DEVELOPMENT OF RELIABLE PROTOCOLS FOR DETECTING AND CONTROLLING LILY VIRUSES

By

TING-CHIN DENG

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KEY TO ABBREVIATIONS

ATCC	the American Type Culture Collection (Rockville, MD)
ATCC PV-291	ATCC accession number of a TBV isolate from 'Texas Flame' tulip contributed by D.H.M. Van Slogteren
ATCC PV-475	ATCC accession number of a TBV isolate from 'Texas Flame' tulip contributed by A.F.L.M. Derks
ATCC PVAS559	ATCC accession number of a polyclonal antiserum against an isolate of TBV from 'Preludium' tulip contributed by J. Hammond
ATCC PVAS732	ATCC accession number of a TBV-specific MCA produced by H. T. Hsu against a TBV isolate from 'Texas Flame' tulip
ATCC PVAS733	ATCC accession number of a different clone of ATCC PVAS 732
ATCC PVAS734	ATCC accession number of a different clone of ATCC PVAS 732
ATCC PVAS675	ATCC accession number of a different clone of ATCC PVAS 732
ATCC PVAS676	ATCC accession number of a different clone of ATCC PVAS 732
ATCC PVAS766	ATCC accession number of the PTY10 potyvirus group cross-reactive MCA: clone contributed by R. Jordan
ATCC PVAS767	ATCC accession number of the PTY8 MCA clone contributed by R. Jordan
ATCC PVAS768	ATCC accession number of the PTY4 MCA clone contributed by R. Jordan
ATCC PVAS769	ATCC accession number of the PTY3 MCA clone contributed by R. Jordan
ATCC PVAS770	ATCC accession number of the PTY2 MCA clone contributed by R. Jordan

CMV	cucumber mosaic cucumovirus
CMV-2100	a CMV isolated from *Commelina diffusa* in Florida
CMV-2148	a CMV isolated from *Cucurbita* sp. in Florida
CMV-J12	a CMV isolate isolated in this study from lily
CMV-K45	a CMV isolate isolated in this study from lily
CMV-WB	a CMV isolate from winged bean in Florida
DIECA	sodium diethyldithiocarbamate
ELISA	enzyme-linked immunosorbent assay
ACP-ELISA	antigen coated plate ELISA
EPPO	European and Mediterranean Plant Protection Organization
IgG	immunoglobulin G
ISEM	immunosorbent electron microscopy
KDa	kilodalton
LiMV	lily mottle virus (synonymous with *Lilium longiflorum* strain of TBV, or TBV-L)
LSV	lily symptomless carlavirus
LSV-Sir1	the lily symptomless virus isolated in this study from 'Sirocco' lily
LVX	lily X potexvirus
MCA	monoclonal antibody
PAGE	polyacrylamide gel electrophoresis
PCA	polyclonal antibody
PTY1	a potyvirus group cross-reactive MCA produced by R. Jordan, and marketed commercially by Agdia, Inc.
RD60	antiserum against the RTBV isolate of TBV which was originally isolated from 'Jack Laan' tulip by A. F. L. M. Derks

RE102	antiserum against TBV, which was originally isolated from 'Texas Flame' tulip by A. F. L. M. Derks
RTBV	Rembrandt tulip breaking virus, an isolate of TBV from 'Jack Laan' tulip by A. F. L. M. Derks, and synonymous with TBV-JL and TBV-lily
SDS	sodium dodecyl sulfate
TBV	tulip breaking potyvirus
TBV-FA	a TBV isolate isolated from lily in this study
TBV-JL	an isolate of TBV recovered from 'Jack Laan' tulip by A. F. L. M. Derks, which is synonymous with RTBV and TBV-lily
TBV-L	*Lilium longiflorum* isolate of TBV, recovered from Easter lily by A. F. L. M. Derks, and synonymous with LiMV
TBV-lily	lily strain of TBV recovered isolated by A. F. L. M. Derks from 'Jack Laan' tulip and synonymous with TBV-JL and RTBV
TBV-tulip	tulip strain of TBV isolated by A. F. L. M. Derks from 'Texas Flame' tulip and synonymous with TBV-TF and by some to "tulip breaking virus"
TBV-PPVB5	a TBV isolate isolated in this study from 'Pink Perfection' Aurelian lily
TBV-T25	a TBV isolate isolated in this study from 'Toscana' lily
TBV-TF	an isolate of TBV recovered from 'Texas Flame' tulips by A. L. F. M. Derks and synonymous with TBV-tulip and with some "tulip breaking viruses"
TBV-V19	a TBV isolate, isolated from 'Vivaldi' lily in this study
TBBV	tulip band-breaking potyvirus, an isolate of TBV recovered from 'Lucky Strike' tulip by C. J. Asjes
TCBV	tulip chlorotic blotching potyvirus, a virus isolated from tulips, but serologically closely related to TuMV

TTBV	tulip top breaking potyvirus, a virus isolated from tulip, but closely related serologically to TuMV
TuMV	turnip mosaic virus
TuMV F815	antiserum against a TuMV isolated from Florida, provided by D. E. Purcifull
TuMV-L12	turnip mosaic potyvirus isolated from 'Lincolnshire' tulip by J. Hammond and closely related serologically to TuMV
UT478	antiserum against LiMV in *N. benthamiana*, provided by A. F. L. M. Derks
WaTV	Washington tulip potyvirus, a virus isolated from tulip by J. Hammond closely related serologically to TuMV

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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By

Ting-Chin Deng

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Chairman: F. W. Zettler
Cochairman: E. Hiebert
Major Department: Plant Pathology

Cucumber mosaic cucumovirus (CMV), lily symptomless carlavirus (LSV), and tulip breaking potyvirus (TBV) were isolated from naturally infected lilies (*Lilium* spp.). Single isolates were characterized biologically and serologically. Antisera against each of these three viruses were produced, and reliable protocols for their detection in lilies were established.

Potyvirus-group, cross-reactive monoclonal antibody PTY1 and seven other cross-reactive PTY antibodies failed to react with any of the TBV isolates tested in this study. Two of seven TBV-derived monoclonal antibodies, however, did react with TBV isolates. In contrast, polyclonal antiserum against a lily strain of TBV reacted with all six TBV isolates tested.

Uneven distribution of the three viruses in different

plant parts and its relation to detection were established. Virus distribution varied according to temperature and plant developmental stage. At high temperatures (>30C) or in freshly harvested bulbs, concentrations of all three viruses were very low. In such cases TBV and CMV were often undetectable. In most instances, the lowest leaves and outermost scales of lilies had the highest virus concentrations.

A survey of commercial Easter, Asiatic and Oriental lilies, consisting of 1001 total samples from Holland and North America, was conducted; 55.6% were infected with LSV, 13.7% with TBV, and 1.2% with CMV. All Easter lilies were from North America and all 117 samples were infected with both LSV and TBV.

Under controlled conditions, a yield trial was conducted comparing LSV-infected and healthy 'Sirocco' lilies. The vigor of healthy plants was much better than LSV-infected plants grown under identical conditions. Plant height, leaf size, and bulb yields were also higher, and these differences were all statistically significant at the P=0.01 level.

CHAPTER 1
INTRODUCTION

Despite its considerable economical potential, lily bulb production in the southern U. S. is extremely limited, and growers continue to import planting stock grown elsewhere, especially the Netherlands.

Recently, a preliminary attempt was made to establish a lily bulb industry in southern Florida, taking advantage of the effects of high growing temperature and long day lengths to prevent bolting and thereby decreasing the time required to produce a flowering-sized bulb (169). Recognizing the potential of a lily bulb industry, J. F. Tammen (Advanced Horticultural System, Inc., Lemont, PA) convinced several Florida growers to initiate a feasibility study towards this end. To be commercially successful, such a program would necessitate producing and maintaining virus-free planting stock. However, comparable programs are already underway in the Netherlands. Dutch programs endeavor to reduce incidences of lily symptomless (LSV), tulip breaking (TBV), and lily X (LVX) viruses. In Florida, cucumber mosaic virus (CMV) was an added concern considering that this virus and LSV played a large part in eliminating a flourishing Easter lily industry

several decades ago (75,145). While most virus research has been on Easter lilies, comparatively little is known about Asiatic and Oriental lilies, which is unfortunate since these now have become the lilies with the greatest market potential. Virus problems in Asiatic and Oriental lilies could be avoided by obtaining and maintaining indexed virus-free bulbs, but such stock is not commercially available.

Imported bulbs, while often of high quality, are frequently infected with viruses such as LSV and TBV. Since diseased plants cannot be recognized by symptoms alone, control by roguing infected field-grown plants is impossible, and thus alternative measures must be used to acquire healthy plant material for local propagation.

For the past 40 years, research on lily viruses was conducted primarily at only three centers located in Beltsville, MD (USDA Florist and Nursery Crops Center), Corvallis, OR (Plant Pathology Department, University of Oregon) and Lisse, the Netherlands (Flowering Bulb Research Center). Most of the recent published research has emanated from the latter. Information on lily viruses that applies to conditions peculiar to the Southeastern U.S. has been lacking.

The objectives of this study were: 1) to identify the viruses being encountered in Florida and to determine the relative incidences in commercial lily stocks with emphasis on Asiatic and Oriental lilies; 2) to establish reliable protocols for virus indexing of plant material to be grown for

certification, with primary emphasis in CMV, LSV, and TBV; 3) to develop a commercially feasible protocol for eliminating viruses in U. S. - grown stock through tissue culture or other means; and 4) to evaluate the performance of virus-free lily plants grown under commercial conditions, whereby the value of a virus control program can be assessed.

