroxymethyl) aminomethane and 7.56 gm of sodium sulfate in 1 liter distilled water as a final volume, pH 8.5 (0.3 gm/ml) and then extracted according to Dellaporta *et. al.* (1983) (protocol 2). The

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DNA amplified products were checked on agarose gel electrophoreses 90 min thereafter.

3.4.12. Time threshold of TYLCV detection from tomato by PCR, ELISA and Biology

Ninety-six healthy tomato plants were grown under glasshouse conditions (25-30°C). Whitefly-mediated inoculation was employed using adult insects raised for two generations on TYLCV-immune plants (eggplant (*Solanum melongena* L.), cauliflower (*Solanum melongena* L.) and pumpkins (*Solanum melongena* L.)). Accordingly, eight plants were inoculated by viruliferous whiteflies at 3-4 true leaf stage. Virus was acquired by the whiteflies after an access period of 48 hr on TYLCV-infected tomato plants. Inoculation was done by caging the third top leaf of each plant with ten whiteflies using leaf cages or perforated plastic bottles. After 24 hr feeding access, the whiteflies were killed.

Samples were collected from the youngest top leaves of the inoculated plants at daily intervals. Similarly, leaf tissues were collected from the inoculated leaves up to five days post inoculation. The leaf samples were kept frozen at –20 C and then tested by PCR and the leaf samples obtained from the top leaves were also tested by TAS-ELISA.

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3.5. Application of serology and PCR

3.5.1 TYLCV fate in the whitefly vector (molecular level)

3.5.1.1.Whitefly rearing

First generation

Adult whiteflies were collected from eggplant fields grown in the Ghore El-Safi using a portable electric insect hover. The whiteflies were cultured in woody cages under a glasshouse on TYLCV-nonhosts including eggplants (*S. melongena* L.), cauliflower (*B. oleraceae* L.) and pumpkins (*C. moschta* L.).

Second generation

The second generation of *B. tabaci* was obtained by transferring plants with large numbers of the insect immatures on their leaves into other cage. *B. tabaci* was identified depending on the pupal stage (Gerling, 1980; Avidov and Harpaz, 1969).

3.5.1.2. TYLCV acquisition by the whitefly

Whiteflies emerged within the same 48 hr were obtained and caged with TYLCV-infected tomato source plants. The insects were allowed 48 hr acquisition-access period.

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3.5.1.3. DNA retention by the whitefly vector

The viruliferous whiteflies that had been given 48 hr acquisition-access were transferred and reared on pumpkin (TYLCV-immune plant) for the rest of their life. Whitefly samples (5 insects each) were taken daily up to 22 days using aspirator and stored frozen at –20 C for later analyses. The retention of the viral genome was tested by PCR. Rearing host in whitefly culture were continuously renewed every 10 days to avoid any nonviruliferous insect emerged by the progeny.

3.5.1.4. Capsid protein retention by the whitefly vector

Insect samples (25 insects each) were removed from the viruliferous whiteflies reared on the immune plant every day and frozen at -20C for later analyses. The retention of TYLCV capsid protein was studied using TAS-ELISA as recommended by Adgen (Scotland, Ltd., U.K.). Both monoclonal and polyclonal antibodies were cross-absorbed with acetone-washed non-viruliferous whiteflies for an overnight period at 4 C. whitefly samples were prepared by grinding 25 insects in 0.2 ml of TYLCV grinding buffer. Samples of similar number of non-viruliferous whiteflies were used as a negative control. Quantitative meaning of the results was also obtained using ELISA-Reader at 405 nm 1hr after the substrate incubation.

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3.5.1.5. Nonspecific acquisition of TYLCV by *T. vaporariorum*

Adult whiteflies of *T. vaporariorum* raised for several generations on pumpkin plants were obtained from Acarology laboratory. The whiteflies were given 48 hr acquisition-access on TYLCV-infected tomato source plants. The whiteflies were removed and frozen at –20 C. for latter analysis.

The virus acquisition was checked by PCR for the DNA and by ELISA for the capsid protein.

3.5.2. Occurrence of TYLCV in tobacco fields

Tobacco plants were assayed for TYLCV infection in five locations including the Moadi nursery in the Jordan Valley and Al-Baqua, Irbid, Al-Karak and Amman regions (Table 7). The samples were collected during summer months. Symptomatic and symptomless plants were covered in the collection without any bias from the perimeter and then on the diagonals. The top 3-4 inches of foliage of each plant were collected using suitable precautions against cross-contamination. Within 24 hrs, TAS-ELISA and PCR were used to check the virus infection and the percentage of infection was calculated for each location.

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3.5.3. Occurrence of TYLCV in bean fields

Leaf samples were collected from bean fields grown in the Jordan Valley and the uplands including Al-Baqua region (Table 8). The samples were obtained from the tip leaf of both symptomatic and symptomless plants. The samples were tested for TYLCV infection using TAS-ELISA and PCR and the percentage of infected plants was calculated for each location.

3.5.4. Occurrence of TYLCV in pepper and eggplant fields

Two hundred leaf samples of both pepper and eggplants were tested for TYLCV infection. The samples were collected from fields established near tomato fields inflecting high incidences of TYLCV in the Jordan Valley and Al-Baqua regions. TAS-ELISA and PCR were employed for all samples.

3.5.5. Field indexing of different weed species and volunteer tomato plants

Different weed species and volunteer tomatoes grew in or within a radius of 200 meters around the tomato fields or greenhouses with high incidence of TYLCV infection were sampled and tested for TYLCV using

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4.1. Virus identification

The tentative identification of TYLCV based on the field symptoms was confirmed *in vitro* depending on symptom expression on the indicator plants, transmissibility, serology and PCR.

The results of the experimental diagnostic assay plants and the symptoms associated with hosts were similar to that reported for TYLCV by Mansour and Al-Musa (1992) (Plate 1 and 2).

TAS-ELISA showed that the isolate reacted specifically with both monoclonal and polyclonal TYLCV-specific antibodies. Such reaction can be easily discriminated from that obtained from the healthy sap extract (Section 4.3.). In addition, TBIA reacted clearly with the stem or petiole segments obtained from TYLCV-infected plant. No reaction was observed with healthy tissue (Section 4.3).

The results of gel electrophoresis showed that TYLCV-specific primers could amplify the genome of the virus isolate. The reaction was exemplified by formation of sharp and distinctive bands on agarose gel (Section 4.4)

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Plate 1. Symptoms of yellowing and curling of TYLCV on tomato plants.

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Plate 2. Symptoms of mosaic and vein banding, with slight leaf malformation caused by TYLCV on *Datura. stramonium* L..

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4.2. Purification of TYLCV

4.2.1. Determining of TYLCV propagative host plants

Quantitative results of TAS-ELISA at 405 nm. supported that both jimsonweed (*D. stramonium* L). and tomato were suitable propagative hosts for TYLCV, since they contained a good virus content. No significant difference was obtained between the virus content of these plants.

4.2.2. Determination of the proper time of harvesting infected tissue

The results of quantitative TAS-ELISA showed that the virus was detected in the tip leaves of tomato 3 wks post inoculation and reached the maximum concentration during the fourth and the fifth wks. Statistical analysis showed that the virus content during these periods was highly significant.

A slight reduction in the virus content was observed during the sixth week after inoculation (Fig 2).

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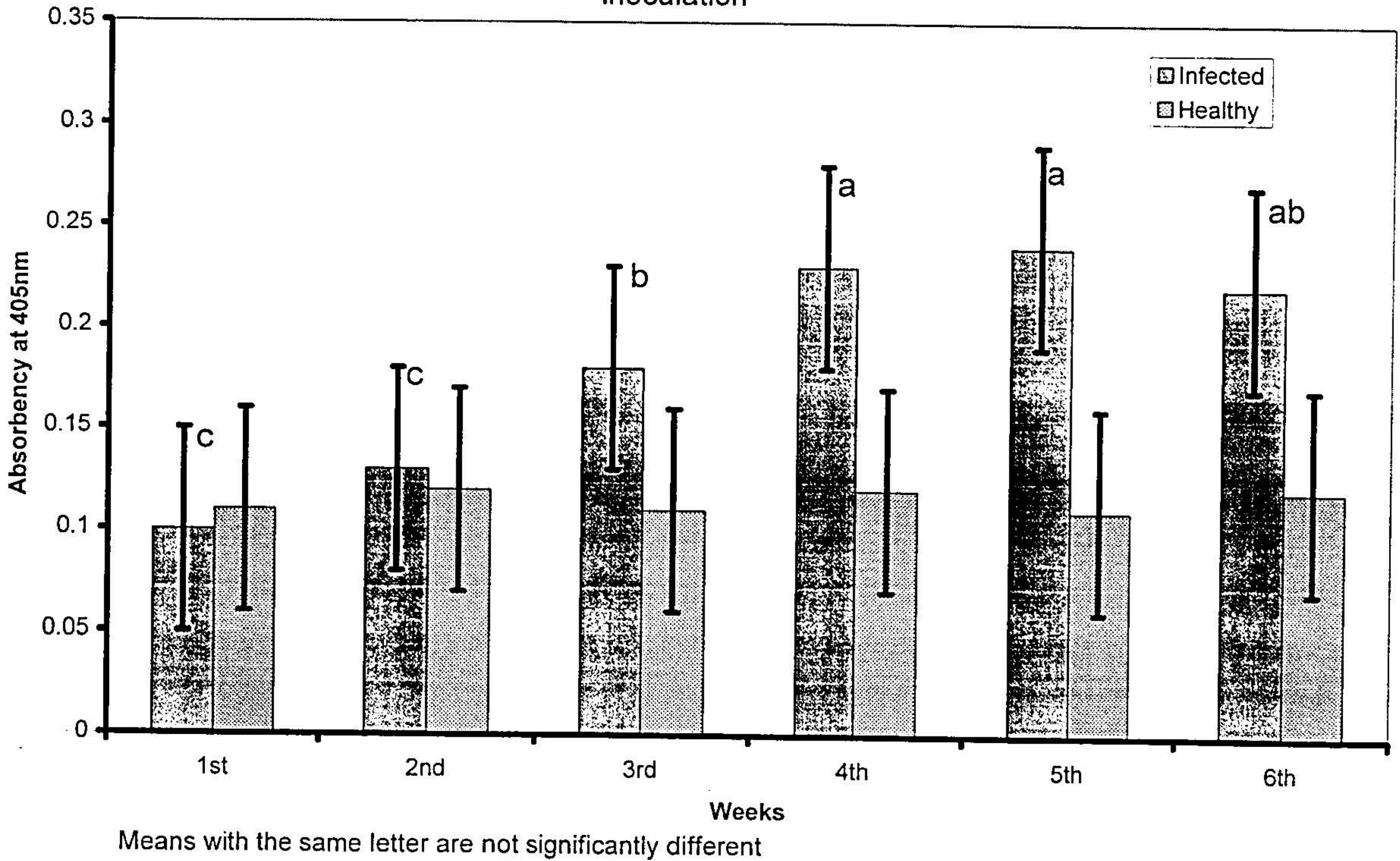
Fig 2. ELISA quantification of TYLCV in tomato plant following different periods after inoculation



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4.2.3. Determination of virus concentration on different parts of tomato plant

Quantitative TAS-ELISA revealed that tip leaves of tomato plants have the maximum virus content followed by tip petiole, upper portion of stem, middle-age leaves and flowers. Organs of middle-age petiole, lower leaves, lower petioles, middle-age stem, lower portion of stem and root have lower virus contents. Virus content in the tip leaves was highly significant compared with others (Fig 3).

4.2.4. Viron purification

4.2.4.1. Comparison of extracting media

The results of the quantitative TAS-ELISA showed that media 7 and 4 accomplished with the maximum TYLCV release from infected tomato tissue. Both media achieved absorbency values at 405 nm at least 3.5 times greater than their negative control. Virus content achieved by medium 7 was highly significant compared with others. Medium 6 achieved good virus release that is about 2.5 times greater than the healthy tissue. Media 1 and 5 provided nearly half amount of the virus yield released by media 7 and 4. The virus yields obtained from media 2 and 3 were the lowest (Fig 4).

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Fig 3 . ELISA quantification of TYLCV in different tomato parts

