Lisianthus Leaf Curl a New Disease of Lisianthus Caused by Tomato Yellow Leaf Curl Virus

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ABSTRACT

Cohen, J., Gera, A., Ecker, R., Ben Joseph, R., Perlsman, M., Gokkes, M., Lachman, O., and Antignus, Y. 1995. Lisianthus leaf curl a new disease of lisianthus caused by tomato yellow leaf curl virus. Plant Dis. 79:416-420.

In 1992 viruslike symptoms appeared on lisianthus plants grown at different locations in Israel. The symptoms included distortion of the growing tips, cup-shaped leaves, and swelling of veins on the lower surface of the leaves. Plants infected at an early stage did not yield flowers, but late infections did not impair flowering. The viral causal agent of the disease was purified from infected plants and identified with a molecular probe prepared from cloned tomato yellow leaf curl virus (TYLCV) DNA and a specific antiserum against the virus. Purified preparations were infectious to healthy lisianthus in transmission experiments with *Bemisia tabaci*. These data confirmed that lisianthus leaf curl is caused by TYLCV. This devastating disease has become a limiting factor in the expansion of lisianthus cultivation in Israel.

Lisianthus (*Eustoma grandiflorum* (Raf.) Shim.), also known as "prairie gentian" a member of the Gentianaceae, is a relatively new cut-flower that has become very popular worldwide (11,19). The plant is grown on a large scale in the Netherlands and Japan. In Israel, lisianthus is planted during late summer or autumn and harvested during the winter. Extensive research and breeding has been carried out in Israel to create varieties adapted to the local conditions (9). During 1992 viruslike symptoms appeared on plants at different locations in Israel. The symptoms included distortion of the growing tips, cup-shaped leaves, and swelling of veins on the lower surface of the leaves. The disease is devastating in plants infected at an early stage, and late infections significantly reduce flower quality. Lisianthus is susceptible to as many as 10 viral diseases, but none is of any economic significance in Israel (10,16). The association of these symptoms with high populations of the sweetpotato whitefly (*Bemisia tabaci* Gennadius) indicated that the disease might be caused by tomato yellow leaf curl geminivirus (TYLCV). This virus, first described in Israel by Cohen and Harpaz in 1964 (5), infects plants from six botanical families, but the major damage is to tomato (3). The virus was cloned, sequenced, and assigned to the geminivirus group (2,7). TYLCV-like viruses cause devastating diseases in tomato crops around the Mediterranean basin, Africa, the Far East, and Australia (1,3,6,8,14,17). However, there are no records of any of these viruses infecting any member of the Gentianaceae. Our work was initiated to characterize the causal agent of this new disease of lisianthus.

Accepted for publication 5 December 1994.

MATERIALS AND METHODS

Virus sources. Naturally infected plants of lisianthus cv. Royal Purple (Takii, Kyoto, Japan) were collected in commercial greenhouses and served as source plants in transmission experiments and for virus purification. The originally described culture of TYLCV (3,5) served also for direct inoculations under greenhouse conditions.

Host range. The following test plants were used for mechanical and whitefly inoculation: *Gomphrena globosa* L., *Nictoiana glutinosa* L., *N. benthamiana* Domin., *N. tabacum* L. cvs. Samsun and Xanthi-nc, *N. sylvestris* Speg. & Comes, *Datura stramonium* L., *N. clevelandii* Gray, *Chenopodium amaranticolor* Coste & Reynier, *C. quinoa* Willd., *Cucumis sativus* L. cv. Bet Alpha, *Lycopersicon esculentum* Mill. cvs. Marmand and CT-751, *Physalis floridensis* Rybd., *Capsicum annuum* L., and *E. grandiflorum*. Plants were grown from seed in an insect-proof greenhouse and sprayed periodically with insecticides.

Mechanical inoculation. Plants were dusted with Carborundum before being rubbed with crude plant extracts in 1% K_2HPO_4.

Insect transmission. Colonies of *B. tabaci* were reared on cotton plants (*Gossypium hirsutum* L.) in muslin-covered cages kept in an insectary greenhouse. Transmission of TYLCV used adult females of *B. tabaci*. Insects were introduced to source plants for a 48-h acquisition access period followed by a 48-h inoculation feeding period on test plants (5). Whiteflies were allowed an overnight membrane feeding on partially purified virus before transfer to test plants for a 48-h inoculation access period (3).