Lilies and the Lily Industry

The genus *Lilium* is in the family *Liliaceae* and consists of some 80 species and hundreds of cultivars, mostly interspecific hybrids (51). A horticultural classification of lilies was adopted by the North American Lily Society and the Royal Horticultural Society, in which these plants were grouped into 9 divisions, some with subdivisions, according to their geographic origin, genetic derivation, and flower form (89). In certain parts of the world the Madonna (*L. candidum*) and Easter (*L. longiflorum*) lilies have been popular for many years for use in festivals. In Asia, lily bulbs have also been used as edible and medicinal plants. More recently, it has been reported that certain lilies may have potential as a control for cancer (124). In prosperous industrialized countries, however, lilies are best known as flowering (129). In recent years, Asiatic and Oriental hybrid lilies have become increasingly popular because of their versatile and colorful flowers and lovely fragrance (164). Lilies are sold commercially, as cut flowers, potted plants and forcing bulbs.

Potted lily production in the U.S. has almost trebled since 1949, and in 1992-93, about ten million were produced with a wholesale value of $40,000,000 (Table 1-1). The commercial value of the U.S. lily industry would total more than 100 million dollars if bulb and cut flower markets were included. Since 1980, the number of lily bulbs imported into the U. S. has increased sharply to about 95 million in 1992 (Fig. 1-1). Holland is the main source of lily bulbs imported into the U. S., and the U. S. is their third largest customer, second only to Italy and Japan (Table 1-2). In addition, about 229,000 wild-collected *Lilium* bulbs were exported from Turkey in 1983-1987 (142).

Table 1-1. Commercial production of potted lilies in the U S.[1)]

Year	No. of producers	Production area (hectares)	Yield (1000 pots)	Values (1000 dollars)
1949	NA[2)]	NA	2825	3556
1959	NA	NA	4119	5780
1970	NA	NA	5359	10066
1980	1291	479	7105	19766
1985	1493	466	7071	25485
1990	1013	603	10068	38293
1991	1006	611	10075	38599
1992	1294	655	10367	39882
1993	1333	670	10158	39979

[1)] Source: Floriculture Crops Summary. Agricultural Statistics Board, NASS, USDA (1).

[2)] NA: Data are not available.

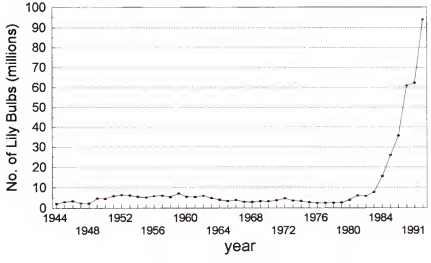

Fig. 1-1. Quantity of lily bulbs imported to USA annually. (Commodity Economics Division, USDA)

Table 1-2. Quantity of *Lilium* bulbs exported from the Netherlands in 1990-1992 [1)]

Importer	June 90 - May 91 (1,000,000 bulbs)	June 91 - May 92 (1,000,000 bulbs)	Fluctuation (%)
Italy	152.4	150.5	- 1.25
Japan	82.3	94.8	+15.19
U.S.A.	56.3	84.6	+50.27
France	43.3	41.6	- 3.93
U. K.	36.6	33.4	- 8.74
Spain	21.1	19.3	- 8.53
Germany	20.5	19.4	- 5.37
Taiwan	11.1	19.8	+78.38
Others	80.7	96.0	+18.96
Total	504.3	559.4	+10.93

[1)] Source: International Bloembollen Centrum. Hillegom, The Netherlands.

In the U. S., commercial flower production increased substantially, shifting since 1970 from the "Snow Belt" to the "Sun Belt" (149). Today, growers of specialty cut flowers may still be found throughout the country, although the greatest concentration is in Florida, California and the Midwest (15).

Propagation of Lilies and Virus Diseases

Lilies can be propagated by either sexual or vegetative means, although most commercially grown lily cultivars are multiplied by only the latter method. Bulbs are propagated for market by dividing mature bulbs or by harvesting new side bulbs from old ones. For commercial growers, stocks can be increased from underground bulblets, aerial bulbils, and/or adventitious bulblets developed from detached scales.

Because they are usually propagated vegetatively, lilies are vulnerable to epidemics of virus disease. Such viruses of lilies induce serious qualitative and quantitative yield losses (50). Some varieties are more susceptible to certain viruses than others and as a result are soon discontinued from commercial production (90). Viruses also reportedly reduce the vase life of certain lily cultivars (26). Virus-free lilies are likely to be bigger and taller than virus-infected ones, and their capacity to produce bulbils somewhat is increased (5). Although procedures for producing virus-free lilies through micropropagation have been developed (4, 5, 7, 10, 11, 21, 25, 36, 38, 44, 60, 100, 104, 125, 134, 143, 145,

153, 156, 157, 172), and although cultural control methods have been put into practice (8, 12, 16, 17, 19, 20, 22, 24, 27, 78, 105, 128, 148, 173), viruses continue to damage this crop and remain important limiting factors in the commercial production of lilies.

Viruses Infecting Lilies

Viruses are considered among the most important and widespread pathogens of lilies (148). At least 13 viruses have been reported to infect *Lilium* spp. (Table 1-3), but only three, cucumber mosaic cucumovirus (CMV), lily symptomless carlavirus (LSV), and tulip breaking potyvirus (TBV), are commonly found in North America (6). Lily X potexvirus has been reported from the Netherlands and Japan (101,152), but little is known about the occurrence of this virus elsewhere.

Cucumber Mosaic Cucumovirus

Many strains of CMV occur naturally. They are found throughout the world and all have wide host ranges (82). Since the 1970's, infections of lilies by CMV were reported in Belgium (163), Great Britain (127), the Netherlands (26), Italy (35), Korea (55), Japan (85, 86), and Taiwan (54). While CMV is ignored by European and Mediterranean Plant Protection Organization's (EPPO) "Scheme for production of certified pathogen-tested material of lily" (78), it continues to be a

Table 1-3. Viruses reported to occur in lilies.

Name [1)]	Symptoms	*Lilium* Species	Location	Year/Reference
Unidentified	rosette	*longiflorum*	Bermuda	1927 (130, 131)
"Transmissible virus"	mosaic	*auratum*	USA	1928 (84)
"Color-removing virus" (Tulip breaking virus)	mosaic	*speciosum*	USA	1932 (115)
"Celery mosaic 1 virus" (Cucumber mosaic virus)	necrotic spots	*longiflorum*	USA	1935 (168)
"Lily mosaic virus" (Cucumber mosaic virus)	mosaic	*giganteum*	USA	1937 (136)
"Tulip virus 1" or "Color-removing virus" (Tulip breaking virus)	latent	*candidum* *tigrinum* *longiflorum*	USA	1937 (117)
(Cucumber mosaic virus & lily-symptomless virus)	necrotic fleck	*longiflorum*	USA	1944 (47)
"Lily mottle virus" (Tulip breaking virus)	mottle	*longiflorum* *longiflorum* *tigrinum*	USA	1944 (48)
"Lily-rosette virus"	rosette	*longiflorum* 13 other species	USA	1945 (49)
"Lily ringspot virus" (Cucumber mosaic virus)	faint mottling necrosis	*regale* *tigrinum*	England	1950 (150)

Table 1-3. (continued)

Name [1]	Symptom	*Lilium* species	Location	Year/Reference
"Unnamed virus of corn" (Cucumber mosaic virus)	fleck	*speciosum*	USA	1954 (122)
(Tobacco ringspot virus)	latent	*longiflorum*	USA	1957 (155)
"Curl stripe" (Lily symptomless virus)	curl stripe	*longiflorum*	USA	1964 (120)
"Streak mottle virus"	streak mottle	*speciosum*	USA	1968 (77)
(Tobacco rattle virus)	curling	*hollandicum*	Holland	1975 (64)
(Citrus tatter leaf virus)	yellowing	*longiflorum*	Japan	1979 (93)
Lily virus X	latent	Hybrid lily	England	1980 (152)
(Arabis mosaic virus)	necrosis, mosaic	*tigrinum*	Holland	1983 (27)
Broad bean wilt virus	color breaking	*tigrinum*	Korea	1987 (55)
Narcissus mosaic virus	stunting			
(Strawberry latent ringspot virus)	mosaic, flower distortion	Hybrid lily	Israel	1995 (61)

[1] The names of the diseases or the viruses described by respective authors are in quotes. The names in parentheses are the viruses implicated as listed by the International Committee for the Taxonomy of Viruses (80).

potential threat to lily production in many countries.

CMV is transmitted by *Aphis gossypii*, *Myzus persicae* and many other aphid species (82). Several diseases of lily, fully or in part caused by CMV strains, have been described.

Celery mosaic. In 1924, a serious mosaic disease of celery occurred in Florida (79). Wellman (167) identified the causal agent as celery virus 1 in 1934. In the following year, Wellman found many cultivated Easter lily plants to be infected with this virus, and he determined that both *L. auratum* and *L. longiflorum* could contract this disease through inoculation by aphids (168). Later, celery-mosaic virus was determined to be a strain of cucumber mosaic virus by Price (135).

Lily mosaic. The earliest description of a virus-like disease of Easter lily was by Stewart in 1896 (132,151). Guterman (84) first reported the mechanical transmission of the disease agent from infected plants to *L. auratum* in 1928. When Price (136) classified the viruses causing lily mosaic symptoms in 1937, he indicated that the lily disease described by Stewart was caused by cucumber mosaic virus.