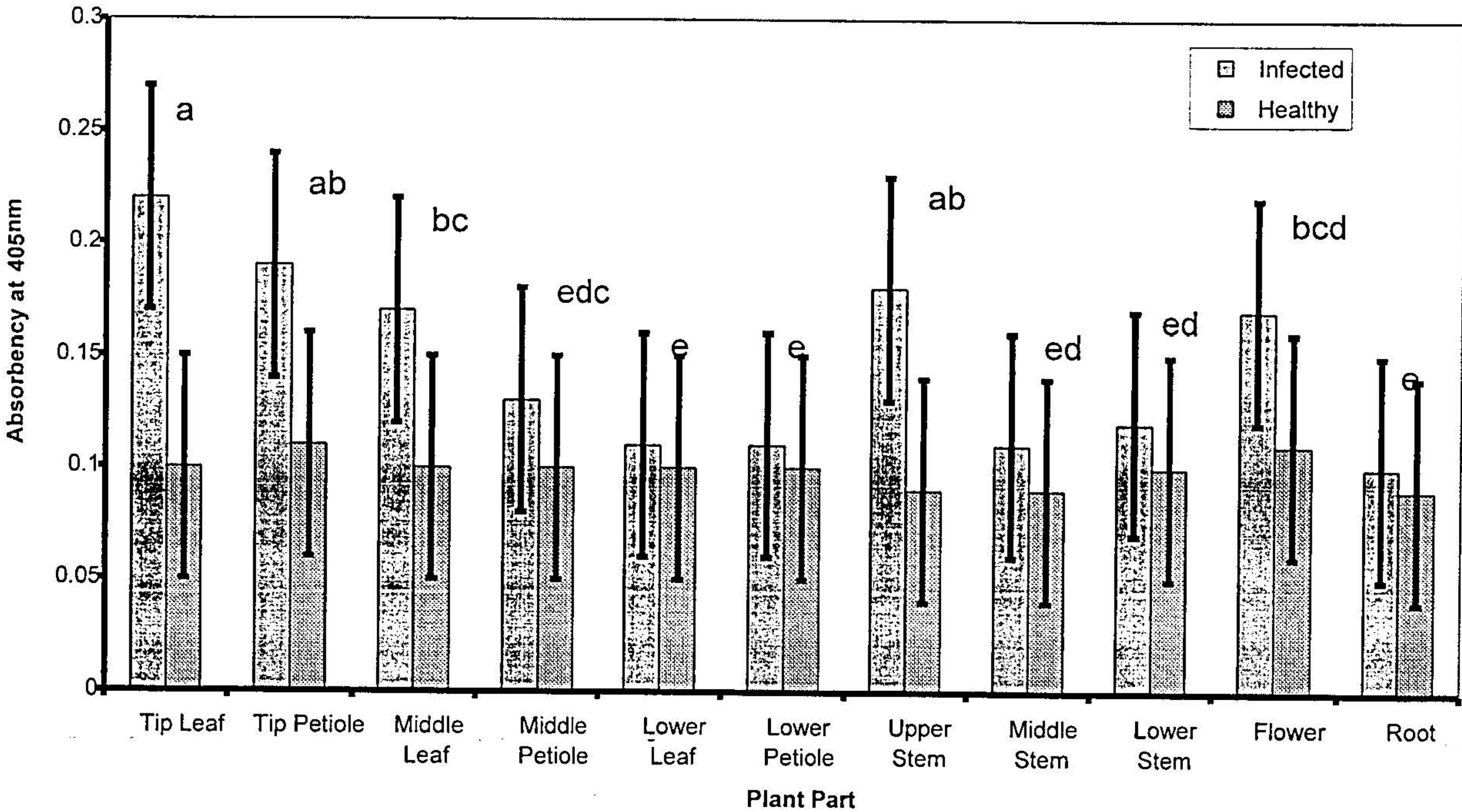

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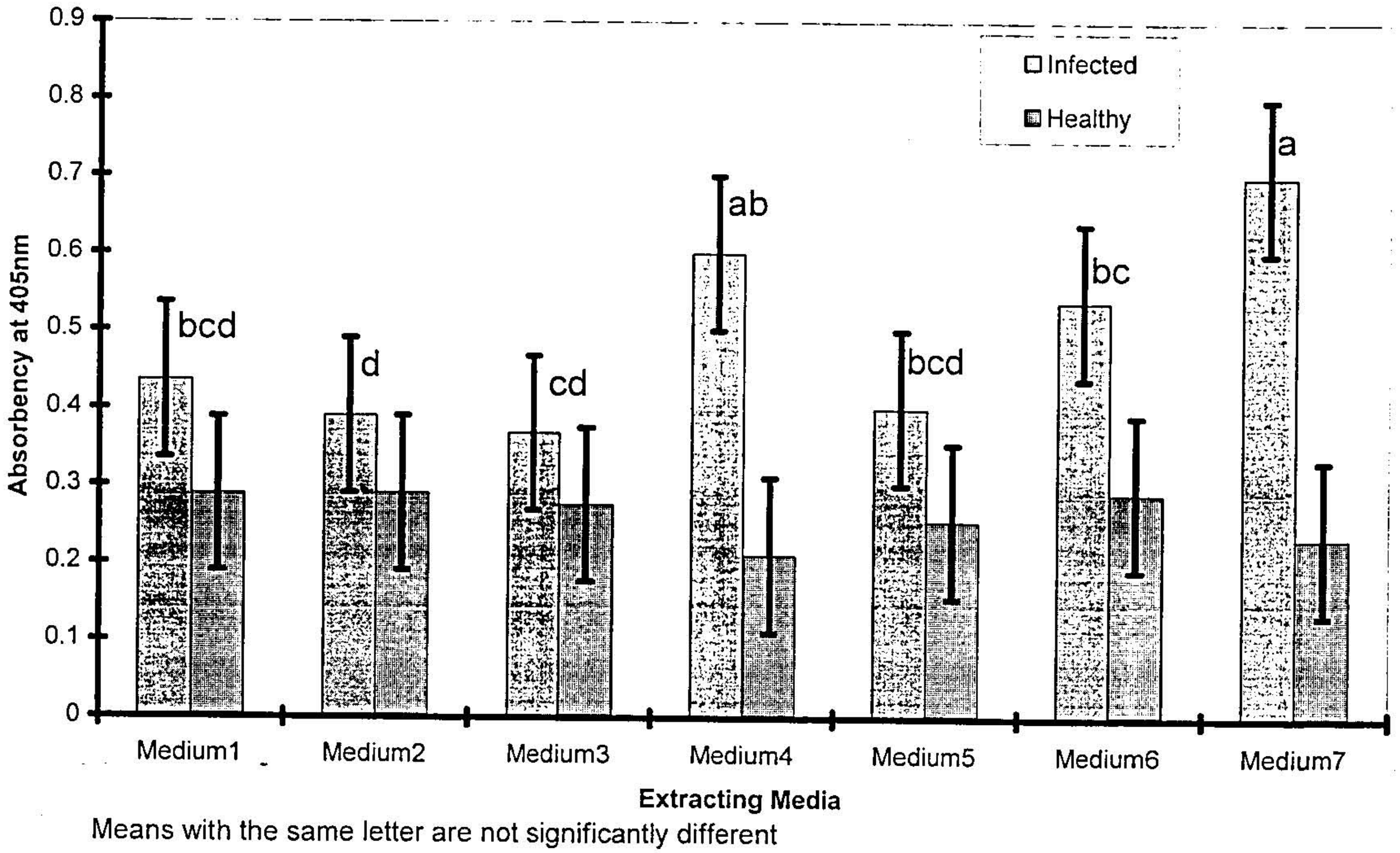
Fig 4.TAS-ELISA quantification of TYLCV in different extraction media



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The aqueous phase obtained after chloroform clarification was the clearest when media 7 and 4 were used for TYLCV extraction from infected tomato tissue. In addition, these media gave clearest pellets with medium volumes after ultra-centrifugation.

4.2.4.2. Effect of different clarification treatments

Chloroform 10% (v/v), 10% CCl4, a combination of chloroform 10% (v/v) and CCl_4 10% (v/v) and low speed centrifugation at 8,000 *g* gave minimum virus loss following the low speed centrifugation. Pellets obtained by these treatments have maximum virus contents. The chloroform clarification was more significant than the others.

Few to some virus loss was achieved by the clarification treatments of 8.5% n-butanol (v/v), overnight freezing at –15 °C, acidification to pH 4.5, filtration through a celilte pad, charcoal, Cl3F3C2 10% (v/v), and a combination of chloroform 10% (v/v) and n-butanol 8.5% (v/v) (Fig 5).

Beside their minimum virus loss, low speed centrifugation for the chloroform 10% (v/v) emulsion provided the clear and the large supernatants and pellets, respectively. Moreover, this treatment resulted in developing the smallest pellet collected after ultra-speed centrifugation (120,000 *g* for 5 hrs).

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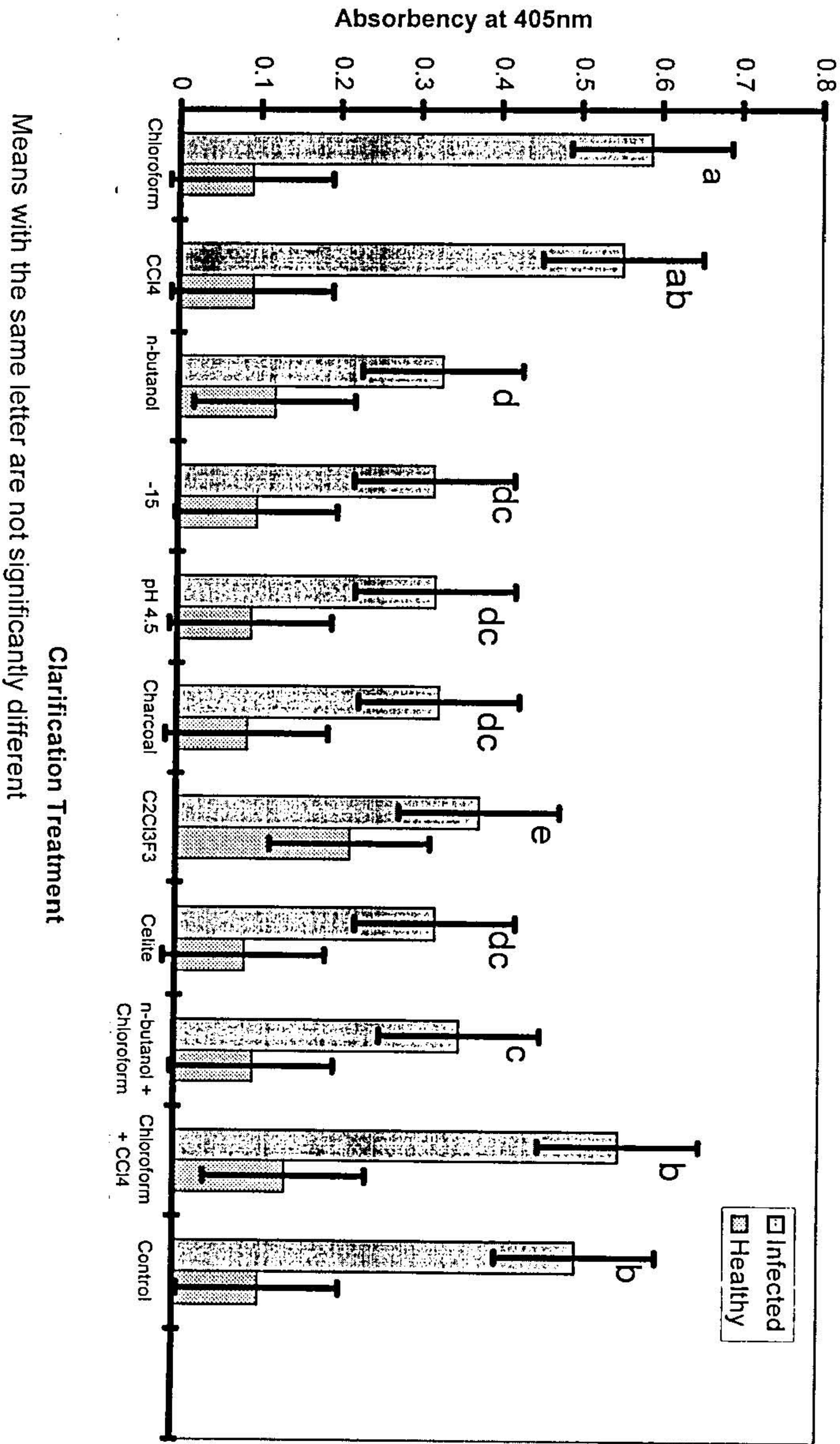
Fig 5. TYLCV clarification methods and ELISA quantification of the virus in the pellets obtained after low speed centrifugation



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ultra-speed centrifugation gave clear precipitates with medium volumes.

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Fig 6. ELISA quantification of TYLCV following different precipitation agents

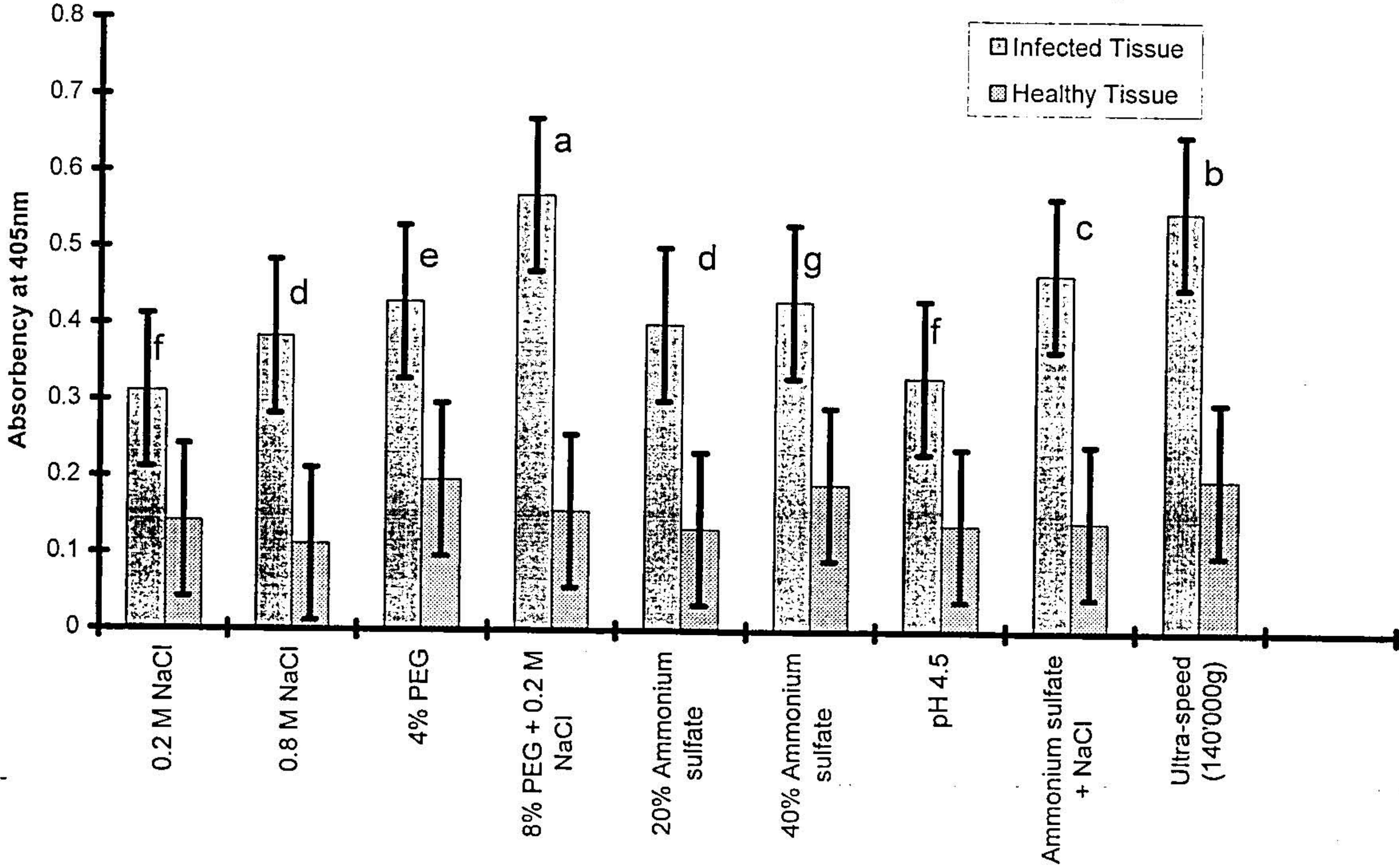

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Fig 7. ELISA detection of TYLCV in the gradient fraction at 405nm