Virus purification. Partial purification of the virus was carried out as described by Czosnek et al (7). The extraction buffer contained 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.

Electron microscopy. Dip samples of virus preparations were stained with 1% uranyl acetate and screened for virus in a JEOL 100CX II electron microscope (Tokyo) (7).

Extraction of plant total DNA. DNA was extracted from lisianthus, *D. stramonium*, and tomato plants as described by Rom et al (20).

DNA analysis. DNA was electrophoresed in 1% agarose gels and transferred to nylon membranes (Zeta probe, Bio-Rad Laboratories, Richmond, CA) by alkaline transfer (18). Preparation of sap extracts for dot spot and Southern hybridizations was carried out as described by Rom et al (20).

Polymerase chain reaction (PCR). Primers for amplification of TYLCV DNA from different plant sources were designed on the basis of a published sequence of TYLCV (2) and synthesized by Biotechnology General, Nes-Ziona, Israel. Three primer pairs were designed to amplify the full-length viral DNA, the virus sense, and the complementary sense coding regions, respectively:

- 1.5′ AAGGTTCGCCGAAGGCTG 3′ (PTYv522 [vNo—nucleotide number on the viral sense strand]) and 5′ GACGGGCGTGGAAATGAT 3′

(PTYc2273 [cNo—nucleotide number on the complementary sense strand]).
- 2.5′ GTCACTGTCAACCAATC 3′ (PTYv225) and 5′ GAGTATTTAGATATGAAG 3′ (PTYc1401).
- 3.5′ ACGCATCTATTTCTATG 3′ (PTYv1220) and 5′ CGAAATCCGTGAACAGA 3′ (PTYc2425).

The PCR reaction was carried out in 100 μl of reaction buffer. The reaction buffer was supplied by Appligene, Illkirch, France, and consisted of: Tris-HCl 10 mM, pH 9; 50 mM KCl; 1.5 mM $MgCl_2$; 0.1% Triton X-100; and bovine serum albumin at 0.2 mg/ml. The reaction mix contained 0.2 mM dNTPs (final concentration), 1 μM primer, 0.5 unit of *Taq* polymerase (Appligene), and 1 μl of sample DNA. Presumed viral DNA was amplified by 36 cycles of melting, annealing, and DNA extension conditions: 1 min at 95 C, 1 min at 48 C, and 5 min at 71 C. A final cycle with an extension step of 5 min ended the run before holding at 4 C.

Preparation of radiolabeled probe. A clone of TYLCV served for the preparation of a ^{32}P-labeled riboprobe (2,20).

Western blots. Samples from sucrose gradient fractions of the partially purified virus were separated on 10% sodium dodecyl sulfate-polyacrylamide gels (15). Protein bands were transferred to reinforced nitrocellulose (Schleicher and Schuell, Inc., Keene, NH) and immunostained with a specific antiserum against TYLCV (2,12).

RESULTS

Symptomatology and epidemiology. The most prominent symptoms in lisianthus were found during the early stem elongation period, but symptoms could be observed at all growth stages. Symptoms included stunting, distortion of the growing apex (Fig. 1A), cup-shaped leaves, and swelling of veins on the lower surface of the leaves (Fig. 1B). In some cases, yellowing of leaves also was observed. In early infections, plants remained stunted, did not flower, or produced a short flower that sometimes exhibited a color break. Flowers of this type are of no commercial value. Plants with symptoms were found in all lisianthus-growing areas in Israel. High disease incidence (50–100%) was always associated with large populations of the sweetpotato whitefly. Therefore, a higher disease incidence was observed in early plantings (August and September) compared with plantings during the late autumn (October) (data not shown). In many of the early planted greenhouses surveyed during March, disease incidence reached approximately 100% (data not shown).