Necrotic flecking. Although Wellman (168) suspected that Easter lilies were affected by at least two viruses in Florida, it was not until 1939 that Brierley (41) provided definitive evidence for more than one virus being involved in the mosaic complex. In 1940, Brierley (42) conducted a survey of garden lilies, and found CMV to be present in 18 species or

varieties from 9 localities (42). Brierley and Smith (47) found that the debilitating necrotic fleck disease in Easter lilies was due to a combined infection by CMV and LSV, whereas CMV alone or CMV combined with TBV, which they referred to as "lily mottle virus," did not induce this symptom (47). Because of the severity of symptoms expressed by lilies infected with both CMV and LSV, necrotic fleck became an important factor in the demise of the U. S. Easter lily industry in the 1930's (75,146). In Florida, *Commelina nudiflora* was identified as the principal reservoir of CMV inoculum for lilies (76,168). Since the 1970's, changes in cultural practices and shifts in crop production are believed to have controlled CMV (174). Based upon 1988 survey results (140), CMV was detected in only 13 out of 549 cucurbit samples collected in Florida. Nevertheless, *Commelina* spp. are still considered important potential sources of CMV inoculum for crops such as celery, gladiolus, and Easter lilies (28).

Lily ringspot. In England, Smith (150) reported that certain shipments of hybrid lilies imported from U. S. had a disease he described as "lily ringspot." The causal agent of this disease was determined to be CMV. Later, in 1962, Brierley also found CMV to be present in Easter lilies from Georgia with symptoms similar to the lily ringspot symptoms described by Smith (43).

Tulip Breaking and Other Potyviruses of Lilies and Tulips

Unfortunately, much confusion exists in the literature regarding the nomenclature of potyviruses that infect lilies and tulips. Unlike tulips, which are commonly infected with two distinct potyviruses, turnip mosaic and TBV, lilies are susceptible only to the latter. Nevertheless, differences between isolates of TBV have caused authors, even in recent years, to apply various names to distinguish them from one another. Another inherent problem in defining potyviruses of lilies is that much of the antisera used to diagnose them is derived from preparations purified from tulips before the discovery in 1985 of strains of turnip mosaic occurring in this host (125).

Because tulips with flower break symptoms like those induced by TBV were illustrated by Dutch artists of the Renaissance, this virus has sometimes been referred to as the "oldest plant virus disease" (111). Cayley (53) in 1928 first reported that color break in tulips was caused by an infectious agent which could be transmitted to healthy plants. This agent was also proved to be transmissible by at least three species of aphids (110). Subsequently, at least four other species of aphids were determined to be vectors of TBV (46, 112, 113, 114, 160). In 1932 McWhorter (115) transmitted TBV from *L. speciosum* to tulips, which then developed symptoms indistinguishable from those noticed before in TBV-infected tulips (117). Brierley and Doolittle (45) showed that *L.*

formosanum plants respond to TBV by developing more severe foliar mosaic symptoms than those in tulips. Using *L. formosanum* seedlings as indicator plants for detecting TBV in commercial Easter lily plantings, Brierley (42) determined that this virus was present nearly everywhere they were grown. In addition to TBV which he called "lily latent virus," other more severe viruses were described, namely a "strong mottle virus" and a "more virulent virus" infecting Easter lily (41,48). Other early synonyms of TBV were "tulip mosaic" (137,170) and McWhorter's "color-adding" and "color-removing" viruses, which he later referred to as "Tulip Viruses I and II," respectively (116,117,118). While McWhorter considered his two viruses to be distinct, Van Slogteren (160) considered them both to be strains of TBV, which he referred to as "severe" and "mild," respectively.

Lily latent virus. The virus isolated from an apparently healthy lily that caused breaking in inoculated tulips was named "lily latent virus" (45,117). Originally, McWhorter considered the latent virus to be identical to his Tulip Virus I (117). However, he later distinguished them based upon the absence of viroplasts in tulips infected with lily latent virus (119).

Jack Laan, Texas Flame, and Easter lily isolates of TBV. Polyclonal antiserum against isolates of TBV from Jack Laan (TBV-JL) and Texas Flame (TBV-TF) tulips reacted differently when tested against lily isolates of TBV. Whereas the TBV-JL

antiserum reacted strongly to lily isolates of TBV, the TBV-TF did not (33,73,83). These differences led Boonekamp *et al.*(39) to distinguish the -JL and -TF serotypes as "lily- " and "tulip-strains," respectively. Yet another TBV strain (TBV-L), originally isolated from Easter lily, was described in Holland in 1985 (66). This strain proved similar to a previously described lily isolate in Israel (14), which cross-reacted against both lily and tulip serotypes of TBV (63). However, a monoclonal antibody for TBV-L reacted with neither the -JL nor the -TF isolates (40).

Langeveld *et al.* (106) mistakenly referred to a virus they tested from 'Flevo' Easter lily as "TBV-lily," without regard to the fact that Boonekamp *et al.* (39) previously used that term exclusively for the TBV-JL isolate from tulip. The terms "TBV-lily" and "TBV-tulip" have also been used by other worker without regard to the specific isolates studied by Dutch workers. Ohira *et al.* (133), for example, who described the nucleotide sequences of TBV isolates from Japan, used these terms to describe their respective isolates from lilies and tulips.

In 1993, Dekker *et al.* (63) proposed the name "lily mottle" (LiMV) for the 'Flevo' Easter lily virus described by Langeveld *et al.* (106), Derk's (66) TBV-L, and Alper *et al.*'s (14) Easter lily virus. Dekker *et al.* (63) also renamed the -JL isolate of TBV studied by Derks *et al.* (73) to "Rembrandt tulip-breaking virus" (RTBV). A fourth TBV strain, which was

isolated from 'Lucky Strike' tulip but was not infectious to lily was named "tulip band-breaking virus" (TBBV) (63). Based upon differences in host range, nucleotide sequence, and coat protein serological properties, Dekker *et al.* (63) concluded that TBV-JL, TBV-TF, LiMV, and TBBV should be considered as distinct viruses, despite being closely related to one another serologically.

Tulip infecting isolates closely related to turnip mosaic virus. In 1985, Mowat (126) described a potyvirus distinct from TBV, which he called "tulip chlorotic blotch virus" (TCBV). Later, Hammond and Chastagner (87,88) described two similar tulip viruses, "Washington tulip virus" (WaTV) and "TuMV-L2," which were serologically related to turnip mosaic virus (TuMV). Later, Dekker *et al.* (63) reported that yet another tulip virus, called "tulip top-breaking" was also closely related to TuMV. These four viruses are also related serologically to bean yellow mosaic virus (88) and can infect certain herbaceous dicotyledons. None, however, can infect lilies.

Lily Symptomless Carlavirus

In 1944, Brierley and Smith (47) described lily symptomless virus (LSV). Like CMV, LSV had become widely spread in commercial Easter lilies before being discovered. The virions of LSV have a normal length of about 636 nm, typical of other carlaviruses (3,13,37,57,137).

Besides the direct effects of necrotic fleck in mixed infections with LSV and CMV in Easter lily, these viruses increased the host susceptibility to *Pythium* root rot (141). LSV alone induces leaf curl-stripe symptoms (3,120,121) when plants are grown under cool environmental conditions, whereas at higher temperatures, symptoms, are latent. LSV combined with TBV can induce the formation of brown rings in bulb scales of the Asiatic lily 'Enchantment' (26,65). Although LSV has been isolated from tulips (69), it does not often infect them (23, 26). In 1979, a survey of North American native *Lilium* species for LSV was conducted by Ballantyne *et al.* (29); however, no LSV was found in any of these plants. In contrast, LSV was reported wherever commercial lilies were grown (3,18,67,86,121,127,163). LSV was transmitted by four species of aphids in a non-persistent manner, with *Myzus persicae* proving the most efficient (127). The host range of LSV is restricted to the Liliaceae (3). No reports concerning strain differentiation between LSV isolates have been described.

Lily Virus X

Stone (152) in 1980 described a potexvirus (LVX) isolated from lilies and that infected certain dicotyledonous herbaceous species. LVX was considered to be a potential problem for lilies, and therefore was one of the viruses indexed in Dutch certification schemes (78). The partial

characterization of LVX by nucleotide sequence analysis was recently accomplished (39, 123). No papers concerning the occurrence of LVX in native or cultivated lilies are reported, except for that by Kimura *et al*. (101), who detected the LVX from several lily cultivars grown in Japan.

CHAPTER 2
IDENTIFICATION AND DETECTION OF VIRUSES IN LILIES

Introduction

The first step toward controlling lily virus diseases is to establish a reliable method for virus detection. In a few instances, the symptoms of virus-infected lilies may be distinctive, such as the flower-breaking induced by TBV in certain lily varieties or the necrotic flecking induced by CMV and LSV in mixed infections (6). Otherwise, diagnosing lily viruses based on symptom expression is unreliable. The three viruses that most commonly infect lilies either induce inconspicuous symptoms or are usually latent. Symptoms of TBV-induced flower breaking, while sometimes striking, are likely to appear only on pink- or purple-flowered lilies, rather than white or yellow ones (160).

Lilies infected with certain viruses can also be detected by inoculating indicator plants, such as *Nicotiana tabacum* for CMV (41,168), *N. benthamiana* and *Chenopodium quinoa* for LiMV (14,63), *Tetragonia expansa* for LVX (152), and tulips or *L. formosanum* for TBV (115,41). However, bioassays using indicator plants also depend on symptom development, which in turn can be affected by environmental conditions.

Lily viruses can also be detected by electron microscopy (6). Immunosorbent electron microscopy (ISEM) has been especially helpful for increasing the sensitivity of detecting viruses such as LSV by negatively stained leaf extracts (74).