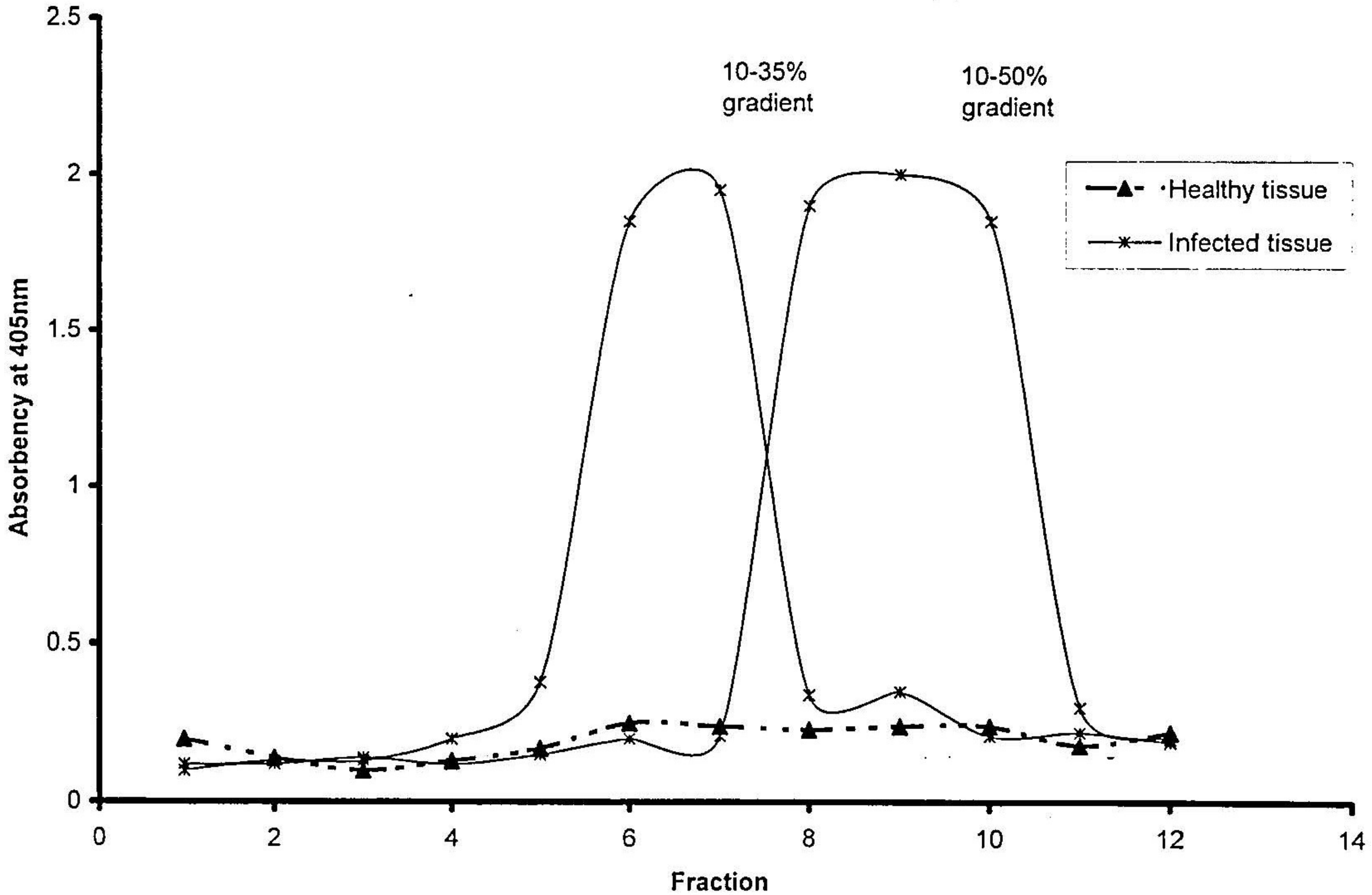

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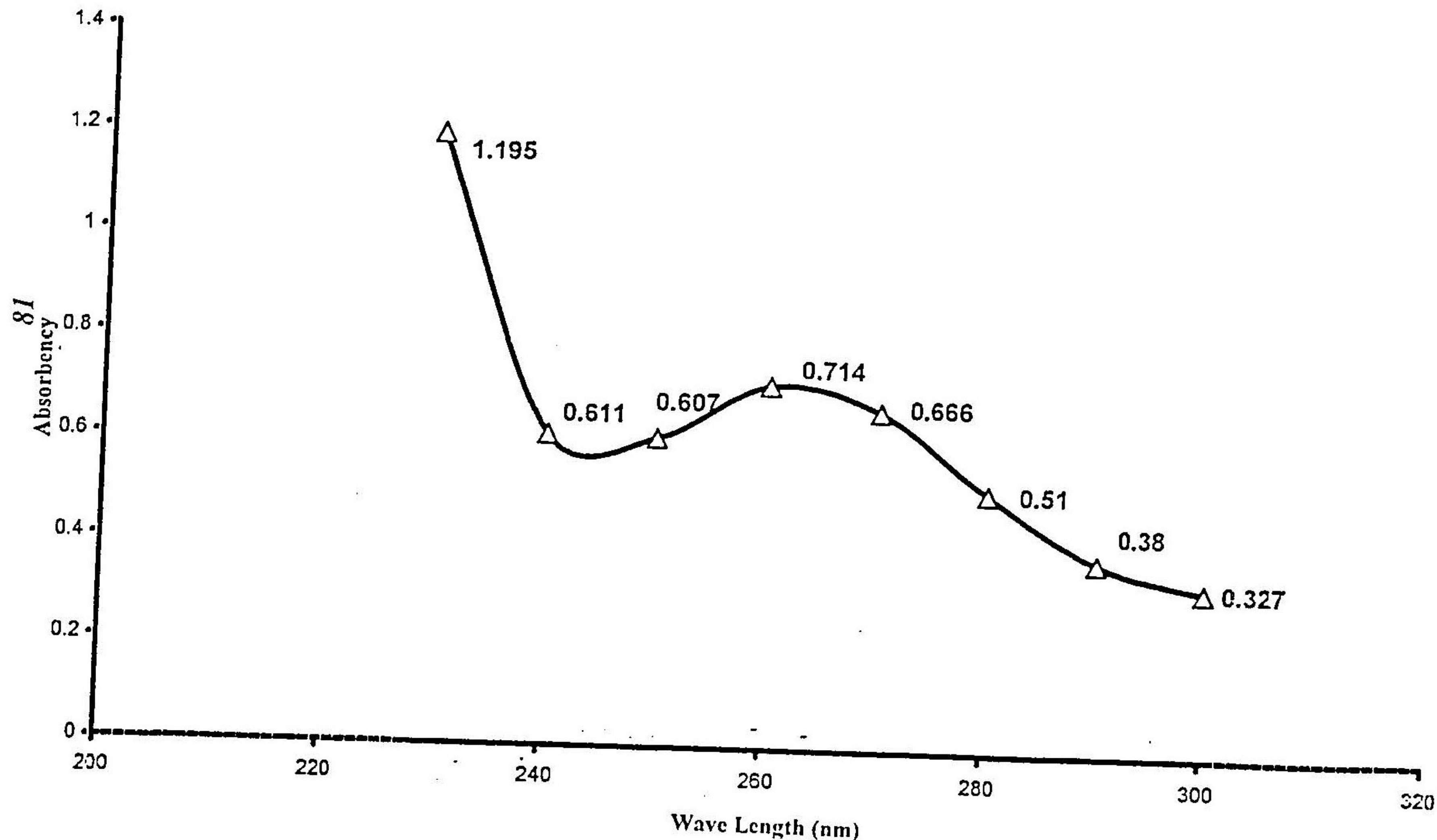
Fig 8. Ultraviolet absorption spectrum of the purified TYLCV from 230 to 300 nm



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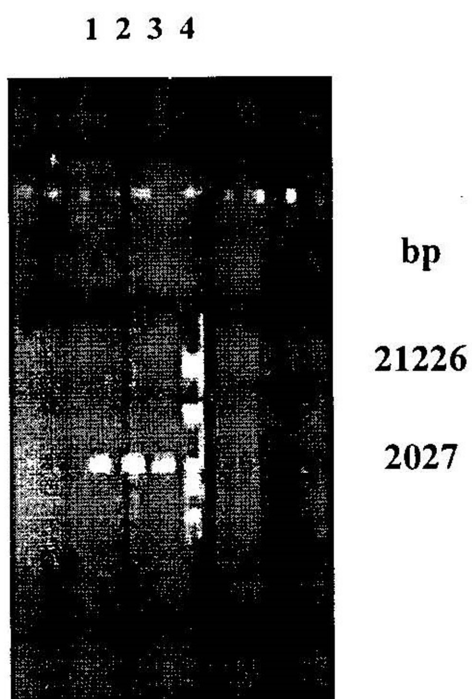

Plate 4. Agarose gel electrophoreses of PCR amplified TYLCV DNA from fractions 8, 9 and 10 obtained from the 10-50% linear sucrose density gradient. Primers are P1V and P4C.

Lane 1: PCR product from fraction 8
Lane 2: PCR product from fraction 9
Lane 3: PCR product from fraction 10
Lane 4: DNA size marker (*Lambda Hind III EcoR1,* 125-21226 bp)

More details about this plate are discussed in section 4.4.

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4.3. Antiserum production and serological tests

4.3.1. Antiserum production

Antiserum prepared against standard purified preparation of TYLCV reacted specifically with TYLCV antigen of the infective crude sap, and did not show any reaction with the healthy plant sap processed similarly in TAS-ELISA. The IgG for TYLCV was detected in all bleedings followed the last injection (Fig. 9). The maximum IgG titer was achieved in the third and the forth bleedings. The later was highly significant.

4.3.2. Immunosorbent assays

4.3.2.1. Triple antibody sandwich enzyme-linked Immunosorbent assay (TAS-ELISA)

The results showed that TAS-ELISA was sensitive enough to detect TYLCV in the crude sap of infected tomato plants. Quantitative study using ELISA-Reader revealed that 1/8 and 1/500 dilutions for the antigen and the antibodies, respectively, were the best to develop a sharp distinction between the positive and the negative controls. In addition, dilutions of 1/2 to 1/16 for the antigen and 1/1000 for the antibodies provided a good discrimination between the negative and the positive controls. One-hour incubation with the substrate was enough to develop a dark yellow color to be distinguished visually.

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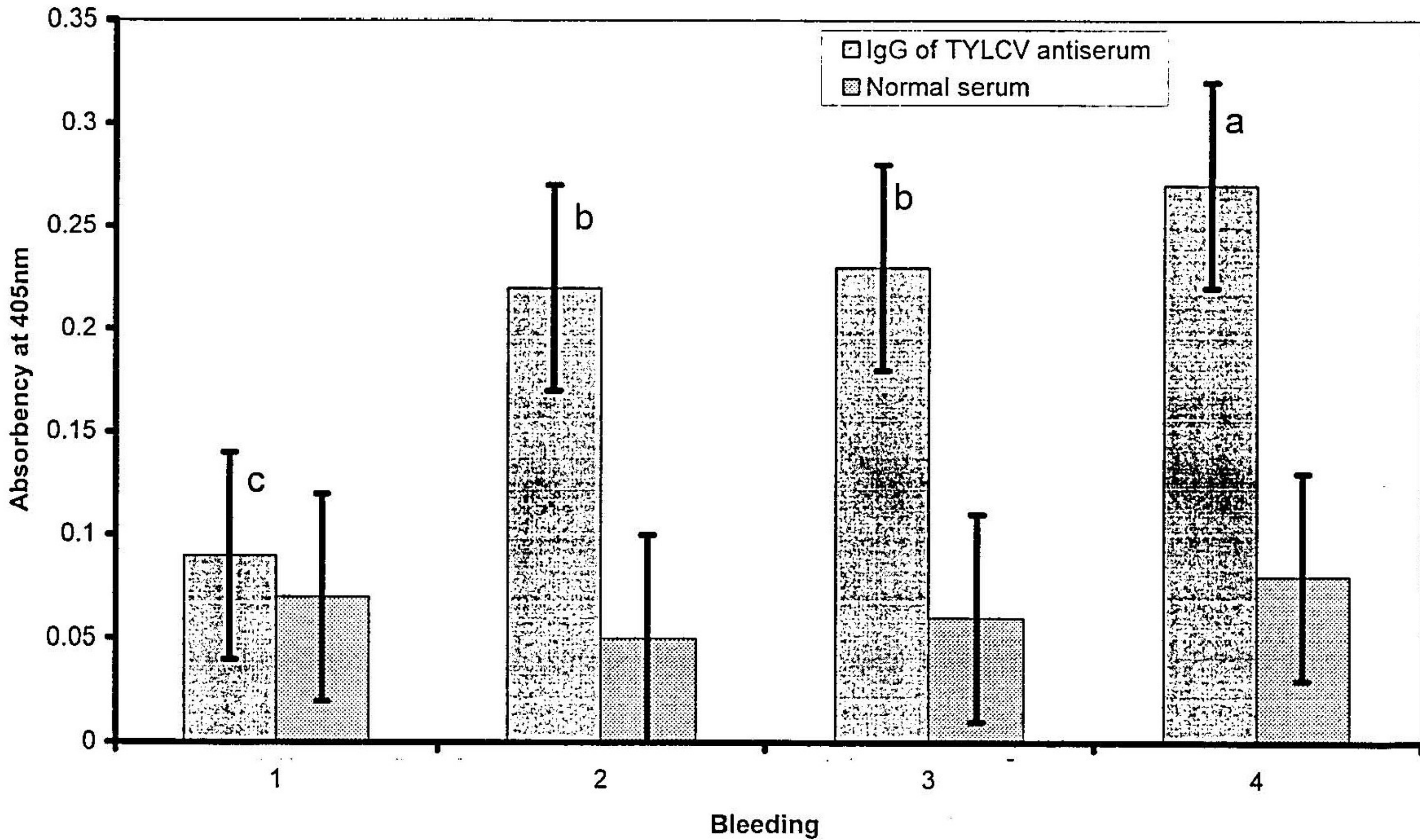
Fig 9. IgG content of different bleedings from a rabbit injected with purified TYLCV.