Virus transmission. All efforts to transmit the disease by mechanical inoculation failed. To test the ability of the virus to be transmitted by whiteflies, nonviruliferous whiteflies were introduced into naturally infected lisianthus source plants collected at a commercial greenhouse. Insects were transferred later for inoculation access to healthy test plants of *L. esculentum* cvs. Marmand and CT-751, *D. stramonium*, *C. annuum*, and lisianthus. The casual agent was transmitted from naturally infected lisianthus to both susceptible and tolerant tomato and lisianthus plants (Table 1). The reaction of both tomato cultivars to

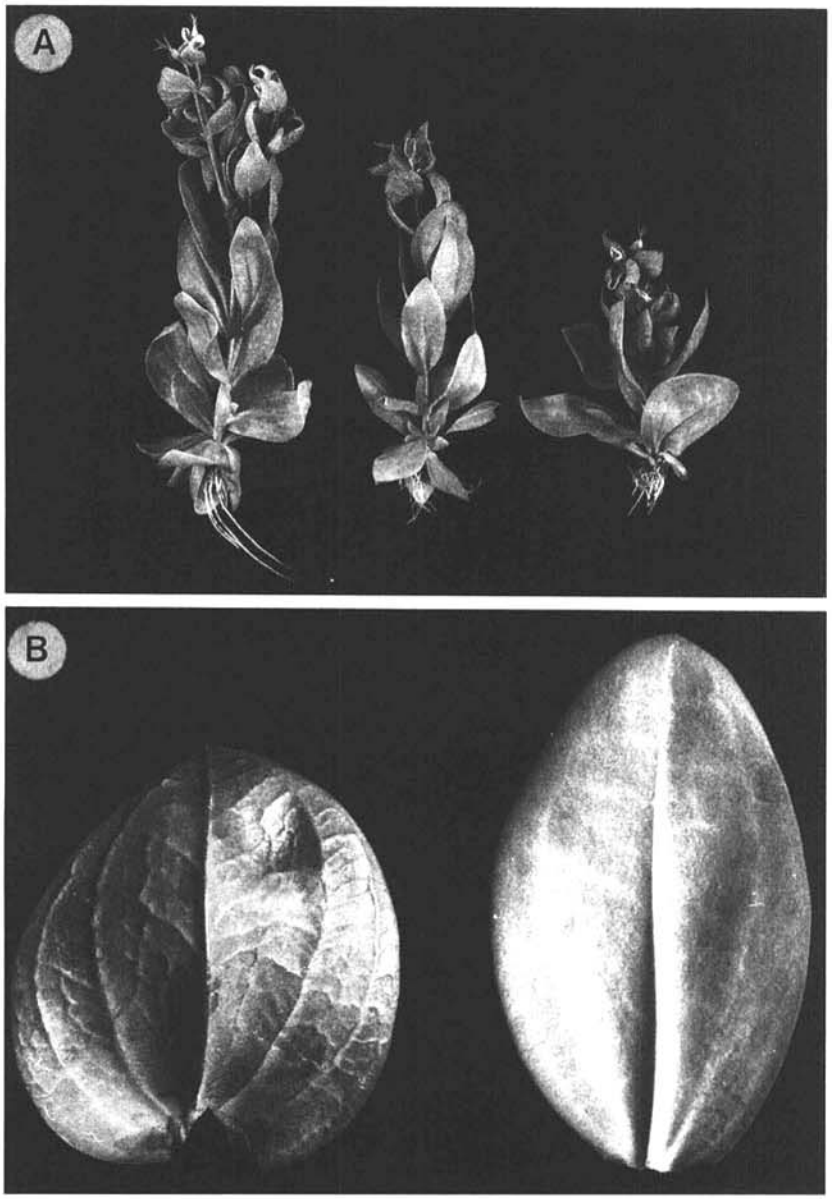

Fig. 1. Symptoms of tomato yellow leaf curl disease in lisianthus. **(A)** Plant stunting and leaf distortion in naturally infected plants; **(B, left)** a cup-shaped leaf with typical vein swelling from an infected lisianthus plant; and **(B, right)** normal leaf from healthy lisianthus.

Table 1. Transmission of lisianthus leaf curl causal agent by *Bemisia tabaci*[a]

Source plant	Test plant	Transmission rates
Infected lisianthus[b]	*Lycopersicon esculentum* cv. Marmand (susceptible)	5/5[c]
Infected lisianthus[b]	*L. lycopersicon* cv. TY-20 (tolerant)	5/5
Infected lisianthus[b]	*Datura stramonium*	5/5
Infected lisianthus[b]	*Capsicum annum* cv. Maor	0/5
Infected lisianthus[b]	Lisianthus	5/10
TYLCV[d]-infected tomato	Lisianthus	5/5

[a]One representative experiment out of three. Each plant was inoculated by 20 sweetpotato whiteflies.
[b]Naturally infected.
[c]Number of infected plants/total number of inoculated plants.
[d]Originally described strain of tomato yellow leaf curl virus (TYLCV) (5).

infection was typical of the reaction of these varieties to TYLCV. Moreover, inoculation of lisianthus with the originally described culture of TYLCV (3) resulted in symptoms identical to those that appear in field-infected plants.