Serological diagnosis is the most feasible method for virus detection in lilies, especially in certification programs where a large number of samples must be indexed in a relatively short period of time. Immunodiffusion once was employed extensively in the Netherlands to test the presence of viruses in lilies (72,162). After 1980, however, ELISA became the major tool for routine tests (31,32,66) and ELISA detection procedures facilitated the commercial production of lilies free of LSV, TBV and LVX in the Netherlands. ELISA tests are also recommended by the European and Mediterranean Plant Protection Organization (EPPO) for its member countries as a standard method for virus indexing in lilies (78). Dot-blot or direct tissue blotting assays have also been employed to detect LSV in lilies. This technique is potentially more sensitive than ELISA but as yet is not as widely used for lilies (92,105).

Several workers have experienced difficulties in detecting lily potyviruses in serological tests, however, especially when they used monoclonal antibodies (40, 39, 66, 83, 91). Although the polyclonal TBV-JL antiserum produced in 1982 by Derks *et al.* (73) was from tulips, it reacted strongly

with lily isolates of TBV, but only weakly with the TBV-TF isolate from tulips. In contrast, TBV-TF antiserum consistently reacted in ELISA more strongly with tulip isolates of TBV than with lily isolates. As a consequence, tulip and lily isolates of TBV were considered to consist of two serotypes (33,73). Later, in 1985, a third type of TBV was found (66). This isolate (TBV-L) was from Easter lily and had characteristics similar to the one described by Alper in 1982 (14). Very weak serological relationships were observed between TBV-L and the TBV-JL and TBV-TF isolates from tulips, which led the authors to conclude that TBV-L should be given a distinct name, lily mottle virus (63). Based on these reports, there are at least 3 serotypes of TBV that exist in lilies.

The serological specificity for isolates of TBV pose possible hazards for routine indexing of lily potyviruses and may limit the effectiveness of certain serological methods for routine diagnosis. Derks's TBV-L isolate could not be detected by polyclonal antisera (PCA) of either the TBV-JL or the TBV-TF isolates (66). Similarly, Boonekamp and Pomp (40) reported that a monoclonal antibody (MCA) derived from TBV-L did not react against either TBV-JL or TBV-TF. When the TBV MCAs of Hsu *et al.* (91) were used, only indirect ELISA was suitable (83,91), and only a few clones could detect lily isolates of TBV (39,66). After testing the MCAs in ELISA, Franssen and van der Hulst (83) concluded that PCA was preferred over MCA for

the detection of TBV in lilies by ELISA. Thus, while TBV is among those viruses reported to react with the potyvirus group-specific PTY1 MCA (99), certain isolates apparently do not (68).

Materials and Methods

Virus Isolates

The LSV-Sir1 isolate was from a 'Sirocco' lily bulb imported from Holland in 1993. The CMV-J12 isolate was derived from a plant of the lily cultivar 'Juanita.' The CMV-K45 isolate was collected in 1994 from a naturally infected lily plant growing in a lily field in Alachua County, Florida. The TBV-FA isolate was found in an unidentified Asiatic lily growing in a greenhouse on the campus of the University of Florida at Gainesville. Other TBV isolates used in this investigation were obtained during virus surveys conducted in 1992-1995. These were isolates TBV-TDG31 isolated from a commercial trumpet lily purchased from Dutch Garden Inc. (Adelphia, NJ); TBV-PPVB5 from a 'Pink Perfection' Aurelian lily purchased from Van Bourgondien, Inc. (Babylon, NY); TBV-V19 isolated from 'Vivaldi' lily provided by the Manatee Fruit Co. (Palmetto, FL); and TBV-T25 isolated from an imported Dutch 'Toscana' lily provided by J. F. Tammen (Advanced Horticultural Systems, Inc., Lemont, PA). Two TBV isolates

(ATCC PV-291 and ATCC PV-475) obtained from the American Type Culture Collection (ATCC) (Rockville, MD) were also used. In addition, isolates of TCBV (ATCC PV-425), and WaTV (ATCC PV-388) were obtained from the ATCC. Dried antigens in leaf tissue of TBV-TF, RTBV, TTBV, TBBV, LiMV, LVX, and LSV were kindly provided by A. F. L. M. Derks at the Bulb Research Center, Lisse, the Netherlands.

Test Plants for Inoculation

The reconstitution of freeze-dried tissues and preparation of inocula were done according to the ATCC's guidelines. Other samples of infected leaves or scales were homogenized in 0.1 M phosphate buffer, pH 7.2, and the extracts were rubbed onto leaves of test plants dusted with 600-mesh carborundum. Seedlings of *Chenopodium amaranticolor* Coste and Reyn., *amaranticolor. quinoa* Willd., *Gomphrena globosa* L., *L. formosanum* Stapf., *L. regale* Wilson, *N. benthamiana* Domin., and *Tetragonia expansa* Thunb. were the test plants used. All test plants were maintained in a greenhouse at <30C.

Virus Purification

LSV. The LSV-Sir1 isolate was purified from its original host, the lily cultivar, 'Sirocco'. All above ground tissues of plantlets generated from scales of a lily bulb singly infected with LSV were collected. Partially purified virus was prepared as described by Derks and Vink-van den Abeele

(72), except that 0.1% sodium diethyldithiocarbamate (DIECA) and 0.3% ascorbic acid were added to the extraction buffer. For further purification, cesium sulfate was added to the virus suspension to make a final concentration of 30% (w/v), and the mixture was centrifuged at 105,800 g for 18 hours at 4C in a Beckman SW 50.1 rotor. The virus zone was collected from the tubes and diluted with equal volumes of 1 mM sodium phosphate buffer (pH 7.0). After a low speed centrifugation to remove the contaminants, the virus suspension was dialyzed against 1 mM sodium phosphate buffer (pH 7.0) at 4C overnight.

CMV. CMV-K45 was transferred by mechanical inoculation to *N. benthamiana* and maintained for purification. Leaf tissues of *N. benthamiana* infected with CMV-K45 were harvested 10 days after inoculation. All protocols for partial purification were as described by Scott (144). Further purification was conducted in 30% cesium sulfate as noted above for LSV, except that the buffer used in the final step was 1 mM borate buffer (pH 8.0).

TBV. The leaves of *L. formosanum* seedlings infected with TBV-FA were collected for purification. The procedures and buffers for the initial stages of purification were as described by Derks *et al.* (73), except that the crude virus preparations were precipitated with 6% polyethylene glycol (PEG MW 6000) and that the final preparations were subject to isopycnic centrifugation in 30% cesium sulfate as described above.

The concentration of purified virus was estimated with a Beckman model 25 spectrophotometer using an extinction coefficient of 3.0 for a 0.1 % LSV solution at 260nm (72), 5.0 for CMV (82), and 2.7 for TBV (73). Virus suspensions were stained in uranyl acetate and observed by electron microscopy (56).

For analysis of purity and determination of coat protein mobility, purified virus preparations were subjected to sodium dodecyl sulfate polyacrylamide gel analysis (SDS-PAGE) as described by Laemmli *et al.* (103).

Antiserum Preparation

Polyclonal rabbit antiserum was prepared by Cocalico Biologicals, Inc. (Reamstown, PA) using four 1 mg injections of purified LSV-Sir1, three 1 mg injections of purified CMV-K45, and four 1 mg injections of TBV-FA as immunogens. The titers of polyclonal antisera (PCAs) collected at different times were determined by immunodiffusion tests as described by Purcifull and Batchelor (139). The selected antiserum was then further tested by indirect ELISA as described by Converse and Martin (62).

Immunodiffusion Test

SDS-immunodiffusion tests (139) were conducted to assess the titers of LSV antisera. For CMV-K45, immunodiffusion medium not containing SDS was used. Instead, the diffusion

medium contained 0.7% Noble agar in 10 mM sodium phosphate (pH7.6) and 0.1% sodium azide. The same medium was also used for reciprocal immunodiffusion tests with other CMV isolates and for detecting the homologous and heterologous reactivities of CMV-K45. In those tests, the antigens and antisera of the CMV-2100 isolate from *Commelina diffusa* (94), the CMV-2148 isolate from cucurbits (94), and the CMV-WB isolate from winged bean (102) were used. The CMV-lily and the CMV-gladiolus antigens used were from single plants of lily and gladiolus, respectively.

Other Polyclonal Antisera and Monoclonal Antibodies

In addition to the above, polyclonal antisera RE102 (derived from TBV-TF), RD60 (from TBV-JL), and UT478 (from TBV-L) were kindly provided by A. F. L. M. Derks (63,71). TBV-specific monoclonal antibodies (MCAs) of ATCC PVAS675, ATCC PVAS676, ATCC PVAS732, ATCC PVAS733, and ATCC PVAS734 (91) as well as potyvirus cross-reactive MCA of PTY2 (ATCC PVAS770), PTY3 (ATCC PVAS769), PTY4 (ATCC PVAS768), PTY8 (ATCC PVAS767), and PTY10 (ATCC PVAS766) were purchased from the ATCC. The PTY1 MCA was purchased from Agdia, Inc. (Elkhart, IN). A MCA pool with an admixture of PTY1, PTY2, PTY4, PTY8, PTY10, PTY21, PTY24, and PTY30 was kindly provided by R. Jordan (95, 96, 97, 98, 99). Antiserum of a turnip mosaic potyvirus (TuMV F815) isolate from Florida was provided by D. E. Purcifull (138).