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4.3.2.2. Sensitivity evaluation of TAS-ELISA

Testing infective crude sap exposed to two-folds dilution revealed that ELISA was able to detect the virus from the crude sap diluted up to 1/64 Any dilution beyond this point provided no significant difference between the positive and the negative controls. The maximum discrimination between the infected and healthy tissue was achieved when the dilution ranged from 1/4 to 1/8. Dilution of 1/8 was highly significant (Fig 10).

4.3.2.3. Double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA)

Quantitative and qualitative tests showed that DAS-ELISA was not sensitive to detect TYLCV from infected tissue. No significant discrimination was found in the absorbency values between the positive and negative controls 1 hr after the substrate incubation. Increasing the incubation to overnight period did not improve results (Fig.11).

DAS-ELISA using monoclonal antibodies was found to be workable. Although the background coloration of the healthy tissue was reduced, the reaction of the positive control was lower compared with TAS-ELISA using plates pre-coated with polyclonal antibodies (Fig 12).

Fig 10. TAS-ELISA detection of TYLCV in crude sap exposed to 2-fold dilutions

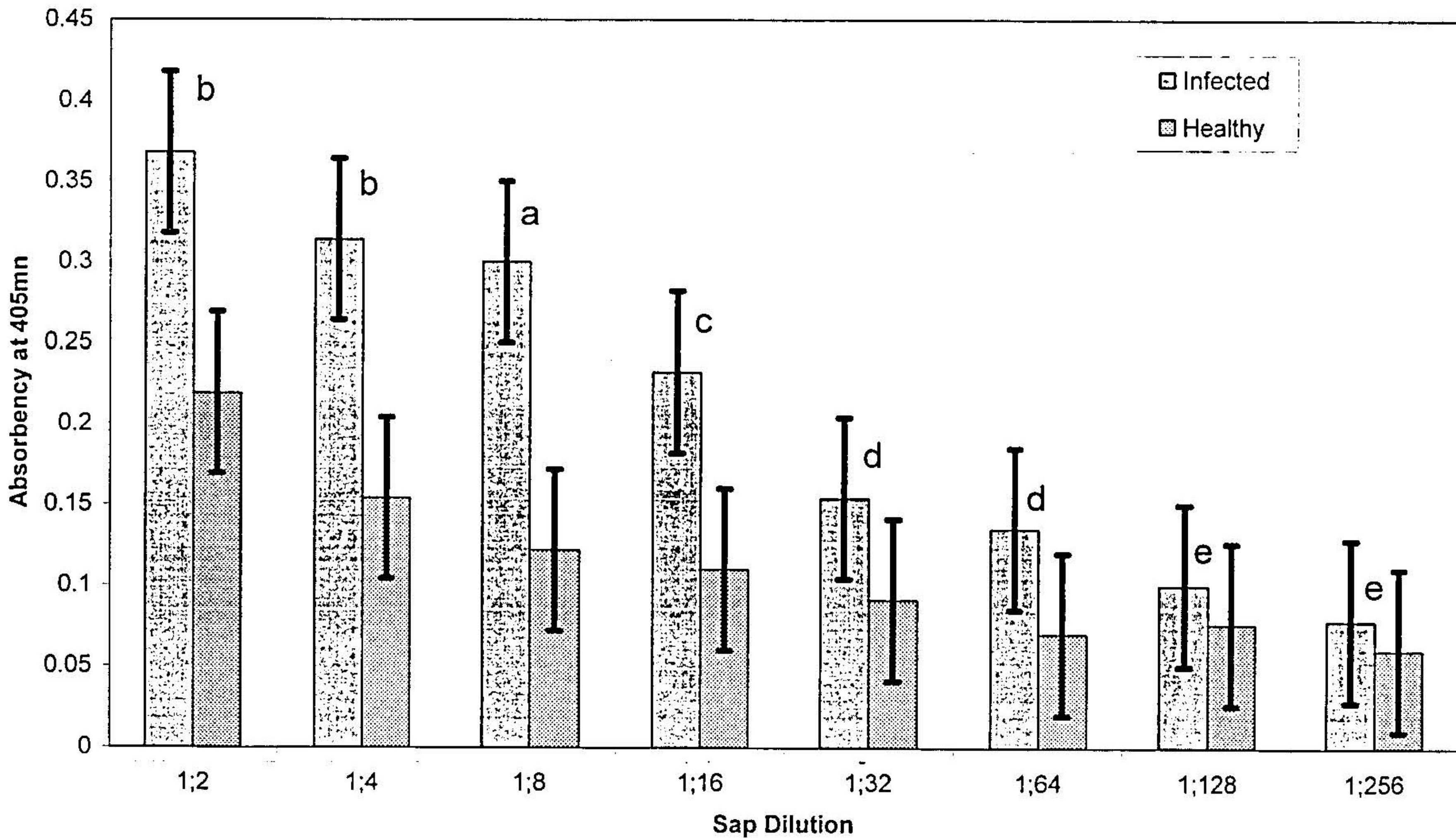

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Fig 11. DAS-ELISA detection of TYLCV following 2-fold dilution of the antigen

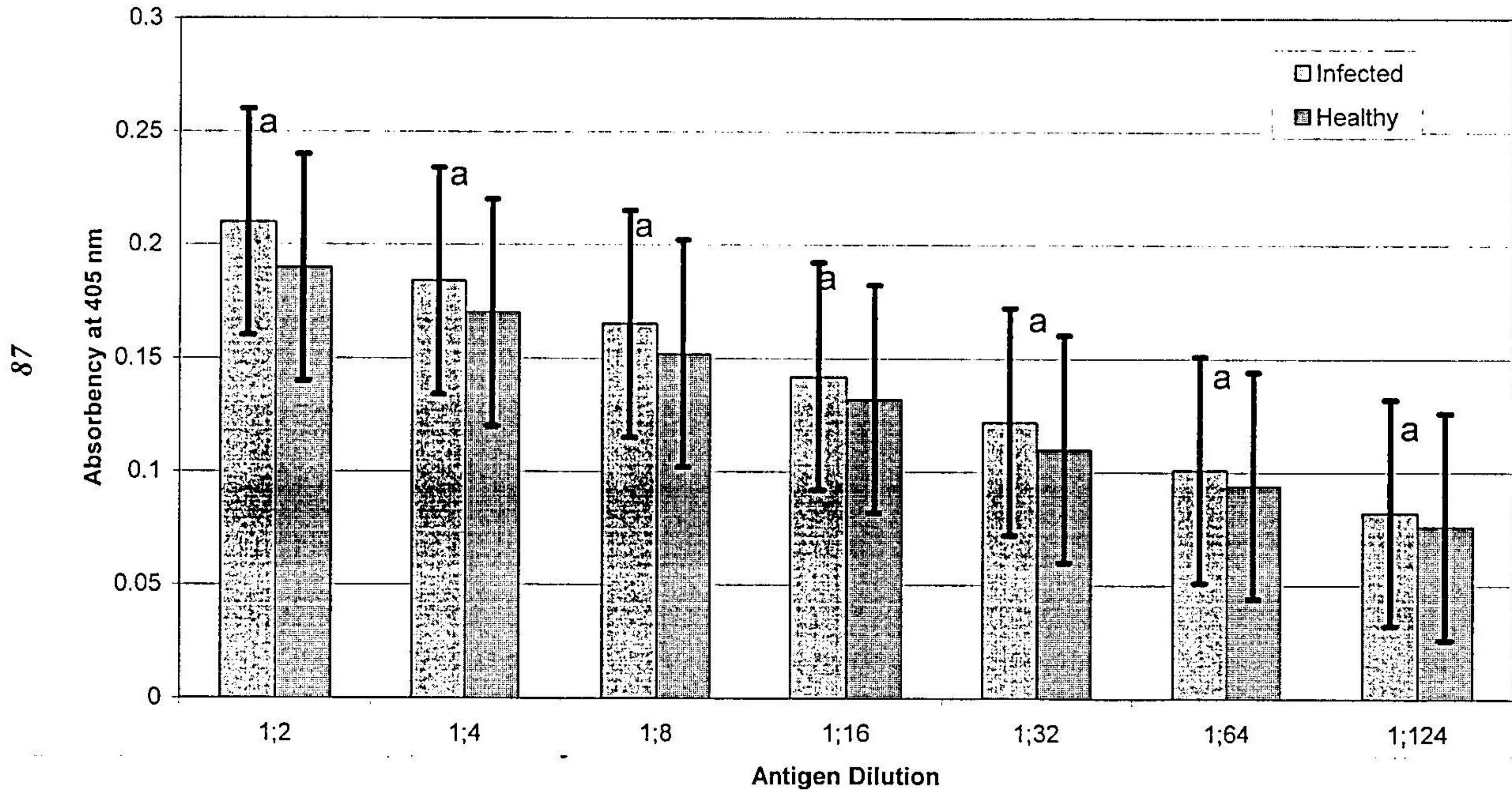

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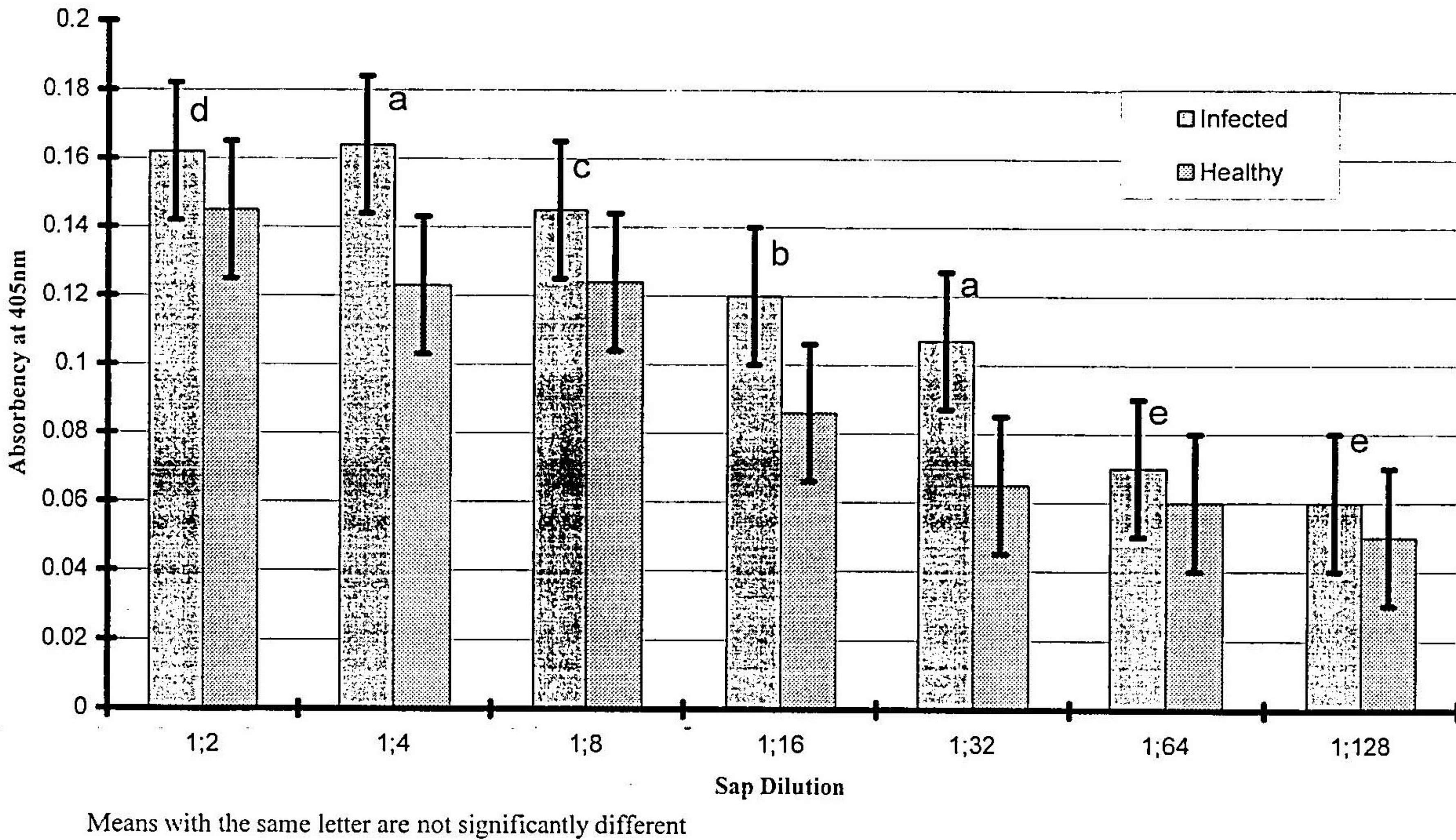
Fig 12. TYLCV detection by DAS-ELISA using monoclonal antibodies



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4.3.2.4. Antigen-coated indirect enzyme-linked immunosorbent assay (I-ELISA)

The result proved that I-ELISA was not suitable to be used in TYLCV detection from the infected sap preparation. No visual discrimination was obtained between the positive and the negative samples. The test failed to react with the virus even in low dilution of both the antigen and the antibody that approached 1/1 and 1/500, respectively. Moreover, prolonging the incubation to an overnight period revealed the same results (Fig.13).

4.3.2.5. Tissue blot immunosorbent assay (TBIA)

The result revealed that using monoclonal antibody in TBIA was applicable for TYLCV detection. Therefore, nitroblue tetrazolium treatment of the nitrocellulose membrane preprinted with fresh segments of stems and petioles of tomatoes revealed a clear-cut discrimination between infected and healthy statuses. The test was sensitive enough to detect the virus up to the antibody dilution of 1/2000. Infected sample was recognized by the coloration of the vascular bundles with a dark pink color 20-60 min after the substrate addition. No or nil coloration was noticed in the TYLCV-free plant (Plate 5).