Virus purification. Lisianthus plants showing symptoms of the leaf curl disease were collected in commercial greenhouses located in the central part of Israel and used for virus purification. Electron microscopy analysis of sucrose gradient fractions indicated the presence of gemini-like virus particles (Figs. 2 and 3). To test the infectivity of the partially purified preparation, sucrose gradient fractions were fed to *B. tabaci* for 24-h through Parafilm membranes followed by a 48-h inoculation access period on *D. stramonium* or lisianthus. Infectivity was correlated with the presence of gemini particles (Fig. 3B). Inoculated *D. stramonium* test plants showed vein yellowing and leaf curl identical to symptoms reported for TYLCV-infected *D. stramonium* (3). Samples from the same fractions were spotted on a nylon membrane and hybridized with a specific TYLCV molecular probe. The presence of TYLCV nucleic acid coincided with infectivity and the presence of gemini particles (Fig. 3C). When the same fractions were probed with a specific antiserum against TYLCV in Western blots, overlap was found between the presence of high amounts of viral coat protein and infectivity (Fig. 3D).

Identification of the viral nucleic acid. To obtain additional evidence of the identity of the geminivirus isolated from lisianthus, total DNA was extracted from naturally infected lisianthus plants, lisianthus plants infected with the originally described TYLCV, and healthy lisianthus plants. TYLCV-infected tomato plants served as a positive control. Southern blot and dot spot hybridizations of the extracted DNA were carried out with a TYLCV-specific molecular probe. TYLCV ssDNA was detected in all the tested samples, except in the healthy control (Fig. 4).

Further confirmation of the identity of the viral nucleic acid was obtained by PCR amplification and restriction fragment length polymorphism (RFLP) analysis. The viral DNA was amplified from DNA preparations extracted from *D. stramonium* infected with the originally described strain of TYLCV (3) as well as from field-infected tomato and lisianthus plants showing TYLCV symptoms. Amplified virus fragments of the same size were obtained in all the compared preparations (Fig. 5A–C). RFLP analysis of one of the fragments (Fig.

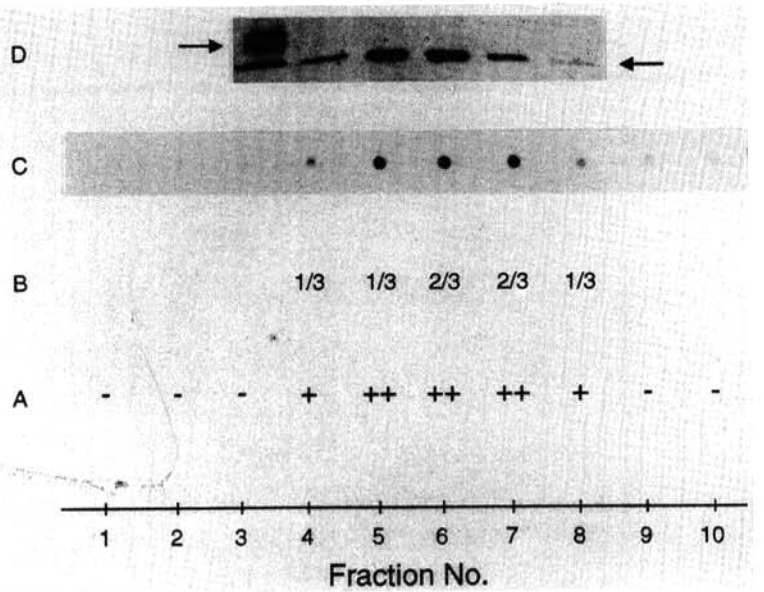

Fig. 3. Identification of the viral agent causing lisianthus leaf curl disease. **(A)** Sucrose fractions of partially purified preparations obtained from naturally infected lisianthus were examined by an electron microscope for the presence of virus particles (++ indicates a high number of particles; − indicates an absence of virions). Fractions were tested for infectivity by feeding to *Bemisia tabaci* using the membrane feeding procedure. **(B)** Healthy lisianthus served as test plants (number of infected plants/total number of inoculated plants). The presence of tomato yellow leaf curl virus (TYLCV) in the infectious fractions was confirmed using a TYLCV-specific molecular probe in **(C)** dot spot hybridization tests and **(D)** Western blots analysis with a TYLCV-specific antiserum. Right arrow marks the viral coat protein band; left arrow marks a 32,500-Da prestained protein marker. The lower band in this lane is a degradation product of the marker.