Indirect ELISA

The purification of immunoglobulin (IgG) was done according to the procedures of Clark and Adams (59). Indirect ELISA with plate-bound antigen protocols (62) were conducted, except that all the reagents were reduced to 100 µl/well, and the incubation periods were reduced to one hour at 35C. Purified viruses or test samples of lily leaf or bulb scale tissues extracted in 10 volumes of coating buffer (50 mM sodium carbonate, pH 9.6) were added to wells of microtiter plates. The plates were incubated overnight at 4C, then washed with phosphate buffered saline (PBS), pH 7.4, containing 0.005% Tween 20 (PBS-T). PCAs or MCAs diluted with conjugate buffer, PBST containing 2% polyvinylpyrrolidone-40 (PVP-40, MW 40,000), and 0.2% bovine serum albumin (BSA) were added and incubated. After washing with PBST, plates were loaded with alkaline phosphatase-labeled goat antirabbit (for PCA) or antimouse (for MCA) immunoglobulins. Plates were incubated and washed as before. Enzyme substrate, *p*-nitrophenyl phosphate, was used at 1 mg/ml in diethanolamine buffer, pH 9.8, and incubated at 35C for color development. Absorbance values (A_{405nm}) were determined with a Bio-Tek automatted microplate reader (Bio-Tek Instruments, Winooski, VT), at fifteen minute intervals for 1 hour or longer.

ACP ELISA. An indirect antigen-coated plate (ACP)-ELISA recommended by R. Jordan (99) was employed to detect the

cross-reactivities of MCAs and PCAs. Plant tissues were directly ground (1:20; w/v) in 0.05 M sodium carbonate-coating buffer containing 2% PVP-40, 0.2% DIECA, and allowed to stand at room temperature for 2 hours before adding to the wells. A blocking step with Tris buffered saline containing 1% dry milk and 0.5% BSA was added to the indirect ELISA procedure, although the subsequent incubation steps were the same as previously described for indirect ELISA.

Western Blot

The Western blot procedure was conducted as described by Towbin *et al.* (154), using a Bio-Rad (Hercules, CA) Mini-Protein II Electrophoresis cell and Bio-Rad Trans-Blot Electrophoretic Transfer cell.

Tested samples were prepared by extracting 1 g of fresh leaf or 0.1 g of dried tissue in 1 ml of Laemmli dissociation buffer (0.1 M Tris-HCl, pH 6.8, 2.5% SDS, 5% 2-mercaptoethanol and 5% sucrose) (103). During electrophoresis at a constant voltage of 150 V for 1 hour, the proteins were separated in a 10% gel. The proteins were transferred to nitrocellulose membranes (0.45 μm pore size) by electrophoretic transfer at a constant voltage of 100V for 1 hour. Nitrocellulose membranes were rinsed three times in Tris buffered saline (TBS, 20 mM Tris, pH 8.2, 15mM NaCl) containing 0.1% Tween 20. After blocking with 5% nonfat dry milk, virus-specific antibodies were added and incubated at 4C overnight. The

dilution for MCA PTY1 was 1:100, and the dilutions for MCAs PTY2, PTY3, PTY4, PTY8, and PTY10 were 1:2000. Dilutions for PVAS388, PVAS559, and admix MCAs were 1:5000, and for all others 1:1000. After the same rinse procedure, membranes treated with PCAs were incubated with alkaline phosphatase conjugated goat anti-rabbit IgG, whereas membranes treated with MCAs were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG for 1 hour at room temperature (both were used at a dilution of 1:1000). After washing as described above, followed by a brief rinse in substrate buffer (0.1M Tris-HCl, pH 9.5, 0.1 M NaCl, 5mM $MgCl_2$), the color of the immune complex was developed in the presence of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Gibco-BRL, Gaithersburg, MD). A sample was considered positive when the band showed in appropriate location that was absent in healthy control.

Cross-Absorption

To eliminate cross-reactivity with healthy lily sap when using antisera of TBV-JL (RD60), TBV-TF (RE102) and TBV-L (UT478), the following procedures were carried out. Leaves of healthy *L. formosanum* were extracted (1:10) in PBS and subjected to dialysis against the same buffer at 4C overnight. Antiserum was mixed with the healthy plant sap (1:10) and stirred at 36C for 4 hours and then at 4C overnight. After centrifugation (5,000 *g*, 10 min) and freezing overnight, the

admixture of antiserum and plant sap was subjected to immunoglobulin (IgG) purification as described by Clark and Adams (59).

Results

Host Reactions

The reactions of indicator plants to LSV, two isolates of CMV, and to TBV and eight other potyviruses are listed in Table 2-1. LSV-Sir1, CMV-J12, CMV-K45 did not produce symptoms in their respective natural hosts, and in the inoculation tests, all three infected *L. formosanum* without producing symptoms. Both CMV-J12 and CMV-K45 induced local lesions in *Chenopodium amaranticolor* and *C. quinoa*, and systemic mosaic symptoms in *N. benthamiana* and *Tetragonia expansa*.

Two types of breaking symptoms were observed on TBV-FA and TBV-V19 infected plants. "Full breaking" symptoms like those described by Van Slogteren (160) developed in TBV-FA infected 'Sirocco' lily flowers (Fig. 2-1 top), whereby some pink pigments failed to form, causing portions of the petals and sepals to have a bleached appearance. A different flower breaking symptom like the "self breaking" symptom described by Van Slogteren (160) developed in TBV-V19 infected 'Vivaldi' lily (Fig. 2-1 bottom). In this instance, the anthocyanin pigments were abnormally high causing irregular purple-colored blotches on parts of the petals and sepals. Similar symptoms

Table 2-1. Indicator plant reactions to 12 virus isolates from lilies or tulips.

Indicator Plant	Virus isolate [1]											
	LSV-Sir1	CMV-J12	CMV-K45	TBV-291	TBV-FA	TBV-V19	TBV-T25	TBV-TDG	TBV-PPVB5	TBV-E33	TCBV-425	WaTV-388
Chenopodium amaranticolor	-[2]	L	L	-	-	-	-	-	-	-	L	L
C. quinoa	-	L	L	-	-	-	-	-	-	-	L	L
Gomphrena globosa	-	-	-	-	-	-	-	-	-	-	-	-
L. formosanum	S	S	S	M	M	M	M	M	M	M	-	-
L. regale	NT	NT	NT	S	-	-	-	S	S	-	-	-
N. benthamiana	-	M	M	-	-	-	-	-	-	-	M	M
Tetragonia expansa	-	LM	LM	-	-	-	-	-	-	-	NT	NT

[1] LSV = lily symptomless virus, CMV = cucumber mosaic virus, TBV = tulip breaking virus. TCBV and WaTV = tulip chlorotic blotch virus and Washington tulip virus, respectively.
[2] - = no infection (confirmed by ELISA)
L = local lesion, S = symptomless (infection confirmed by ELISA), M = mosaic, NT= not tested

Fig. 2-1. Two types of flower breaking symptoms caused by TBV infection on Asiatic lilies. "Full-breaking" on Asiatic lily cv. Sirocco, in which some anthocyanin pigments in petals and sepals are poorly developed (top left). "Self-breaking" on Asiatic lily cv. Vivaldi, in which some anthocyanin pigments in petals and sepals are intensified (bottom left). Healthy counterparts of each variety are on top and bottom right.

were also seen on a 'Toscana' lily infected with the TBV-T25 isolate provided by J. Tammen. These TBV isolates all induced conspicuous foliar mosaic and distortion symptoms on *L. formosanum* (Fig. 2-2). In addition to *L. formosanum*, TBV-291 and the TBV-TDG and TBV-PPVB5 isolates also infected *L. regale*. Of the nine potyviruses tested, only the TCBV-425 and WaTV-388 isolates infected the dicotyledonous hosts, *C. amaranticolor*, *C. quinoa*, *N. benthamiana*, and *T. expansa*.

Virus purification

LSV-Sir1 and CMV-K45 were readily purified. According to ultraviolet absorption spectra (without correction for light-scattering), yields of purified LSV-Sir1 and CMV-K45 were 131 μg and 24 μg from per gram of host tissue, and the A260nm/A280nm ratios were 1.715 and 1.857, respectively. In SDS-PAGE, the capsid proteins of purified LSV-Sir1 and CMV-K45 each migrated as a single band with estimated molecular weights of 33 and 26 kDa, respectively. TBV-FA purification was not successful, however, despite repeated attempts. Although the "virus zone" collected from isopycnic centrifugation had a high absorbance at 260nm, the A260nm/A280nm ratio was 1.982, and no clearly discernible protein band was evident after SDS-PAGE. Also, very few virus-like particles were observed by electron microscopy in those preparations.

Fig. 2-2. Leaf mosaic and distortion in *Lilium formosanum* seedling induced by the FA isolate of TBV.

Virus Morphology

After dilution, three types of virus particles in their respective purified preparations were seen (Fig. 2-3). Particles of CMV-K45 were isometric with a diameter of 28-30 nm in diameter, whereas those of LSV-Sir1 and TBV-FA were flexuous rods ca. 600 and 700-800 nm long, respectively.

Reactivities of Polyclonal Antiserum

CMV. CMV-K45 antiserum titers as high as 1/8 were noted in immunodiffusion tests. The working dilution end points of antiserum that reacted with 0.1 μg viral protein in indirect ELISA tests was 1/640,000. This antiserum could detect CMV at 1/1000 dilutions in lily and 1/100,000 in tobacco (Fig. 2-4). Reciprocal immunodiffusion tests shown in Fig. 2-5 and Fig. 2-6 revealed that the CMV-K45 antiserum reacted with all four strains of CMV tested in this study. However, in reciprocal tests, precipitin lines formed by CMV-K45 with antiserum to CMV-K45 spurred over the precipitin line fromed by CMV-2100. In tests with antiserum to CMV-2100, CMV-2100 and CMV-K45 showed reactions of apparent identity (no spurs detected). The antisera to CMV-2100, CMV-2148, and CMV-WB isolates all reacted with CMV-K45, although the reactions were relatively weak.