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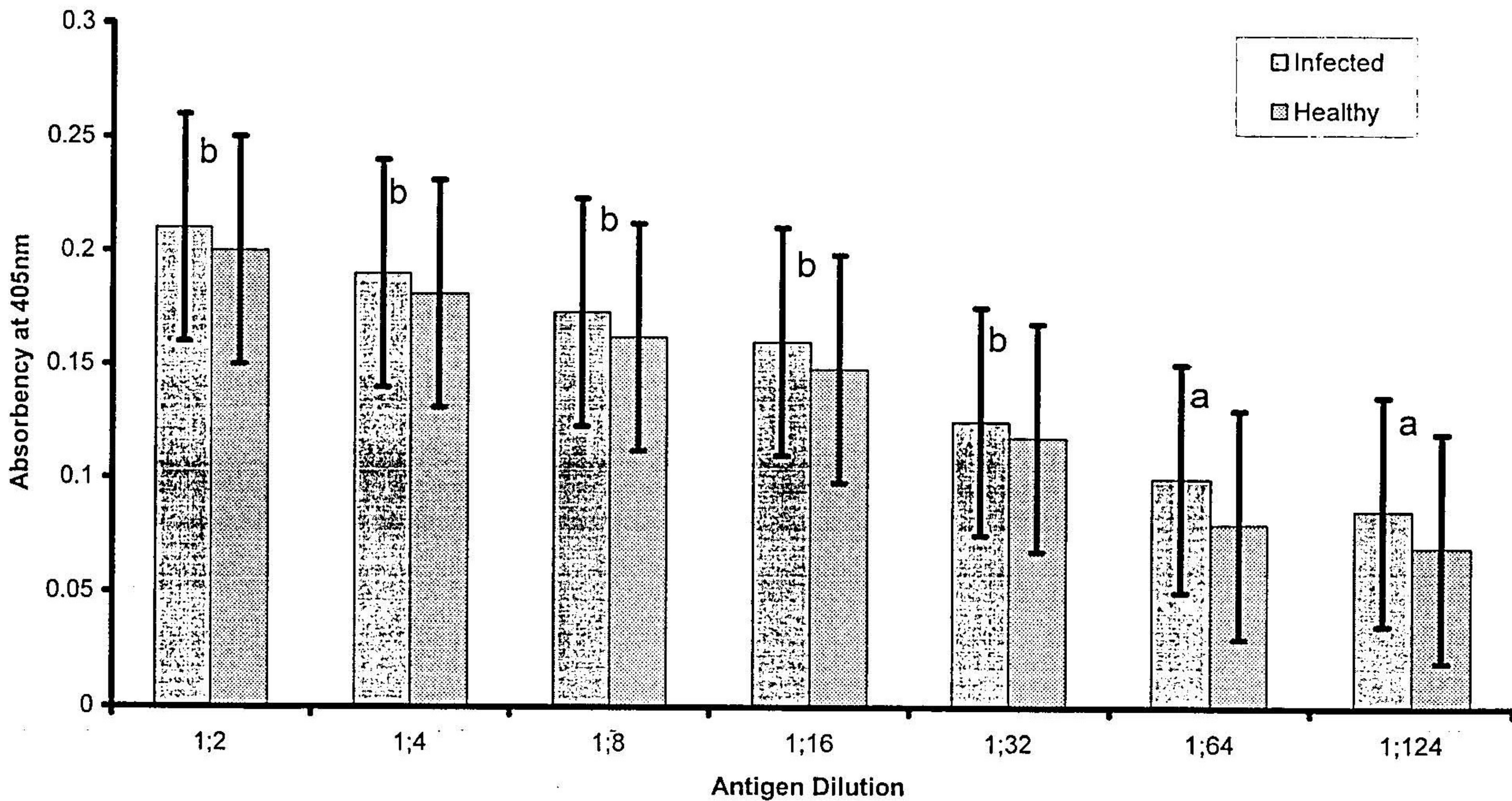
Fig 13. I-ELISA detection of TYLCV following 2-fold dilution of the antigen.



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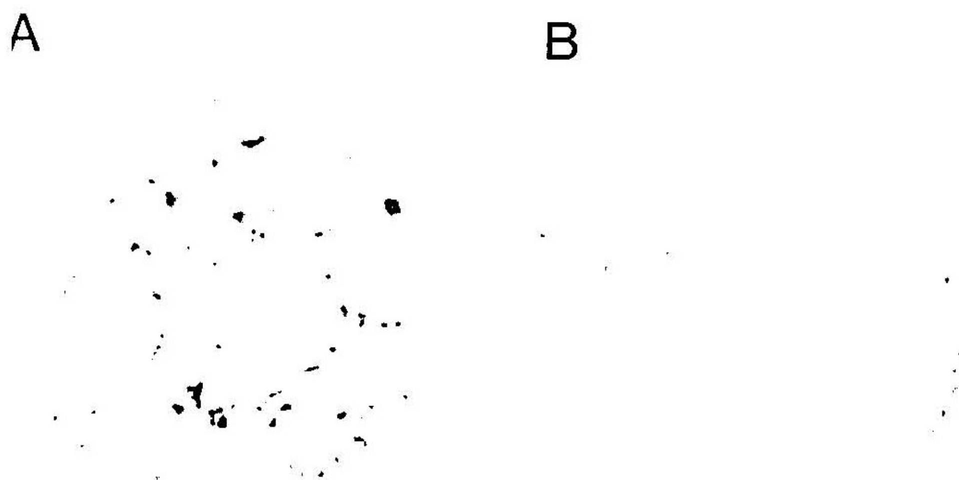

Plate 5. TBIA for the cross sections of petioles obtained from TYLCV-infected and healthy tomato plant

A: Infected plant

B: Healthy plant

4.4. PCR

4.4.1. DNA extraction from viruliferous whiteflies

Both protocols of DNA extraction from viruliferous Whiteflies proved to be efficient and workable for PCR. In case of DNA extraction from single whitefly, both methods resulted with nil or vague DNA pellets after centrifugation. Although the second protocol was short and easy, both of them were applicable for single whitefly testing by PCR.

4.4.2. DNA extraction from TYLCV-infected plant tissue

In the experiments, TYLCV DNA template was extracted according to different methods of DNA extraction. In regard to infected plant tissue, the protocols 1 gave turbid and brownish pellets of DNA after ethanol washing. Adding a further step to precipitate the digested proteinacous materials using TE-saturated phenol (protocol 2) gave clear and small whitish DNA pellets after centrifugation at 10,000rpm.

TYLCV immunocapturing with either polyclonal or monoclonal specific antibodies before protein digestion gave vague pellets with minimum impurities,

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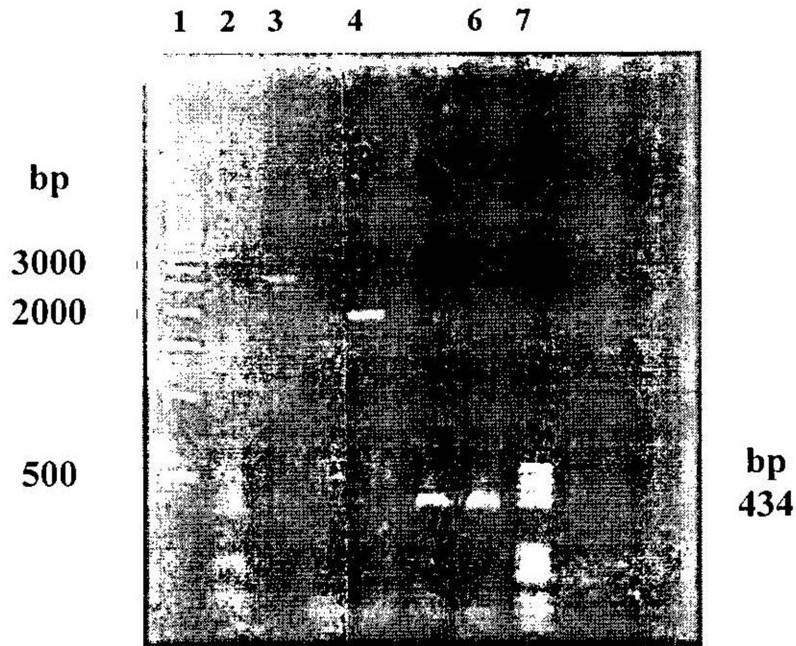

Plate 7. Agarose gel electrophoreses of PCR amplified TYLCV DNA from tomato plant using different primers.

Lane 1: 500 base DNA size marker.
Lane 2: Healthy plant
Lane 3: P1V/P2C
Lane 4: P1V/P4C
Lane 5: P1V/P5C
Lane 6: P1V/P5C
Lane 7: PBR DNA size marker

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4.4.5. Threshold of TYLCV detection in plant sap by PCR

The results showed that PCR using P1V and P4C primers could pick up the TYLCV genome from tomato crude sap exposed to 10-fold dilution up to 10^{-6}. Despite, no PCR was noticed beyond 10^{-6} dilution, the PCR signals remained strong even at the cut-of point dilution. No signal was obtained with healthy tissue (Plate 8).

Incorporation of TYLCV polyclonal antibodies produced against the local isolate increased the sensitivity of PCR one thousand times compared with the normal PCR. The cut-of point for the IC-PCR was 10^{-9} (Plate 9).

4.4.6. PCR detection of TYLCV genome from different plant parts

PCR signals obtained with primers P1V and P4C were very strong for all plant organs tested. No visual discriminations were noticed in the PCR product of the tested organs. All organs dissected from healthy tomato plant showed no PCR amplification signals (Plate 10).

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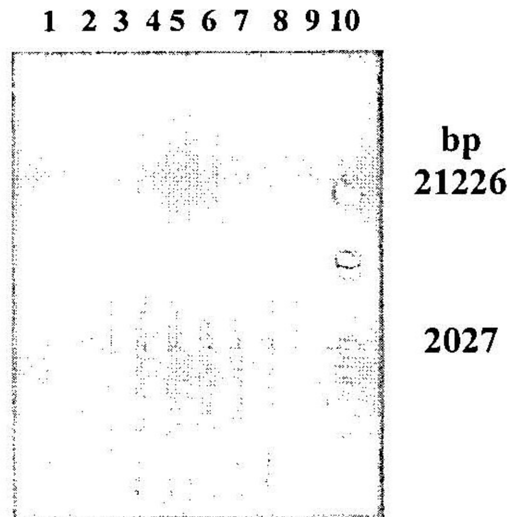

Plate 9. Agarose gel electrophoreses of IC-PCR (using polyclonal antibodies produced against the local isolate of TYLCV) amplified TYLCV DNA from different dilutions of crude sap extracted from TYLCV-infected tomato. Primers are P1V and P4C.

Lane 1: PCR product from 10^{-9} sap dilution
Lane 2: PCR product from 10^{-8} sap dilution
Lane 3: PCR product from 10^{-7} sap dilution
Lane 4: PCR product from 10^{-6} sap dilution
Lane 5: PCR product from 10^{-5} sap dilution
Lane 6: PCR product from 10^{-4} sap dilution
Lane 7: PCR product from 10^{-3} sap dilution
Lane 8: PCR product from 10^{-2} sap dilution
Lane 9: PCR product from 10^{-1} sap dilution
Lane 10: DNA size marker (*Lambda Hind II EcoR1*, 125-21226 bp)

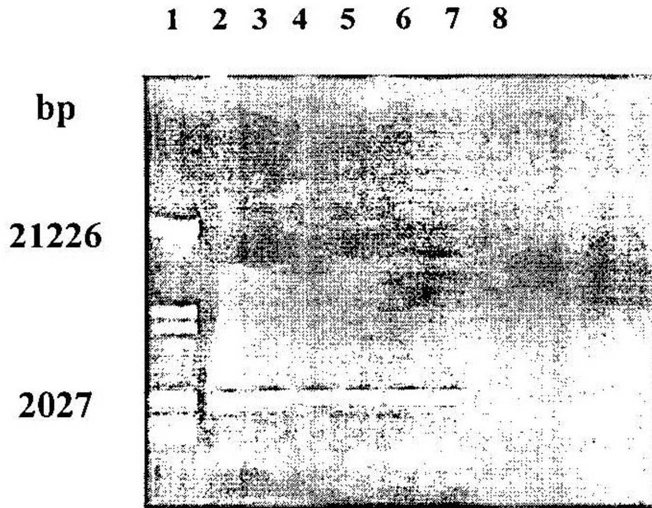

Plate 8. Agarose gel electrophoreses of PCR amplified TYLCV DNA from different dilutions of crude sap extracted from TYLCV-infected tomato. Primers are P1V and P4C.

Lane 1: DNA size marker (*Lambda Hind III EcoR1,* 125-21226 bp)
Lane 2: PCR product from 10^{-1} sap dilution
Lane 3: PCR product from 10^{-2} sap dilution
Lane 4: PCR product from 10^{-3} sap dilution
Lane 5: PCR product from 10^{-4} sap dilution
Lane 6: PCR product from 10^{-5} sap dilution
Lane 7: PCR product from 10^{-6} sap dilution
Lane 8: PCR product from 10^{-7} sap dilution

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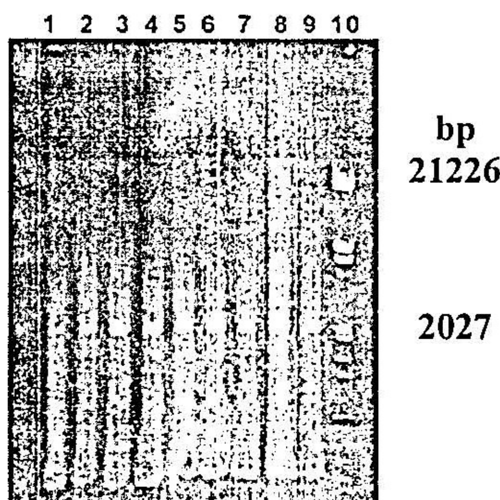

Plate 9. Agarose gel electrophoreses of IC-PCR (using polyclonal antibodies produced against the local isolate of TYLCV) amplified TYLCV DNA from different dilutions of crude sap extracted from TYLCV-infected tomato. Primers are P1V and P4C.

Lane 1: PCR product from 10^{-9} sap dilution
Lane 2: PCR product from 10^{-8} sap dilution
Lane 3: PCR product from 10^{-7} sap dilution
Lane 4: PCR product from 10^{-6} sap dilution
Lane 5: PCR product from 10^{-5} sap dilution
Lane 6: PCR product from 10^{-4} sap dilution
Lane 7: PCR product from 10^{-3} sap dilution
Lane 8: PCR product from 10^{-2} sap dilution
Lane 9: PCR product from 10^{-1} sap dilution
Lane 10: DNA size marker (*Lambda Hind II EcoR1*, 125-21226 bp)

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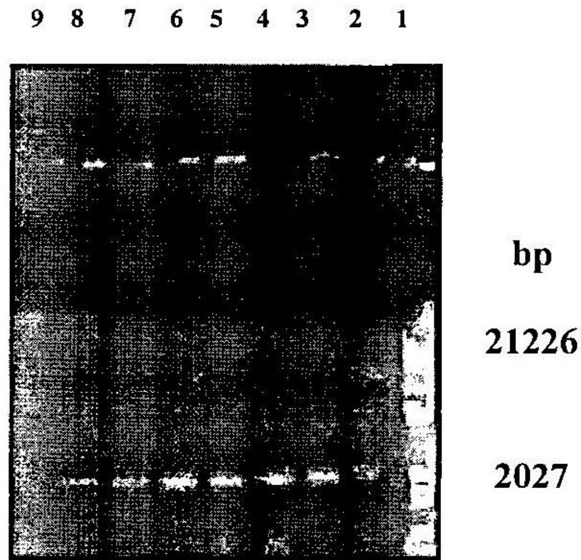

Plate 10. Agarose gel electrophoreses of PCR amplified TYLCV DNA from different plant parts of infected tomato.