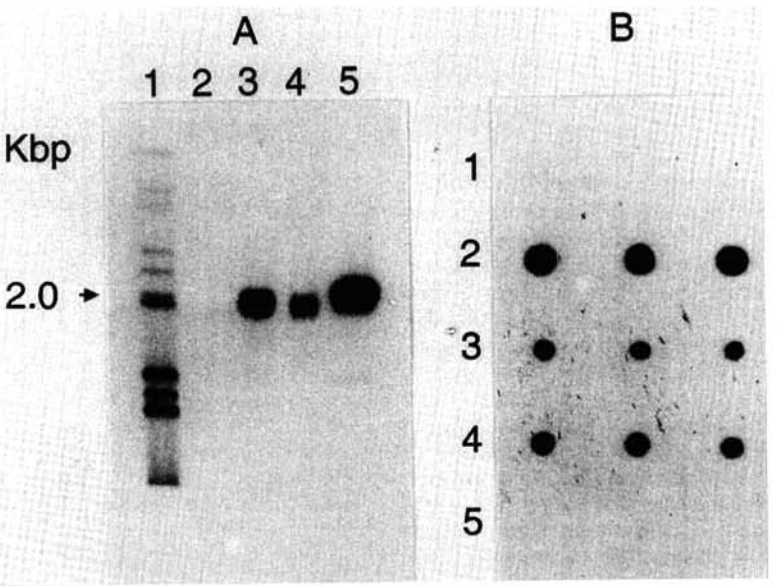

Fig. 4. Detection and identification of the genomic DNA of tomato yellow leaf curl virus (TYLCV) in infected tomato and lisianthus plants. Total DNA was extracted from the tested plants, separated on an agarose gel, or spotted on a nylon membrane. **(A)** Southern blots of the gels and **(B)** dot spots were probed with a radiolabeled molecular probe specific for TYLCV. The following DNA samples were taken for analysis: **(A, lane 1)**, lambda DNA size marker; **(A, lane 2; B, lane 5)**, healthy lisianthus; **(B, lane 1)**, healthy tomato; **(A, lane 3; B, lane 4)**, lisianthus inoculated in the greenhouse with the originally described culture of TYLCV; **(A, lane 5; B, lane 2)**, tomato inoculated with the originally described culture of TYLCV; and **(A, lane 4; B, lane 3)**, naturally infected lisianthus.

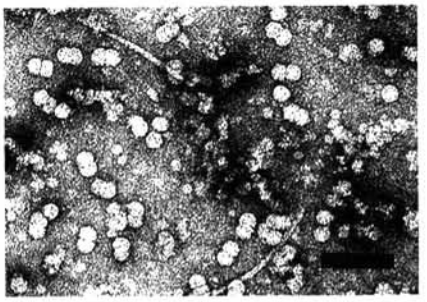

Fig. 2. Electron micrograph of gemini particles in a dip preparation from partially purified virus obtained from naturally infected lisianthus. Staining was done in 1% uranyl acetate. Bar = 100 nm.

5B) with *Bbv*I, *Sfa*NI, *Xba*I, *Eco*RI, and *Nde*II yielded identical RFLP patterns in all the compared samples (Fig. 5D).

Accumulation of TYLCV DNA in infected tomato and lisianthus plants. Tomato plants at the two-leaf stage and lisianthus plants at the stage before stem elongation were inoculated with viruliferous whiteflies in muslin-covered cages under greenhouse conditions.

Following an inoculation access period of 48 h, plants were sprayed and kept in an insect-proof greenhouse. To follow viral DNA accumulation, plants were divided into five groups, each consisting of five tomato and five lisianthus plants. The groups were sampled successively at 1-wk intervals, beginning at 7-days postinoculation. Plants of each group were sampled by removal of a leaf disk from the growing tip. All sampled plants were kept for symptom appearance to confirm a successful infection. Viral DNA was detectable in tomato plants as early as 14-days postinoculation, whereas in lisianthus significant amounts of TYLCV DNA were found only in the plant group sampled 5-wk postinoculation (Fig. 6). In the latter plants, the appearance of detectable amounts of viral DNA coincided with the time of symptom appearance.