LSV The titer of the LSV-Sir1 antiserum was 1/4 in SDS-immunodiffusion tests, and 1/320,000 in indirect ELISA tests.

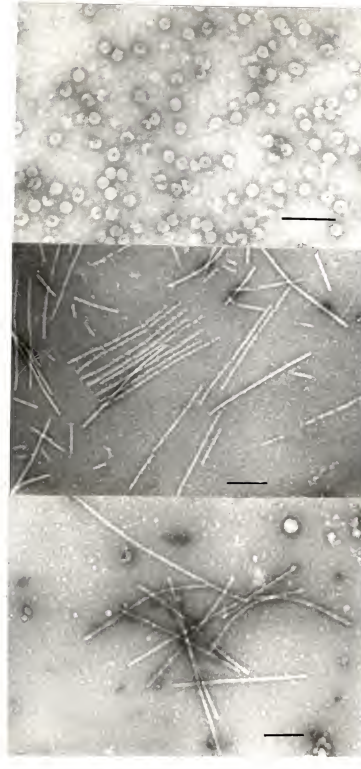

Fig. 2-3. Virus particles observed in purified preparations of the K45 isolate of CMV (top), the Sir1 isolate of LSV (Middle), and the FA isolate of TBV (bottom). Bars represent 200 nm.

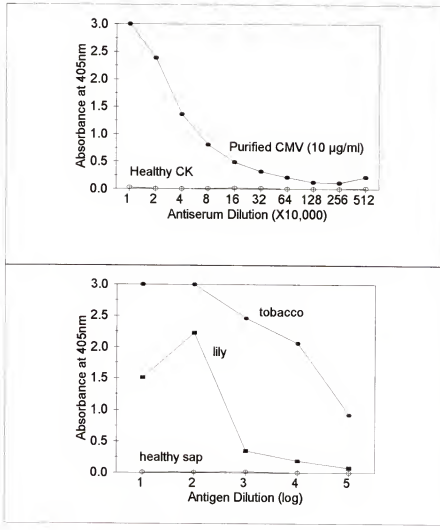

Fig. 2-4. Top: CMV-K45 antiserum titers in indirect ELISA tests determined by reactions with 10 μg /ml of purified homologous antigen and healthy tobacco sap (Healthy CK). Bottom: Antigen dilution end points of CMV-K45-infected tobacco and lily reacting with homologous antiserum in indirect ELISA; compared with extracts from healthy lily sap used as controls.

Fig. 2-5. Serological reactivities of CMV-K45 and its relationship with CMV-2148, CMV-2100, and CMV-WB in reciprocal immunodiffusion tests. Antigens were extracted in 0.5 M sodium citrate (pH 6.5). Agar medium (0.7%) contained 10 mM sodium phosphate (pH 7.6) and 0.1% sodium azide. The center wells contained: CMV-K45 antiserum (1) and CMV-2148 antiserum (2). The peripheral wells contained antigens: H = healthy *N. benthamiana*; K = CMV-K45; C = CMV-2100; 8 = CMV-2148; W = CMV-WB; D = CMV-lily; h = healthy pumpkin; G = CMV-gladiolus.

Fig. 2-6. Serological reactivities of CMV-K45 and its relationship with CMV-2148, CMV-2100, and CMV-WB in reciprocal immunodiffusion tests. Antigens were extracted in 0.5 M sodium citrate (pH 6.5). Agar medium (0.7%) contained 10 mM sodium phosphate (pH 7.6) and 0.1% sodium azide. The center wells contained CMV-2100 antiserum (3) and CMV-WB antiserum (4). The peripheral wells contained antigens: H = healthy *N. benthamiana*; K = CMV-K45; C = CMV-2100; 8 = CMV-2148; W = CMV-WB; D = CMV-lily; h = healthy pumpkin; G = CMV-gladiolus.

The IgG derived from this antiserum could detect LSV in lily extracts with dilutions as high as 1/100,000 (Fig. 2-7).

TBV. Although suitable for Western blot tests, the antiserum against TBV-FA prepared in this study could not be used in indirect ELISA tests due to low antiserum titers and high nonspecific backgrounds. Therefore, antiserum against a lily strain of TBV (TBV-lily) provided by A. F. L. M. Derks in 1989 was substituted for the following tests.

Western Blot

In Western blot tests, potyvirus cross-reactive monoclonal antibodies, PTY1, PTY2, PTY3, PTY4, PTY8, and PTY10, failed to react with either the -TF, -JL, -FA, and -291 TBV isolates, or to RTBV, TBBV, and LiMV (Table 2-2; Fig. 2-8). Some of these antisera did, however, cross react with at least one of the three TuMV-related isolates tested (Table 2-2). Similarly, no reactions were noted for any of the TBV isolates when the admixture of PTY 1 + 2 + 4 + 8 + 10 + 21 + 24 + 30 was used (Fig. 2-9). Five reportedly TBV-specific MCAs were tested against 6 TBV and 3 TuMV-related isolates derived from either lilies or tulips, but only MCA 732 and MCA 733 reacted specifically to any of the six TBV isolates tested (Table 2-3). Interestingly, MCA 676 reacted very strongly with all three of the TuMV-related isolates, but with only one of the six isolates of TBV. In the latter instance, which involved TBV-291, reactions were much weaker than those noted

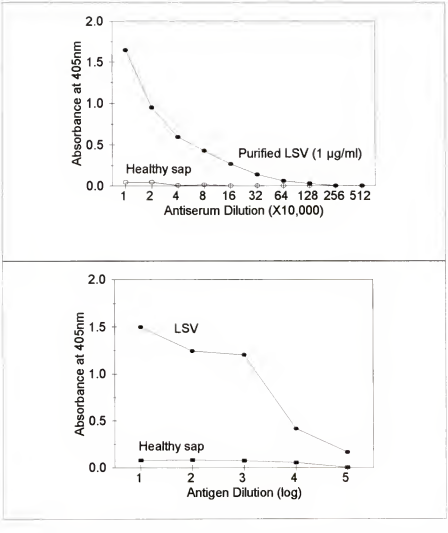

Fig. 2-7. Top: LSV-Sir1 antiserum titers in indirect ELISA tests determined by reactions with 1 µg/ml of purified homologous antigen and healthy lily leaf sap.
Bottom: Antigen dilution end points of LSV-infected and healthy lily extracts reacting with homologous Sir1 IgG in indirect ELISA.

Table 2-2. Cross-reactivities of monoclonal antibodies to potyviruses with viruses isolated from lilies or tulips in Western blot tests

	Antibody						
Antigen [1]	PTY1	PTY2	PTY3	PTY4	PTY8	PTY10	Admix [2]
RTBV	-[3]	-	-	-	-	-	-
TBBV	-	-	-	-	-	-	-
TBV-TF	-	-	-	-	-	-	-
TBV-FA	-	-	-	-	-	-	-
TBV-291	-	-	-	-	-	-	-
LiMV	-	-	-	-	-	-	-
TTBV	+	-	-	-	+	±	+
TCBV	+	±	±	-	+	-	+
WaTV	+	+	+	+	+	-	+
LSV	-	-	-	-	-	-	-
CMV	-	-	-	-	-	-	-
HCK	-	-	-	-	-	-	-

[1] The antigens are RTBV = Rembrandt tulip-breaking virus, TBBV = tulip band-breaking virus, TBV-TF = tulip breaking virus Texas Flame isolate, TBV-FA = tulip breaking virus FA isolate, TBV-291 = tulip breaking virus ATCC PV291 isolate, LiMV = lily mottle virus, TTBV = tulip top-breaking virus, TCBV = tulip chlorotic blotch virus, WaTV = Washington tulip virus, LSV = lily symptomless virus, CMV = cucumber mosaic virus, and HCK = healthy lily sap.

[2] An admixture of monoclonal antibodies of PTY 1, -2, -4, -8, -10, 21, -24, -30 provided by R. Jordan.

[3] Reactivity determined as + when viral protein band is visible in nitrocellulose membrane, - = no reaction, ± = weak reaction.

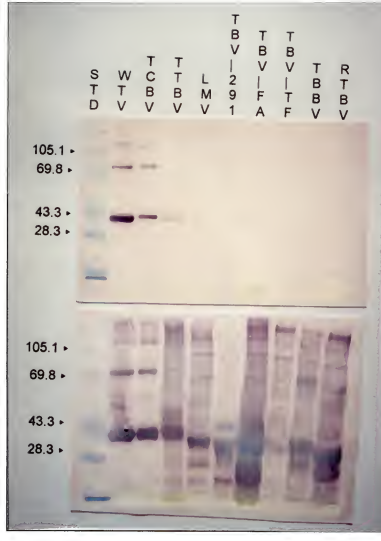

Fig. 2-8. Comparative reactivities of monoclonal antibody PTY1 (top) and TBV polyclonal antibody (bottom) to 9 potyvirus isolates from lilies and tulips in Western blot tests. The labels are STD = standard marker proteins with the numbers to the left indicating the M_r in kilodaltons, WTV = Washington tulip virus, TCBV = tulip chlorotic blotch virus, TTBV = tulip top-breaking virus, LMV = lily mottle virus, TBV-291 = tulip breaking virus ATCC isolate PV291, TBV-FA = tulip breaking virus FA isolate, TBV-TF = tulip breaking virus Texas Flame isolate, TBBV = tulip band-breaking virus, and RTBV = Rembrandt tulip-breaking virus. The multiple bands represent higher oligomers and degradation products.