Lane 1: DNA size marker (*Lambda Hind III EcoR1*, 125-21226 bp)
Lane 2: PCR product from tip leaves
Lane 3: PCR product from middle leaves
Lane 4: PCR product from old leaves
Lane 5: PCR product from upper part of stem
Lane 6: PCR product from middle part of stem
Lane 7: PCR product from lower part of stem
Lane 8: PCR product from root system
Lane 9: PCR product from healthy plant

4.4.7. Time threshold of TYLCV detection from tomato by PCR, ELISA and biology

Time threshold of TYLCV detection from the top leaves of tomato after whitefly-mediated inoculation depended on the method of detection. PCR detected the virus DNA 4 days after inoculation, where as the detection of the coat protein using ELISA delayed 1 days beyond the PCR. In addition, virus detection by back grafting of scions obtained from the upper stem of the inoculated plants into healthy tomato was achieved after 7 days of inoculation.

Furthermore, the first PCR signal of TYLCV infection was obtained from the inoculated leaves 1 days post inoculation.

For the majority of the inoculated plants, the typical symptoms of TYLCV were observed 19-22 days after inoculation. (Fig 14)

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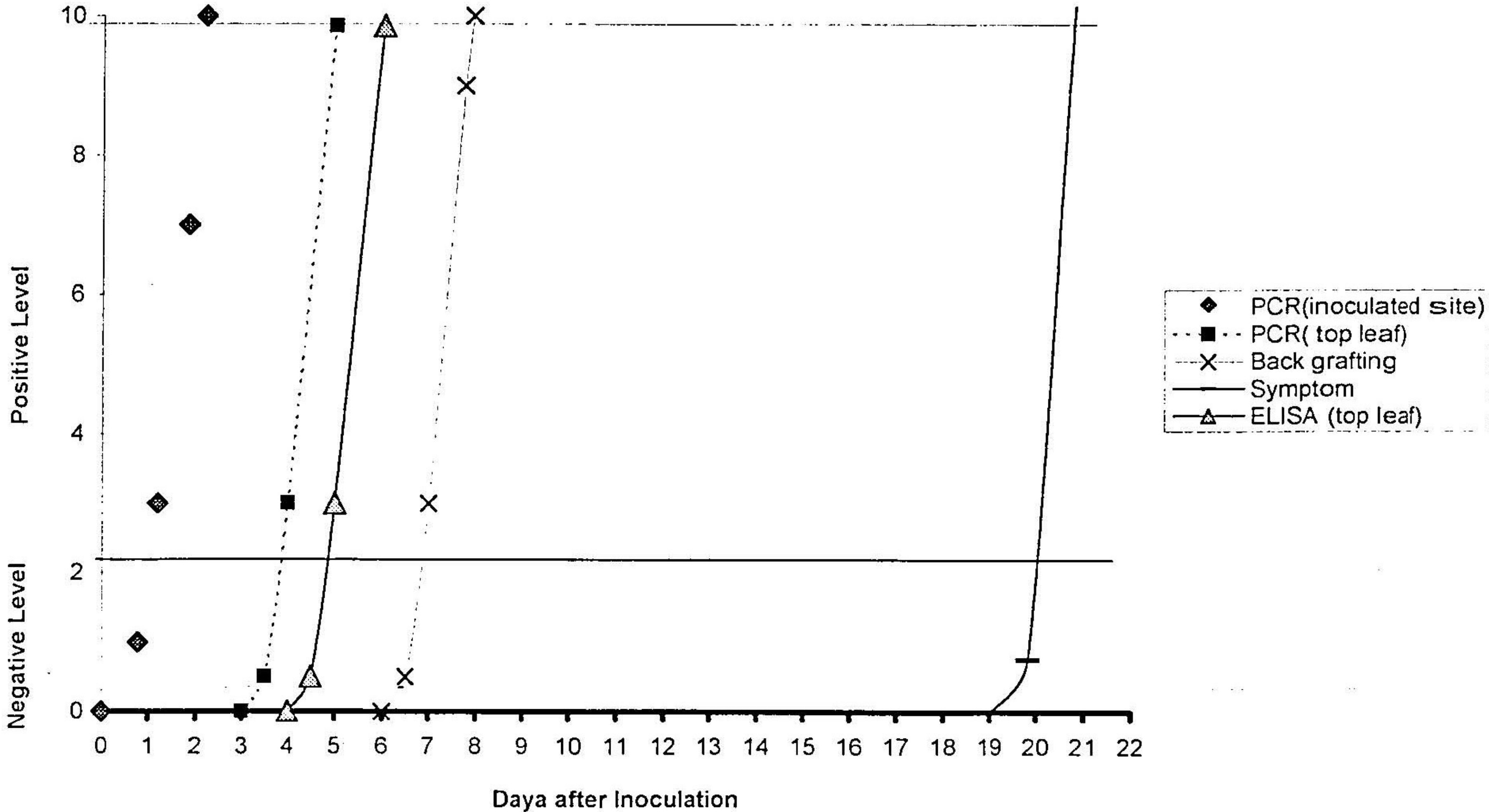
Fig 14. Time threshold of virus detection by PCR, ELISA and biology in resprct to symptom appearance on tomato plants



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4.5. Application of serology and PCR

4.5.1. TYLCV fate in the whitefly vector (molecular level)

4.5.1.1. DNA retention by the whitefly vector

After 48 hrs acquisition-access period, PCR results revealed that the ssDNA of TYLCV was persisted for 22 days in viruliferous *B. tabaci* reared on non-TYLCV host (pumpkin). The fragment size of the amplified product was typical for TYLCV (Fig 15).

4.5.1.2. Capsid protein retention by the whitefly vector

ELISA results showed that coat protein of TYLCV was detected 1 day later in the viruliferous whiteflies reared on pumpkins. In addition, the virus antigen decreased with time until the ninth to tenth days when it completely vanished and became no longer be detected by ELISA (Fig 15)

The above all results indicated that the virus DNA was retained by the insect vector at least 3 wk., which is far beyond the retention of the virus antigen.4.5.1.3. Nonspecific acquisition of TYLCV by *T. vaporariorum*

The results obtained by the PCR showed that the total DNA extract of adult *T. vaporariorum* given 48 hr access on TYLCV-infected tomato source contained the DNA template of TYLCV. In addition, results of agarose gel electrophoreses for the PCR product showed that the insect acquired TYLCV in a high rate, since all the tested insects were found carriers.

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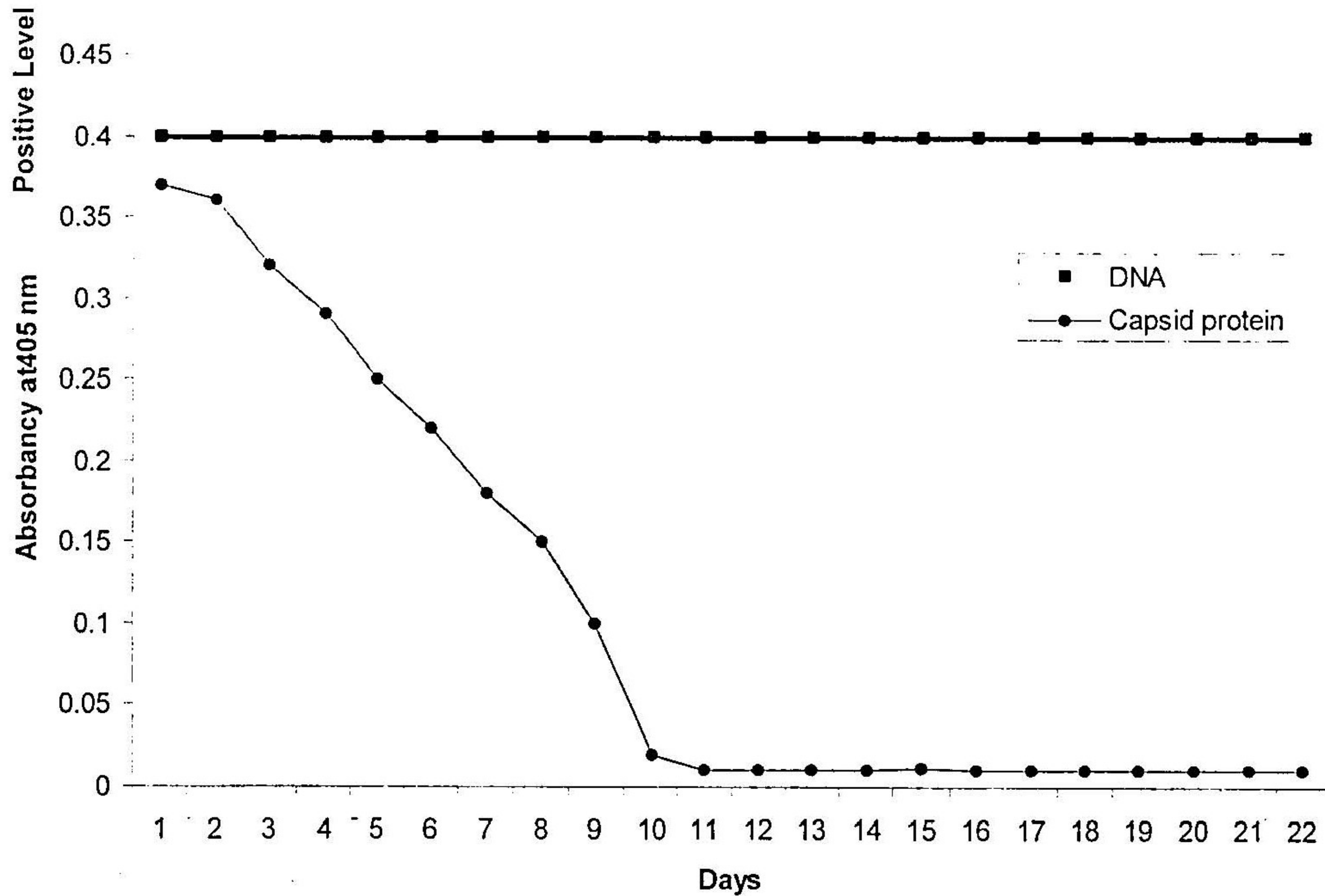
Fig 15. Fate of DNA and capsid protein of TYLCV in the viruliferous whiteflies reared on TYLCV-immune plant



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In addition, serological tests showed that the insect could also acquire the virus antigen after 48 hr acquisition access as indicated by ELISA.

4.5.2. Occurrence of TYLCV in tobacco fields

The maximum percentage of virus-infected tobacco plants were 15% in the Jordan Valley followed by 7% and 6% in Remeimeen and Al-Baqua, respectively. 2% of TYLCV infection was obtained in tobacco grown in Amman, Al-Karak and Irbid regions (Table 4). The results revealed that the percentage of TYLCV infection was at least 2 times greater in the Jordan Valley than in the Al-Baqua region (Plate 11).

4.5.3. Occurrence of TYLCV in bean fields

The serological and molecular tests showed that TYLCV infected bean fields by 7% and 3% in the Jordan Valley including Wadi Shuayb and Al-Karameih, respectively. Beans of Al-Baqua were found to be infected by 4%, whereas, beans of Al-Karak and Madaba were infected by 2%. In addition, 1% infection was found in beans grown in Amman region (Table 5). Therefore, the virus occurrence in beans of the Jordan Valley was nearly two times greater than that in Al-Baqua region, and about three times compared with those grown in Al-Karak and Madaba regions. In addition, the percentage of bean infection in the Jordan Valley was seven times greater than that occurred in Amman County (Plate 11).

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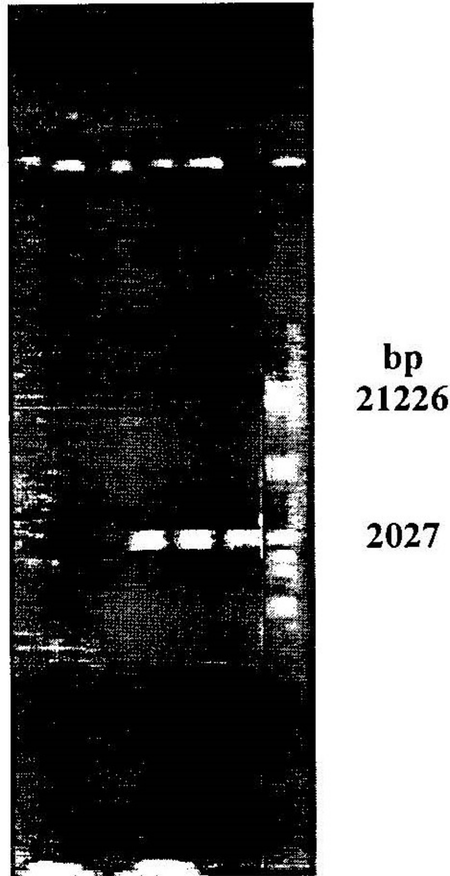

Plate 11. Agarose gel electrophoreses of PCR amplified TYLCV DNA from representative infected tobacco, bean and volunteer tomato collected from the Jordan Valley. Primers are P1V and P4C.

Lane 1: PCR product from infected volunteer tomato
Lane 1: PCR product from infected bean plant
Lane 1: PCR product from infected tobacco plant
Lane 4: DNA size marker (*Lambda Hind III EcoR1*, 125-21226 bp)

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Table 5. Occurrence of TYLCV in bean fields grown in different locations.