DISCUSSION

Lisianthus has been grown in Israel for the last 10 yr; however, severe epidemics of the leaf curl disease were recognized only during the last 2 yr. Lisianthus leaf curl disease has become a limiting factor for lisianthus production in Israel. Incidence was close to 100% in greenhouses located at different geographic parts of the country. No commercial varieties grown in Israel were resistant to the disease. Electron microscope analysis of partially purified preparations, use of specific molecular probe and antibodies, and studies of host range and interaction with the whitefly vector indicate that lisianthus leaf curl disease is caused by TYLCV. This is the first report on the ability of TYLCV to infect a member of the Gentianaceae. To eliminate the possibility that a new strain of the virus is responsible for the spread of this disease, the first originally described culture of TYLCV (3) was used for inoculation of lisianthus plants. This virus induced symptoms identical to those expressed by naturally infected plants. Moreover PCR analysis of viral DNA from lisianthus and tomato naturally infected with field isolates of TYLCV has shown identity in amplification products and RFLP patterns with the viral DNA of the originally described strain of TYLCV(3) (Fig. 5).

Symptoms were visible in lisianthus 5-wk postinoculation compared with an incubation period of 2 wk in tomato plants (20). This significant delay is associated with a delay in the appearance

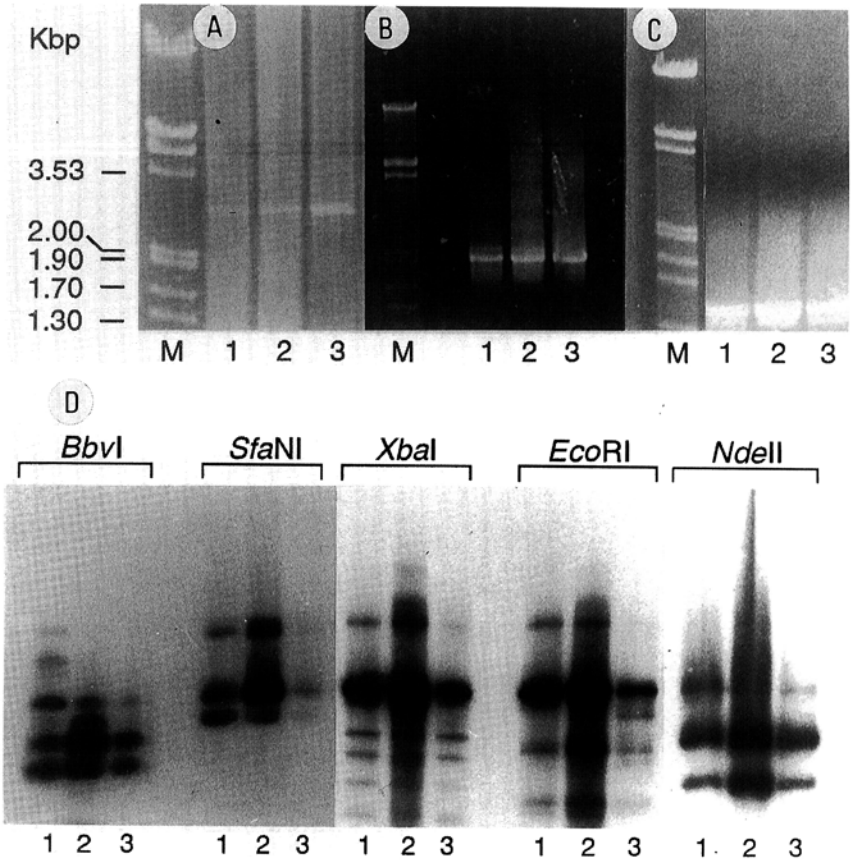

Fig. 5. Characterization by polymerase chain reaction (PCR) of tomato yellow leaf curl virus (TYLCV) DNA from: lane 1, field-infected lisianthus, lane 2, *Datura stramonium* infected with the originally described isolate of the virus, and lane 3, field-infected tomato. Agarose gel of PCR amplified fragments: **(A)** full-length DNA with PTYv522 and PTYc2273, **(B)** virus sense coding region of TYLCV with PTYv225 and PTYc1401, and **(C)** complementary sense coding region of TYLCV with PTYv1220 and PTYc2425. Restriction fragment length polymorphism analysis of the amplification product (fragment shown in **B**) amplified from the above mentioned plant sources. **(D)** Southern blots of the digested preparations were hybridized with a TYLCV-specific probe.