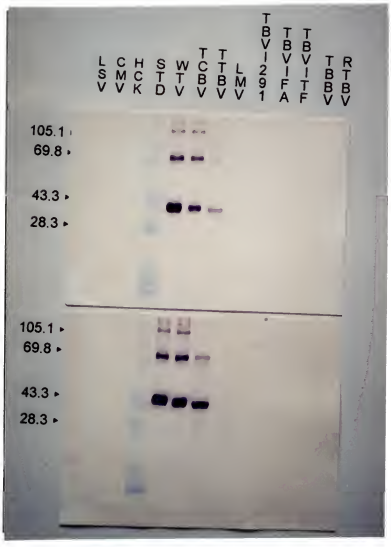

Fig. 2-9. Comparative reactivities of monoclonal antibodies PTY8 (top) and an admixture of PTY 1,-2,-4,-8,-10,-21,-24,-30 (bottom) to 11 viruses from tulips or lilies in Western blot tests. The labels are LSV = lily symptomless virus, CMV = cucumber mosaic virus, HCK = healthy lily sap, STD = standard marker proteins with the numbers to the left indicating the M_r in kilodaltons, WTV = Washington tulip virus, TCBV = tulip chlorotic blotch virus, TTBV = tulip top-breaking virus, LMV = lily mottle virus, TBV-291 = tulip breaking virus ATCC PV291, TBV-FA = tulip breaking virus FA isolate, TBV-TF = tulip breaking virus Texas Flame isolate, TBBV = tulip band-breaking virus, and RTBV = Rembrandt tulip-breaking virus. The multiple bands represent higher oligomers and degradation products.

Table 2-3. Reactivities of one polyclonal antiserum and five monoclonal antibodies to TBV with viruses isolated from lilies or tulips in Western blot tests

	Antisera					
Antigen [2]	PCA 559	MCA 675	MCA 676	MCA 732	MCA 733	MCA [1] 734
RTBV	+[3]	-	-	-	-	-
TBBV	+	-	-	-	-	-
TBV-TF	±	-	-	-	-	-
TBV-FA	+	-	-	+	+	-
TBV-291	+	-	±	±	±	-
LiMV	+	-	-	-	-	-
TTBV	+	-	+	-	-	-
TCBV	+	-	+	-	-	-
WaTV	+	-	+	-	-	-
LSV	-	-	-	-	-	-
CMV	-	-	-	-	-	-
HCK	-	-	-	-	-	-

[1] Polyclonal antiserum (PCA) and monoclonal antisera (MCA) obtained from the American Type Culture Collection (ATCC); the numbers represent their respective PVAS codes.
[2] The antigens are RTBV = Rembrandt tulip-breaking virus, TBBV = tulip band-breaking virus, TBV-TF = tulip breaking virus Texas Flame isolate, TBV-FA = tulip breaking virus FA isolate, TBV-291 = tulip breaking virus ATCC PV291, LiMV = lily mottle virus, TTBV = tulip top-breaking virus, TCBV = tulip chlorotic blotch virus, WaTV = Washington tulip virus, LSV = lily symptomless virus, CMV = cucumber mosaic virus, and HCK = healthy lily sap.
[3] Reactivity determined as + when viral protein band is visible in nitrocellulose membrane, - = no reaction, ± = weak reaction.

for the TuMV-related isolates (Fig. 2-10). PCA 559 reacted with all the tested potyviruses but not with LSV, CMV or the healthy control (Fig 2-11). WaTV antiserum reacted exclusively with the three TuMV-related isolates, whereas TBV PVAS 559 antiserum reacted with all 9 potyviruses tested (Fig. 2-11). LiMV antiserum (UT-478) likewise reacted strongly with both TBV and TuMV-related isolates (Fig. 2-12). None of the tested potyvirus isolates reacted with antisera of LSV and CMV, however (Fig. 2-13). These Western blot results are summarized in Table 2-4.

Effects of Cross-Absorption

After cross-absorption with healthy lily leaf sap, the titer of TBV-lily antiserum was 1/16,000 as determined in indirect ELISA tests by reactions against 1/10 dilutions of TBV-FA-infected lily leaf sap. The other polyclonal antisera obtained from Derks all had high backgrounds. However, cross-absorption eliminated most of the non-specific reactions making them suitable for serodiagnostic work (Fig.2-14).

Evaluation of Antibodies for Serodiagnosis

In ELISA tests, none of the seven cross-reactive PTY MCAs detected any of the seven TBV isolates tested (Table 2-5). PTY1 only reacted with the TuMV-related isolates, TTBV, TCBV and WaTV. Likewise, the admixture of MCAs failed to react with any of the isolates of TBV.

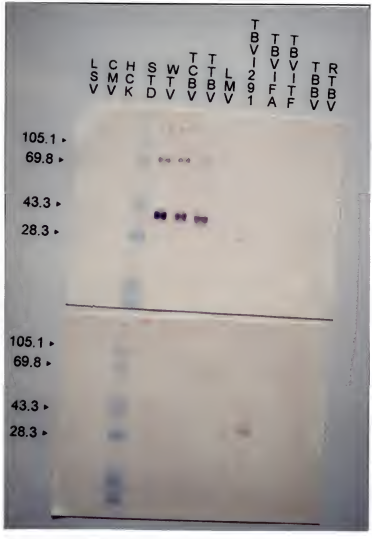

Fig. 2-10. Comparative reactivities in Western blot tests to monoclonal antisera of a tulip isolate of tulip breaking virus, ATCC PVAS 676 (top) and ATCC PVAS 733 (bottom) to 11 viruses isolated from tulips and lilies. The labels are LSV = lily symptomless virus, CMV = cucumber mosaic virus, HCK = healthy lily sap, STD = standard marker proteins with the numbers to the left indicating the M_r in kilodaltons, WTV = Washington tulip virus, TCBV = tulip chlorotic blotch virus, TTBV = tulip top-breaking virus, LMV = lily mottle virus, TBV-291 = tulip breaking virus ATCC PV291, TBV-FA = tulip breaking virus FA isolate, TBV-TF = tulip breaking virus Texas Flame isolate, TBBV = tulip band-breaking virus, and RTBV = Rembrandt tulip-breaking virus. The multiple bands represent higher oligomers and degradation products.

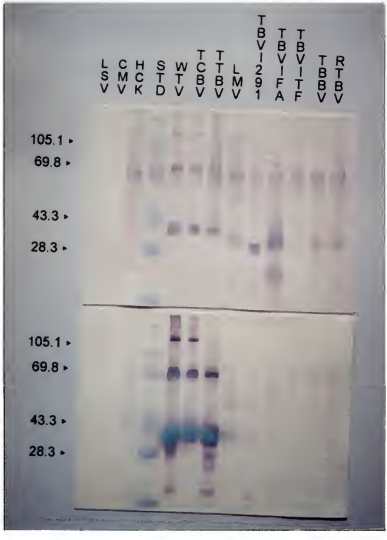

Fig. 2-11. Comparative reactivities in Western blot tests to a polyclonal antiserum of a tulip isolate of tulip breaking virus, ATCC PVAS 559 (top), and a polyclonal antiserum of the Washington tulip virus, ATCC PVAS 388 (bottom) to 11 viruses from tulips or lilies. The labels are LSV = lily symptomless virus, CMV = cucumber mosaic virus, HCK = healthy lily sap, STD = standard marker proteins with the numbers to the left indicating the M_r in kilodaltons, WTV = Washington tulip virus, TCBV = tulip chlorotic blotch virus, TTBV = tulip top-breaking virus, LMV = lily mottle virus, TBV-291 = tulip breaking virus ATCC PV291, TBV-FA = tulip breaking virus FA isolate, TBV-TF=tulip breaking virus Texas Flame isolate, TBBV=tulip band-breaking virus, and RTBV=Rembrandt tulip-breaking virus. The multiple bands represent higher oligomers and degradation products.

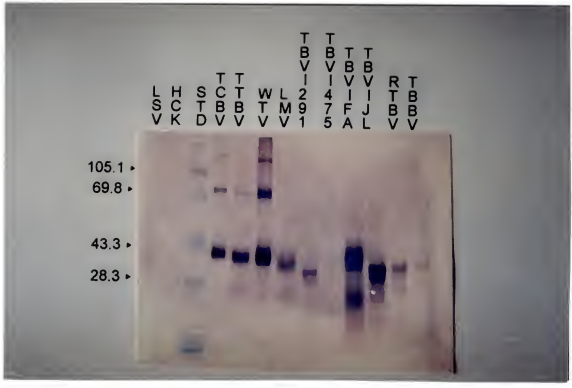

Fig. 2-12. Reactivities of lily mottle virus antiserum (LiMV UT478) to 11 isolates of viruses of tulips or lilies in Western blot tests. The labels are LSV = lily symptomless virus, HCK = healthy lily sap, STD = standard marker proteins with the numbers to the left indicating the M_r in kilodaltons, TCBV = tulip chlorotic blotch virus, TTBV = tulip top-breaking virus, WTV = Washington tulip virus, LMV = lily mottle virus, TBV-291 = tulip breaking virus ATCC PV291, TBV-475 = tulip breaking virus ATCC PV 475, TBV-FA = tulip breaking virus FA isolate, TBV-JL = tulip breaking virus Jack Laan isolate, RTBV=Rembrandt tulip-breaking virus, and TBBV = tulip band-breaking virus. The multiple bands represent higher oligomers and degradation products.