Region	Location	Time of collection	% of TYLCV Infection
The Jordan Valley	Wadi Shuayb	June 9, 1999	7%
The Jordan Valley	Wadi-Shoaib	June 9, 1999	5%
The Jordan Valley	Al-Karameih	June 9, 1999	3%
Al-Baqua	Al-Baqua Campus	August 22, 1999	4%
Al-Baqua	Al-Baqua Campus	June 22, 1999	2%
Madaba	Madaba	August 3, 1999	2%
Al-Karak	Rabba	August 7, 1999	2%
Amman	Western Amman	August 20, 1999	1%

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4.5.4. Occurrence of TYLCV in pepper and eggplant fields

Neither pepper nor eggplants was found to be TYLCV-infected regardless to their location. The results were obtained serologically and molecular biologically.

4.5.5. Field indexing of different weed species and volunteer tomato plants

Of several weed species collected from the tomato growing sites in both the Jordan Valley and the uplands, three weed species were found TYLCV-infected. Accordingly, 20% of *D. stramonium* L. (nightstrade) were found infected. *Malva nicaensis* All. and *S. nigrum* L. were found infected by 13 and 2%, respectively. PCR testing for the infected samples of volunteer tomato, *D. stramonium* L., *M. nicaensis* All. and *S. nigrum* L. provided distinct bands above the infected plants (Plate 11, 12). *D. stramonium* L. has a maximum TYLCV-infection ranged from 15 to 20% in the Jordan Valley (Plate 13). *M. nicaensis* All. was found to be infected by percentages ranged from 0 to 13% depending on the location. The maximum infection of such plant was recorded in the middle Jordan Valley. In addition, 2% infection was recorded for *S. nigrum* L. grew in the

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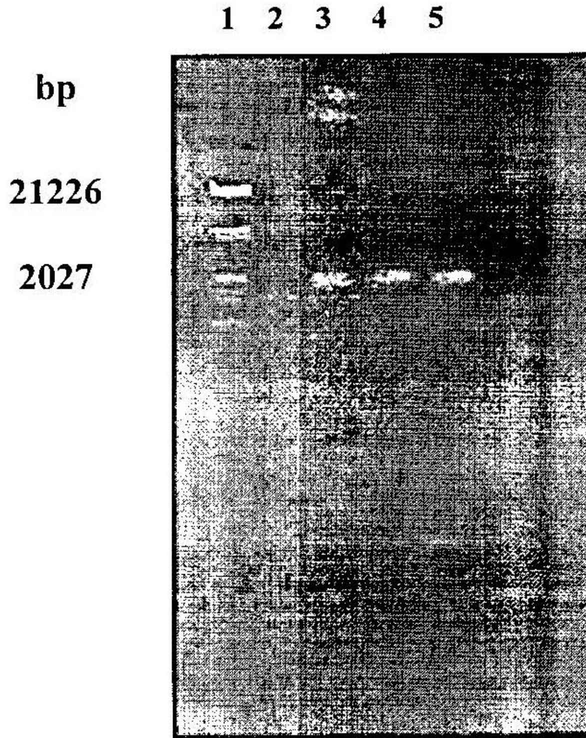

Plate 12. Agarose gel electrophoreses of PCR product of TYLCV DNA obtained by the primers P1V/P4C from infected *S. nigrum*, *M. nicaensis* and *D. stramonium.*

Lane 1: DNA size marker (*Lambda Hind III EcoR1*, 125-21226 bp)
Lane 2: PCR product from healthy *D. stramonium*
Lane 3: PCR product from *D. stramonium*
Lane 4: PCR product from *M. nicaensis*
Lane 5: PCR product from *S. nigrum*

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Plate 13. *Datura stramonium* grew naturally in the Jordan Valley near tomato fields.

Table 6. Occurrence of TYLCV in plants of *D. stramonium* L. *and S. nigrum* L. in different locations

Plant species	Location	% of TYLCV Infection
Datura stramonium L.	Northern Jordan Valley	20%
D. stramonium L.	Middle Jordan Valley	15%
Solanum nigrum L.	Northern Jordan Valley	0%
S. nigrum L.	Middle Jordan Valley	2%
S. nigrum L.	Southern Jordan Valley	0%
S. nigrum L.	Al-Mafraq	0%

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Table 7. Occurrence of TYLCV in plants of *M. nicaensis* All. grew in different locations

Region	Location	% of TYLCV Infection
The Jordan Valley	Northern Jordan Valley	8%
	Middle Jordan Valley	13%
	Southern Jordan Valley	4%
Ghor El-Safi	Ghor El-Mazraa	8%
Al-Baqua	Al-Baqua Campus	4%
Madaba	Madaba	2%
Irbid	Husson	0%
Al-Karak	Qaser	0%
	Rabba	0%
Al-Mafraq	Um El-Jemal	2%

Table 8. Occurrence of TYLCV in volunteer tomato plants grew in different locations

Region	Location	% of TYLCV Infection
The Jordan Valley	Northern Jordan Valley	90%
	Middle Jordan Valley	95%
	Southern Jordan Valley	90%
Ghor El-Safi	Ghor El-Mazraa	90%
	Ghor El-Hadetha	85%
Amman	Western Amman	83%
Al-Baqua	Al-Baqua Campus	90%
Al-Karak	Al-Qaser	50%
	Al-Rabba	57%
Irbid	Al-Husson	50%

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Based on symptomology, biological properties, serological and molecular biological tests, the yellow leaf curl agent appears similar to tomato yellow leaf curl virus (TYLCV), originally described by Cohen and Nitzany (1966) and subsequently reported in the Mediterranean and Middle Eastern countries (Makkouk, 1978; Al-Musa, 1982; Mansour and Al-Musa, 1992).

Different species of the family solanaceae became systemically infected by TYLCV following artificial inoculation by the mean of grafting. Tomato, and *Datura. Stramonium* L. was the only host species, which reacted with clear and severe symptoms.

Irrespective of their apparent reaction, the solanacous hosts supported a high virus concentration, as indicated by the high absorbency value of quantitative ELISA at 405 nm. Such results indicate and, therefore, even if naturally infected, these plants should constitute a rather good source of infection for tomato fields.

Different methods were used for purification of TYLCV to obtain virus preparations of sufficient purity for antiserum production. The modified methods that were used in this work to purify TYLCV from infected tomato plants proved satisfactory in term of rapid and ease as well as in yielding sufficient virus antigen for antibody production as

measured by the quantitative triple antibody sandwich immunoassay (TAS-ELISA) at 405 nm. Spectrophotometry and polymerase chain reaction (PCR) showed that the virus was present in fractions 8-10 out of 17 from 10-50% sucrose gradient or fractions 6 and 7 out of the 10 from the 10-35% sucrose gradient. The ultra-violet absorption spectrum of the purified virus preparation revealed with maximum absorbency at 260 nm and minimum absorbency at 240 nm. The ratio of 260/280 was calculated to be 1.4. Such value agreed with the values of 260/280 of the other geminiviruses including tobacco leaf curl (Osaki and Inouye, 1981), African cassava mosaic virus (Bock and Harrison, 1985) and mung bean yellow virus (Honda and Ikegani, 1986) with values of 1.4, 1.4 and 1.3, respectively.

Chloroform clarification (10% (v/v)) resulted in a minimum virus loss, greater coagulation of host constituents, and clear supernatants. In addition, precipitation of the virus after chloroform clarification gave clean pellets with minimum impurities.

TYLCV was concentrated from partially purified preparation by using a combination of 0.2 M NaCl and 8% Polyethylene glycol (PEG) (w/v). Such treatment revealed clear pellets with a good virus precipitate that was difficult to be performed by other precipitation treatments. Using such method of virus precipitation has a lot of

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advantages including the simplicity and availability of this method was the major factor of choosing it. Moreover, it resulted in good virus yield at relatively lower speed centrifugation. This became clear after the serological testing of the virus precipitate using a quantitative TAS-ELISA. Hebert (1963) showed that certain plant viruses could be preferentially precipitated in a single-phase (PEG) system, although some host DNA may also be precipitated. Since that time, precipitation with PEG has become one of the commonest procedures used in virus isolation.

Other researchers used the method of PEG precipitation to concentrate other geminiviruses; Muniyappa *et. al.* 1991 used PEG (70 gm/liter) to concentrate the Indian tomato leaf curl virus from chloroform-clarified sap (1 ml/3 gm leaf). Similarly, Cohen *et. al.* 1983 precipitated the squash leaf curl virus (SLCV) from partially purified preparation by adding PEG (12 gm/100 ml) and 0.2 M NaCl to the aqueous phase.

The correct diagnosis of any viral disease is a prerequisite of control. The more rapidly and accurately the causal organism is identified, the sooner the proper controls can be instituted.

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Apathy toward research on ecology and control of viral disease is due to large part of difficulties in identifying the viruses. The use of symptomology and other biological tests to detect and identify TYLCV with large number of samples was not entirely practical, in term of numbers of plants and space needed and this leads to confusion particularly in mixed infections. Also, the tests were often lacking the accuracy, difficult to interpret, and required weeks to months to complete. Therefore, the use of other methods of virus detection including serology and molecular biology was essentially needed.

The use of serological techniques for identification and detection of TYLCV are almost precise and peculiar tools that is indispensable for epidemiological and pathological studies of the virus. Moreover, such techniques provided opportunity for quick and wide disease monitoring and thereby these techniques may be beneficial for cooperative and synchronized disease management programs

In addition, the tests were highly versatile, detecting TYLCV in both purified preparations and untreated extracts of herbaceous and infected crop plants.

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The ELISA procedure is particularly suited to the large scale testing of field samples, such as might be required, for example, in surveys of the disease or for epidemiological investigation. Its sensitivity for the direct detection of the virus in plant extracts should facilitate field studies of TYLCV for which adequate and practical methods of detection have not been available. Also, the ELISA was used to compare virus concentrations in different plants and plant parts and to assay TYLCV content obtained by different steps of virus extraction and purification. In addition, the test was practical for TYLCV study, since, it could elicit such information so easily, particularly for large numbers of samples or for very small amounts of tissue

Various alternative ELISA formats have been used to improve the limit of detection. Incorporation of monoclonal antibodies has proved improvement, for example, by decreasing non-specific background reactions and providing more accurate discrimination. The tests showed that the monoclonal antibodies specific for TYLCV could be used in TAS-ELISA to detect the virus in crude and partially purified sap extracts. The test was specific, and sensitive to discriminate between the infected and healthy samples up to the concentration of 1/1000 and 1/64 of antibodies and antigen, respectively. One-hour

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incubation was enough for the test to show the visual differentiation between the infected and virus-free samples.

Moreover, TAS-ELISA was sensitive to detect the virus in hosts other than tomato including the symptomatic and symptomless crops, weeds and natural flora. ELISA values of virus-free plant extracts were substantially lower than virus-containing plants and this validated the test.

Tissue blot immunoassay (TBIA) allowed sensitive detection without the need of any sample preparation that was used for other serological techniques. The tissue-blot proved to be good that is sensitive, simple, reliable and quick method to detect the virus in infected plants. When monoclonal antibodies were used in TBIA, the test was highly sensitive to detect the virus up to the IgG dilution of 1/2000. This procedure eliminates the time-consuming extraction of the viral antigen needed in ELISA and other serological techniques, and therefore, offers the possibility of assaying a large number of samples in a short period of time and with lower costs (Abamburu *et. al.* 1996). The test might be helpful in epidemiology to forecast TYLCV-induced disease as has been done with other persistently transmitted viruses as, for example, barely yellow dwarf virus (Foster *et. al.* 1993).

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PCR was characterized by its: selectivity, sensitivity, and speedy for TYLCV detection (Arnheim, 1992). The test provided a method by which TYLCV was detected in plants and in vector insect, especially, identification and characterization of such virus has been hampered by restricted host range, lack of mechanical transmission, difficulties with nucleoprotein particle purification. The availability of means for its detection facilitates studies of their ecology and epidemiology. A PCR-based method was chosen because it should provide sufficient sensitivity to detect TYLCV in individual whiteflies, and this indeed proved to be so. Such property of the PCR facilities studies of the virus ecology and epidemiology and provides a good monitoring for virus dispersal and incidence and determines the sites that should be the target for chemical sprays.

PCR-based detection method eliminates the need for time consuming detection method such as squash-blot and dot-blot hybridization that is used for plant viruses. Because PCR amplifies nucleic acids, the technique could be useful in overcoming many of the current difficulties such as low titer of antigen, limited availability of antibodies against TYLCV, and the cross-reaction of antibodies with

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heterologous antigen associated with serological detection methods (Mehta, *et.al*, 1994)

PCR-based detection procedure greatly facilitate both epidemiological and diseases management studies of TYLCV (Mehta *et.al.* 1994). The test was applicable to detect TYLCV from infected tomatoes, bean, tobacco and weed species grown near tomato producing sites, which provides good information about the virus source that should be considered before conducting any management program.

PCR and ELISA can detect the virus in inoculated tomato plant during early stage of infection since, the former can detect the virus from inoculated site 1 day after infection. In addition biological detection by back grafting from upper stem of inoculated plants into healthy ones can detect the virus 7 days post infection. Similarly, Rom *et. al.* (1993) reported that the TYLCV DNA could be detected by dot blot hybridization 1 or 2 weeks before the first symptom appearance in susceptible tomato plants. Such feature of PCR provides advantages for indexing and screening of tomato seedlings in the nurseries. In addition, it gives a helpful way for early forecasting of TYLCV in the field. Breeding for TYLCV resistance is often restricted by a lack of

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reliable and efficient screening procedures. Resistant and tolerant individuals may be identified by symptomology, but sometimes, the visual symptoms are not precise and reproducible. In such case, PCR procedure may be used for accurate detection of TYLCV within the plant.

Studying the virus fate in the whitefly vector showed that the retention of the virus nucleic acid was much longer than the retention of the virus capsid protein. Mansour and Al-Musa (1992) reported that the whitefly vector stayed infective for TYLCV until eleven days after 24 hr acquisition access. Therefore, the results indicated a good correlation between the detection of the virus antigen in the whitefly vectors and their ability to transmit the virus. Similar results were obtained by other researchers on other geminiviruses. Polston *et al* (1990) reported that the DNA of squash leaf curl geminivirus (SLCV) was retained in the whitefly vector for at least 120 hr after 48 hr acquisition access. In addition the virus antigen was retained for about six days and its concentration after six days was one-third of its initial level (Cohen *et. al* 1989)

The difficulty or impossibility to detect the virus antigen after ten days may be due to its interaction with the membrane of the accessory salivary glands of the insect or at least part of the antigen is available for

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antibodies binding since the other part is blocked by interaction (Cohen *et.al* 1989)

The results showed that *T. vaporariorum* could acquire TYLCV within 48 hr acquisition access. Similarly, Cohen *et. al.* (1989) found that the *T. abutilonea* can acquire the antigen of SLCV nonspecifically in a higher rate compared with its vector *B. tabaci* also, Polston *et. al* (1990) reported similar virus acquisition by *T. vaporariorum*

The PCR technique cannot be used alone in studying the epidemiology of TYLCV by determining the rate of inoculative vectors in the whitefly population, because inoculativity stayed shorter days than the persistence of the DNA in the vector. In addition, *T. vaporariorum*, which is not TYLCV vector, can acquire the virus nonspecifically and therefore hampered the epidemiological studies which depend on monitoring the virus in the whitefly vector. However, *T. vaporariorum*, which is common in the area of TYLCV, can be used by epidemiologists as an indictor for the presence of TYLCV in the region.