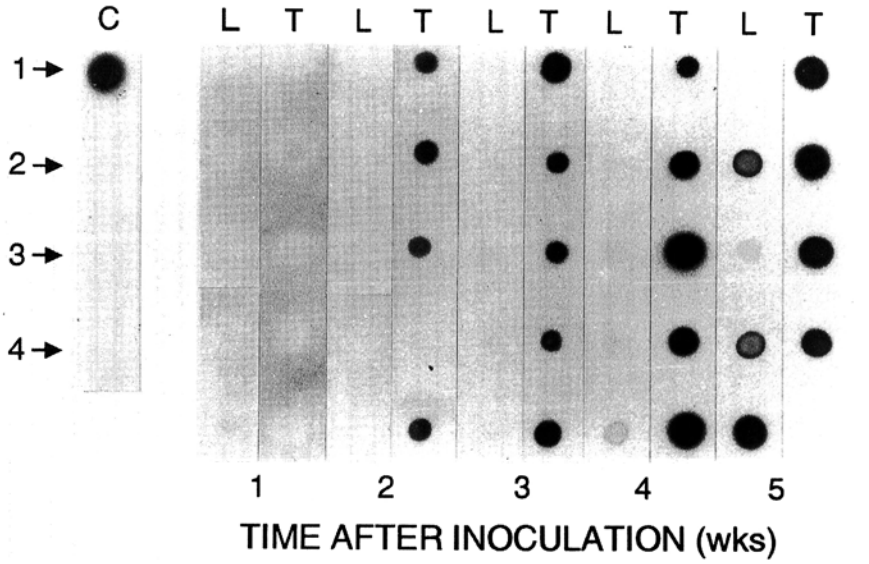

Fig. 6. Accumulation of tomato yellow leaf curl virus (TYLCV) DNA in tomato and lisianthus plants evaluated by dot spot hybridization tests with a TYLCV-specific probe. Plants were inoculated with viruliferous whiteflies. To follow viral DNA accumulation, plants were divided into five groups, each consisting of five tomato and five lisianthus plants. The groups were sampled successively at 1-wk intervals starting 7-days postinoculation. Individual plants of each group were sampled by removal of a leaf disk from the growing tip. DNA accumulation in lisianthus between 1- and 5-wk postinoculation is demonstrated in lanes L; viral DNA accumulation in tomato during the same period is presented in lanes T. To demonstrate the specificity of the molecular probe the following controls were used: lane C1, TYLCV-infected *Datura stramonium*; lane C2, healthy *D. stramonium*; lane C3, healthy tomato; and lane C4, healthy lisianthus.

of detectable amounts of viral DNA. This supports our former findings on the positive correlation between symptom severity and viral DNA accumulation in tomato plants (20). The long incubation time required for symptom appearance in lisianthus reduces the chances of early detection of infected seedlings before they are transferred from nurseries to greenhouses.

The sweetpotato whitefly represents a mixture of biotypes differing in host preference, larval development, transmission efficiency of viruses, and induction of silverleaf symptoms on squash (4). Therefore, it can be hypothesized that the recent spread of the leaf curl disease of lisianthus may be due to a new biotype of *B. tabaci* with better adaptation to lisianthus. Experiments under greenhouse conditions with a greenhouse culture of *B. tabaci* have shown that this biotype is able to complete its life cycle on lisianthus, but transmission of TYLCV from infected to healthy lisianthus was less in comparison with the ability of *B. tabaci* to transmit TYLCV from tomato or *D. stramonium* source plants (Table 1). The dramatic increase of the whitefly populations in vegetables and flower crops in Israel and the whitefly's resistance to insecticides (13) do not leave many alternatives for control of the disease in Israel. Growing lisianthus under 50-mesh screens seems to be a practical solution, but it will increase humidity and temperatures that might be conducive to an increased incidence of fungal diseases. Consequently, the optimal solution seems to be the introduction of resistance against the virus or its vector into commercial lisianthus cultivars.