The virus was readily recovered from infected, oversummered tomato plants, even from those that had been partially destroyed by nature and from plants in old, abandoned crops with very little green

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crops can serve as a major means of virus and an efficient source of primary infection in the following season.

Field surveys showed that *D. stramonium* L., *Malva nicaensis* All. and *Solanum nigrum* L. growing during the summer months in irrigated fields or along ditches and irrigation canals of the Jordan Valley were infected with TYLCV. *M. nicaensis* All. and *S. nigrum* L. were found in different locations of the Jordan Valley and the uplands, while *D. stramonium* L. was found in certain sites in the northern and middle Jordan Valley. Therefore, wherever they grow, *D. stramonium* L., *M. nicaensis* All. and *S. nigrum* L. can serve as natural reservoirs of TYLCV, playing a supplementary role in the oversummering of the virus. Such finding agreed with the results of other researchers: Wilson *et. al.*(1981) reported that *S. nigrum* L. is a good symptomatic host for TYLCV in Iraq, and so, its role as a potential source of inoculum for infection on tomato was suggested. Makkouk and Laterrot, (1983) reported that both *D. stramonium* L. and *M. nicaensis* All. are potentially important natural reservoirs of the virus in Mediterranean region. Similarly, Al-Musa (1986) reported that these plants might act as primary sources of TYLCV in Jordan. In addition, Makkouk, (1978) reported that the jimsonweed is a good symptomatic host for the Jordanian isolate of TYLCV under lab conditions.

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A similar role can be ascribed to tobacco, which is grown commercially in some areas of the Kingdom including Al-Baqua, Irbid, western Amman and Al-Karak counties. Although no systemic survey of such crop was conducted, the virus was recovered from completely symptomless *Nicotiana tabacum* L. and so, the ability of this crop to harbor the virus under field conditions was possible, especially, when they grown in the same regions together with diseased tomato. In regard to *in vitro* experiments, the susceptible cultivars, although completely symptomless (Makkouk, 1978, Mansour and Al-Musa, 1992), they might be efficient sources of infection for tomato plants (Al-Musa, 1986). Ioannou and Hadjinicolis (1991) recovered TYLCV from the complete symptomless *N. tabacum* L. planted near tomato fields in Cyprus, and thereby, the crop was recorded as a secondary host plant that could potentially serves as a natural reservoir for TYLCV.

Similarly, the virus was recovered from tobacco nursery in the Jordan Valley. This situation may facilitate the virus transmission through infected seedlings to the upland where they grown. The infected seedlings may act as a primary source of infection for tobacco and tomato fields established nearby. Al-Musa and Takrouri (1996)

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reported that the occurrence of diseases inside the nurseries is an important factor in disease epidemiology and effect, since they can spread to the field and participate in crop failure.

TYLCV was detected serologically and molecular biologically in different bean fields established near tomato growing areas in the Jordan Valley, Al-Baqua and Madaba regions. Although no apparent symptoms were noticed on these plants, they are ascribed to be a cryptic virus source for tomato fields. Cohen and Nitzany (1966) reported that the *Phaseolus vulgaris* L. 'Bulgarit' was a host for TYLCV under lab condition. In addition, Navot *et. al.* (1992) detected TYLCV in whiteflies collected from bean fields and so, emphasized the role of bean cultivars to harbor the virus under field condition in Israel.

The chronological pattern of tomato planting in the Jordan Valley from south to north may strategize the location of Wadi Shuayb in TYLCV epidemiology. In August, whiteflies that oversummer on bean planted there migrate to nearby newly established tomatoes. The infected tomato plants in the south add to the inoculum potential already present in volunteer and wild hosts at different locations in the Jordan Valley (Al-Musa, 1986).

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The high level of TYLCV-infected beans in Wadi Shuayb during the summer months suggested that these plants might act as a good source of primary inoculum of TYLCV to nearby newly established tomatoes grown from August to October.

Seasonal carry over, in particular, appears to be primarily accomplished on oversummered susceptible crops and weeds. Such plants are the main sources of primary inoculum for the infection of new tomato plantings and are of major importance for the perpetuation of TYLCV, since the virus is neither seedborne nor transovarially transmissible in its whitefly vector (Cohen & Nitzany, 1966; Ioannou, 1985).

The discovery of a large number of naturally infected secondary hosts is very important for virus control and to obtain a satisfactory level of disease management. Ioannou, (1987) reported that a significant decrease in disease incidence is to be expected if sources of infection are eliminated from within and near crops. Therefore, the discovery of natural hosts for TYLCV such as *D. stramonium* L. *M. nicaensis* All. and black nightstrade (*S. nigrum* L.), is of great importance for the control of the damage caused by TYLCV in Jordan.

Rouging or eradication should come first in disease management of TYLCV because each infected plant may act as a

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source for further spread if the whitefly vectors are around. It is part of normal crop hygiene if the virus accompanies a small proportion of planting stock, or if virus invades from outside sources.

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6. Conclusions

8. References

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Appendix 1. Buffers used in Molecular Biology

1-10X Tris-Boric EDTA (TBE):

108gm Tris base

55gm boric acid

7ug or 40ml of 0.5M EDTA, pH 8.0

1liter distilled water as a final volume

2- DNA extraction buffer (1):

100mM Tris-HCl, pH 8.0

50mM EDTA

500mM NaCl

10 mM B-mercaptoethanol

1% SDS

3- Tris-EDTA buffer (TE):

10mM Tris-HCl, pH 8.0

1 mM EDTA.

4-DNA extraction buffer (2):

0.1M Tris-base, pH 9.0

0.1M EDTA

1% SDS

5-Salt Tris EDTA buffer (STE):

0.1M NaCl

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10mM Tris.Cl, pH 8.0

1mM EDTA, pH 8.0

6-Coating buffer, pH 9.6:

1.59 gm Sodium Carbonate

2.93 gm Sodium hydrogen carbonate

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Appendix 2. Weeds collected from the northern Jordan Valley during June,1999

Region	Time of Collection	Weed Species
Northern Jordan Valley	15.June.1999	
		Amaranthus retroflexus L.
		Anthemis palestina Reut.
		Beta vulgaris L.
		Capsella bursa-pastoris (L.) Medik
		Carthamus tenius Bornm
		Chenopodium murale L.
		Cichorium pumilum Jacq
		Convolvulus arevensis L.
		Conyza bonariensis (L.) Cronquist
		Lactuca syriaca (L.)Cass
		M. sylvestris L.
		Malva nicaensis All.
		Melilotus indicus (L) All.
		Prosopis farcta Macbride
		Sinapis arvensis L.
		Solanum nigrum L.
		Sonchus oleraceus L.
		Tribulus terrestris L.
		Vicia ervilla (L.) Willd
		Withania somnifera (L.) Dun.
		Xanthium spinosum L.

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Appendix 3. Weeds collected from the middle Jordan Valley during June, 1999

Region	Time of Collection	Weed Species
Middle Jordan Valley	15.June.1999	
		A. gracillis Desf.
		A. palestina Reut.
		Ammi majus L.
		Anthemis palestina Reut. -
		Avena sreilis L.
		B. vulgaris L.
		Beta vulgaris L.
		C. album L.
		C. arevensis L.
		C. bonariensis (L.) Cronquist
		C. murale L.
		C. pumilum Jacq
		C. tenius Bornm
		Cardaria draba L.
		Carthamus tenius Bornm
		C. murale L.
		Cichorium pumilum Jacq
		Convolvulus arevensis L.
		Conyza bonariensis (L.) Cronquist
		Cynodon datylon L.
		Eruca subive MiV.
		Heliotropium europaeum L.
		Hordeum liporium Link
		L. syriaca (L.)Cass
		Lactuca syriaca (L.)Cass
		M. sylvestris L.
		M. nicaensis All.
		Medicago sativa
		Notobasis L.
		Ordeum liporium Link
		P. farcta Macbride
		Partulaca oleracea L.
		Plantago llaceolata L.
		Prosopis farcta Macbride
		Rubus tomentosus Borhk
		S. arvensis L.
		S. nigrum L.
		S. oleraceus L.
		Sinapis arvensis L.
		Sisymbrium Irio L.
		Solanum nigrum L.
		Sonchus oleraceus L.
		T. terrestris L.

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Appendix 4. Weeds collected from the southern Jordan Valley during June, 1999

Region	Time of Collection	Weed Species
Southern Jordan Valley	15.June.1999	
		A. gracillis Desf.
		Alhagi maurorum Medik
		Anthemis palestina Reut.
		Avena sreilis L.
		B. vulgaris L.
		Beta vulgaris L.
		C. album L.
		C. arevensis L.
		C. murale L.
		C. murale L.
		Cardaria draba L.
		Carthamus tenius Bornm
		Cichorium pumilum Jacq
		Eruca sutiva MiV.
		Heliotropium europaeum L.
		Hordeum liporium Link
		L. syriaca (L.)Cass
		M. nicaensis All.
		M. sylvestris L.
		Notobasis L.
		Ordeum liporium Link
		P. farcta Macbride
		Polypogon monspeliensis (L.) Desf.
		Prosopis farcta Macbride
		Rubus tomentosus Borhk
		S. arvensis L.
		S. nigrum L.
		S. oleraceus L.
		Sinapis arvensis L.
		Sisymbrium Irio L.
		S. nigrum L.
		X. spinosum L.

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تنقية وإنتاج أمصال مضادة لفيروس تجعد واصفرار أوراق البندورة والكشف عنه باستخدام الطرق البيولوجية والجزيئية

إعداد

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المشرف

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المشرف المشارك

الأستاذ الدكتور محمد الخطيب

تم الحصول على عزلة فيروسية من عينات جمعت من نباتات البندورة المظهرة لأعراض فيروسية والمزروعة في غور الأردن. عرفت هذه العزلة الفيروسية على أنها فيروس تجعد واصفرار أوراق البندورة، وذلك اعتماداً على النباتات المخبرية الكاشفة وطرق النقل وكل من الفحوصات المصلية وفحص البوليميريز المتسلسل.

تم تنقية الفيروس من الأوراق القمية لنبات البندورة المصاب وذلك بعد ٣٠-٤٠ يوم من العدوى. فقد أعطت طريقة التنقية المحسنة ناتجا عاليا من الفيروس النقي، حيث كانت هذه الطريقة معتمدة على مادة الكلوروفورم كطريقة للتنقية الجزئية ومادتي كلوريد الصوديوم والبولي ايثلين

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جلايكول كوسيلة لترسيب الفيروس . وعند إجراء التنقية النهائية للفيروس باستخدام مدرج السكروز، وجد بأن الفيروس يتواجد في حزمة غير مرئية بالعين المجردة تحت الضوء العادي .

هذا وقد تبين أن فحص الإدمصاص المناعي ثلاثي الأجسام المضادة (TAS-ELISA) باستخدام المصل المضاد المنتج محليا كان فعالا في الكشف عن هذا الفيروس حتى التخفيف (٦٤١١) ، في حين وجد بأن فحص الادمصاص المناعي على غشاء النيترو سيلولوز (TBIA) كان فعالا بالكشف عن الفيروس من سيقان وسويقات أوراق النبات المصاب .

وعلى النقيض من ذلك، فقد فشلا فحصا الادمصاص المناعي ثنائي الأجسام المضادة (DAS-ELISA) والادمصاص المناعي غير المباشر المغلف بالفيروس (I-ELISA) في الكشف عن الفيروس .

كذلك ، تم مضاعفة الحامض النووي للعزلة الفيروسية بوساطة تفاعل البوليميريز المتسلسل وذلك باستخدام بادئات من الحامض النووي ذات القواعد النيتروجينية التي لها تطابق على بعض الأماكن في الحامض النووي التابع للفيروس . كذلك وجد بأن هذا التفاعل ليس قادرا فقط على كشف الفيروس من جميع أجزاء النبات المصاب بل تعدى ذلك ليكشف الفيروس من السائل الخام المخفف حتى $١٠^{-٦}$ ، إضافة إلى كذلك كان هذا الفحص فعالا في الكشف عن الفيروس المحمول بوساطة الذبابة البيضاء .

عند اجراء دراسة لمصير الفيروس داخل الناقل الحشري تبين ان الحامض النووي للفيروس يبقى محمولا لفترة تزيد عن العشرين يوما في حين يبقى الغلاف البروتيني للفيروس لمدة عشرة أيام فقط .

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كانت الفحوصات المصلية وفحص البوليمريز المتسلسل فعالة في الكشف عن الفيروس في الحقل، حيث تم الكشف بوساطة فحص البوليمريز المتسلسل على أن نبات عنب الثعلب كان مصاباً بنسبة (٢٪) و الخبيزة (١٣٪) والداتورا (٢٠٪) . كذلك تم الكشف عن اصابة الفيروس لنبات التبغ بنسبة تتراوح من (١-١٥٪) ونبات الفاصوليا من صفر إلى (٧٪) ، كذلك بينت هذه الفحوصات أن نباتات البندورة الطوعية النامية في غور الأردن كانت مصابة بنسبة (٩٥٪) .

وعلى النقيض من ذلك، تبين خلو حقول الباذنجان والفلفل المزروعة بجانب حقول البندورة من الإصابة الفيروسية.

عند إجراء مقارنة بين الفحص البوليمريز المتسلسل والفحوصات المصلية تبين أن فحص البوليمريز المتسلسل كان أكثر حساسية من الفحوصات المصلية. كذلك وجد بان تضمين المصل المضاد المنتج محليا ضد الفيروس المذكور لفحص البوليمريز المتسلسل يزيد حساسيته بمقدار الف مرة .

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