ACKOWLEDGMENTS

Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel, number 1348-E, 1994 series.

LITRATURE CITED

1. Abdallah, N. A., Aref, N. M., Fauquet, C. M., Madkour, M., and Beachy, R. N. 1993. Nucleotide sequence and genome organization of an infectious DNA clone of tomato yellow leaf curl virus isolated from Egypt. Abstr. 9th Int. Congr. Virol. Glasgow, Scotland.
2. Antignus, Y., and Cohen, S. 1994. Complete nucleotide sequence of an infectious clone of a mild isolate of tomato yellow leaf curl (TYLCV). Phytopathology 84:707-712.
3. Cohen, S., and Antignus, Y. 1994. Tomato yellow leaf curl virus (TYLCV), a whitefly-borne geminivirus of tomatoes. Adv. Dis. Vector Res. 10:259-288.
4. Cohen, S., Duffus. J. E., and Liu, H. Y. 1992. A new *Bemisia tabaci* biotype in the Southwestern United States and its role in silverleaf of squash and transmission of lettuce infectious yellows virus. Phytopathology 82:86-90.
5. Cohen, S., and Harpaz, I. 1964. Periodic, rather than continual acquisition of a new tomato virus by its vector, the tobacco whitefly (*Bemisia tabaci* Gennadius). Entomol. Exp. Appl. 7:155-166.
6. Crespi, S., Norris, S., Accoto, G. P., and Vaira, A. M. 1993. A new isolate of tomato yellow leaf curl virus from Sicily. Abstr. 9th Int. Congr. Virol. Glasgow, Scotland.
7. Czosnek, H., Ber, R., Antignus, Y., Cohen, S., Navot, N., and Zamir, D. 1988. Isolation of tomato yellow leaf curl virus, a geminivirus. Phytopathology 78:508-512.
8. Dry, I. B., Rigden, J. E., Krake, L. R., Mullineaux, P. M., and Rezaian, M. A. 1993. Nucleotide sequence and genome organization of tomato yellow leaf curl geminivirus. J. Gen. Virol. 74:147-151.
9. Ecker, R., and Barzilay, A. 1993. Quantitative genetic analysis of growth rate in Lisianthus. Plant Breed. 111:253-256.
10. Gera, A., and Cohen, J. 1990. The natural occurrence of bean yellow mosaic, cucumber mosaic and tobacco mosaic viruses in lisianthus in Israel. Plant Pathol. 39:561-564.
11. Halevy, A. H., and Kofranek, A. M. 1984. Evaluation of lisianthus as a new flower crop. HortScience 19:845-847.
12. Hibi, T., and Saito, Y. 1985. A dot immunobinding assay for the detection of tobacco mosaic virus in infected tissues. J. Gen. Virol. 66:1191-1194.
13. Horowitz, A. R., Forer, G., and Ishaaya, I. 1994. Managing resistance in *B. tabaci* in Israel with emphasis on cotton. Pestic. Sci. 42:113-122.
14. Kheyr-Pour, A., Bendahamane, M., Matzeit, V., Accoto, G. P., Crespi, S., and Gronenborn, B. 1991. Tomato yellow leaf curl virus from Sardinia is a whitefly transmitted monopartite geminivirus. Nucleic Acids Res. 19:6763-6769.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.) 227:680-685.
16. Lisa, V., Vaira, A. M., d'Aquilio, M., Dellavalle, G., Masenga, V., Milne, R. G., and Boccardo, G. Characterization of an Ilavirus from Lisianthus (*Eustoma grandiflorum*). Acta Hortic. In press.
17. Navot, N., Picherski, E., Zeidan, M., Zamir, D., and Czosnek, H. 1991. Tomato yellow leaf curl virus: A whitefly transmitted geminivirus with a single genomic component. Virology 184:151-168.
18. Reed, K. C., and Mann, D. A. 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. 13:7207-7221.
19. Roh, M. S. M., and Lawson, R. H. 1984. The lure of lisianthus. Greenhouse Manager 2:103-121.
20. Rom, M., Antignus, Y., Gidoni, D., Pilowsky, M., and Cohen, S. 1993. Accumulation of tomato yellow leaf curl virus DNA in tolerant and susceptible tomato lines. Plant Dis. 77:253-257.