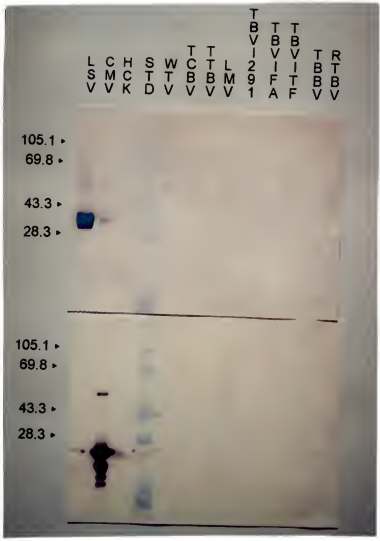

Fig. 2-13. Reactivities of antisera to lily symptomless virus (top) and cucumber mosaic virus (bottom) to 11 viruses isolated from tulips or lilies in Western blot tests. The labels are LSV = lily symptomless virus, CMV = cucumber mosaic virus, HCK = healthy lily sap, STD = standard marker proteins with the numbers to the left indicating the M_r in kilodaltons, WTV = Washington tulip virus, TCBV = tulip chlorotic blotch virus, TTBV = tulip top-breaking virus, LMV = lily mottle virus, TBV-291 = tulip breaking virus ATCC PV291, TBV-FA = tulip breaking virus FA isolate, TBV-TF = tulip breaking virus Texas Flame isolate, TBBV = tulip band-breaking virus, and RTBV = Rembrandt tulip-breaking virus.

Table 2-4. Reactivities of seven polyclonal antibodies to potyviruses with viruses isolated from lilies or tulips in Western blot tests

	Antisera[1)]						
Antigen [2)]	TBV-lily	TBV-AS559	RTBV-RD60	TBV-FA	LiMV-UT478	WaTV-AS388	TuMV-F815
RTBV	+ [3)]	+	+	±	+	-	+ [3)]
TBBV	+	+	-	NT	+	-	-
TBV-TF	±	±	NT	NT	NT	-	NT
TBV-FA	+	+	+	+	+	-	+
TBV-291	+	+	+	+	+	±	-
TBV-475	+	+	+	+	-	NT	-
LiMV	+	+	-	-	+	±	-
TTBV	+	+	+	+	+	+	+
TCBV	+	+	-	-	+	+	+
WaTV	+	+	-	-	+	+	+
LSV	-	-	-	-	-	-	-
CMV	-	-	-	-	NT	-	-
HCK	-	-	-	-	-	-	-

[1)] TBV-lily = antiserum against lily strain of tulip breaking virus (TBV) provided by A. F. L. M. Derks in 1989; TBV-AS559 = TBV antiserum of ATCC PVAS559 isolate, RTBV-RD60 and LiMV-UT478 provided by Derks in 1994; WaTV-AS388 = WaTV antiserum of ATCC PVAS 388; TuMV-F815 = Antiserum of a turnip mosaic potyvirus (TuMV F815) isolate from Florida was provided by D. E. Purcifull.

[2)] The antigens are RTBV = Rembrandt tulip-breaking virus, TBBV = tulip band-breaking virus, TBV-TF = tulip breaking virus, Texas Flame isolate; TBV-FA = tulip breaking virus, FA isolate; TBV-291 = tulip breaking virus, ATCC PV291 isolate; TBV-475 = tulip breaking virus, ATCC PV475 isolate; LMV = lily mottle virus; TTBV = tulip top-breaking virus; TCBV = tulip chlorotic blotch virus; WaTV = Washington tulip virus; LSV = lily symptomless virus; CMV = cucumber mosaic virus; and HCK = healthy lily sap.

[3)] Reactivity determined as + when viral protein band is visible in nitrocellulose membrane, - = no reaction, ± = weak reaction. NT=not tested.

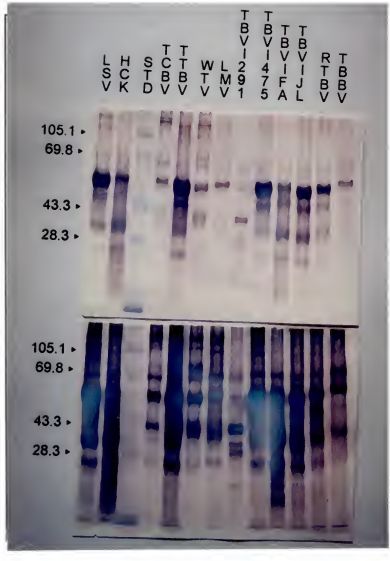

Fig. 2-14. Reactivities of Rembrandt tulip breaking virus RD60 antiserum to 11 viruses of tulips or lilies in Western blot tests. Top : The antiserum was cross-absorbed with healthy lily sap and the IgG was diluted 1/1000. Bottom : Crude antiserum not cross-absorbed with lily leaf sap was used. The labels are LSV = lily symptomless virus; HCK = healthy lily sap; STD = standard marker proteins with the numbers to the left indicating the M_r in kilodaltons; TCBV = tulip chlorotic blotch virus; TTBV = tulip top-breaking virus; WTV = Washington tulip virus; LMV = lily mottle virus; TBV-291 = tulip breaking virus, ATCC PV291 isolate; TBV-475 = tulip breaking virus, ATCC PV 475 isolate; TBV-FA = tulip breaking virus, FA isolate; TBV-JL = tulip breaking virus, Jack Laan isolate; RTBV = Rembrandt tulip-breaking virus; and TBBV = tulip band-breaking virus. The multiple bands represent higher oligomers of viruses and other components from hosts.

Table 2-5. Reactivities in ACP-ELISA of cross-reactive monoclonal antibodies to potyviruses with viruses isolated from lilies or tulips

	Absorbance at 405nm						
Antigen [1]	PTY1	PTY2	PTY3	PTY4	PTY8	PTY10	Admix [2]
RTBV	0.003[3]	0.001	0.000	0.000	0.015	0.003	0.000
TBBV	0.005	0.005	0.000	0.000	0.019	0.003	0.000
TBV-TF	0.007	0.008	0.000	0.000	0.009	0.000	0.000
TBV-FA	0.001	0.001	0.004	0.000	0.005	0.000	0.000
TBV-291	0.007	0.018	0.007	0.005	0.005	0.006	0.000
TBV-475	0.002	0.012	0.000	0.000	0.009	0.004	0.000
LiMV	0.001	0.014	0.000	0.000	0.001	0.004	0.001
TTBV	0.055	0.001	0.001	0.000	0.081	0.009	0.031
TCBV	1.221	0.019	0.000	0.000	1.105	0.043	0.588
WaTV	2.013	0.011	0.216	0.532	0.397	0.006	0.875
LSV	0.002	0.008	0.013	0.011	0.001	0.002	0.000
CMV	0.000	0.009	0.001	0.003	0.018	0.003	0.000
HCK	0.008	0.016	0.007	0.004	0.008	0.006	0.002

[1] The antigens are RTBV = Rembrandt tulip-breaking virus, TBBV = tulip band-breaking virus; TBV-TF = tulip breaking virus, Texas Flame isolate; TBV-FA = tulip breaking virus, FA isolate; TBV-291 = tulip breaking virus, ATCC PV291 isolate; TBV-475 = tulip breaking virus, ATCC PV475 isolate; LiMV = lily mottle virus; TTBV = tulip top-breaking virus; TCBV = tulip chlorotic blotch virus; WaTV = Washington tulip virus; LSV = lily symptomless virus; CMV = cucumber mosaic virus; and HCK = healthy lily sap.

[2] An admixture of monoclonal antibodies consisting of PTY 1, -2, -4, -8, -10, -21, -24, -30.

[3] Absorbances were recorded 90 minutes after addition of substrate, and represent the averages of duplicate wells.

In similar tests involving the use of TBV-specific MCAs, ATCC-PVAS732 reacted with only TBV-FA, whereas ATCC-PVAS676 reacted with only the TuMV-related TCBV, WaTV, and TTBV isolates (Table 2-6). When PCA ATCC-PVAS559 was tested, however, high A_{405nm} values were noted for both TBV-FA and the TuMV-related TCBV.

Although the polyclonal antisera obtained from Holland (TBV-lily, TBV-TF-RE102, RTBV-RD60, and LiMV-UT478) reacted strongly with TBV, strong background values seriously interfered with interpretations of the results (Table 2-7). However, cross-absorption with healthy plant sap eliminated much of the background, while retaining the reactivity of the antiserum to TBV (Fig 2-15). Unexpectedly, the TBV-lily PCA cross-reacted unilaterally with PVY; whereas it reacted with PVY antigen, PTY1 MCA did not react reciprocally with TBV (Fig. 2-15).

The inability of PTY1 MCA to react with TBV-infected tissue was confirmed in additional tests in which leaves and scales as sources of antigens were compared (Table 2-8).

Table 2-6. Reactivities in ACP-ELISA of one polyclonal antiserum and five monoclonal antibodies to tulip breaking virus with viruses isolated from lilies or tulips

	Absorbance at 405nm					
Antigen [2]	PCA [1] 559	MCA 675	MCA 676	MCA 732	MCA 733	MCA 734
RTBV	0.448 [3]	0.001	0.000	0.019	0.007	0.019
TBBV	0.482	0.004	0.000	0.009	0.007	0.010
TBV-TF	0.361	0.006	0.004	0.006	0.006	0.011
TBV-FA	3.000	0.029	0.000	0.463	0.020	0.020
TBV-291	0.659	0.006	0.012	0.047	0.027	0.015
TBV-475	0.410	0.007	0.019	0.010	0.008	0.012
LiMV	0.289	0.006	0.001	0.015	0.012	0.012
TTBV	0.266	0.002	0.071	0.036	0.004	0.009
TCBV	1.758	0.023	2.563	0.018	0.021	0.017
WaTV	0.542	0.006	0.131	0.030	0.014	0.020
LSV	0.235	0.006	0.011	0.023	0.017	0.017
CMV	0.186	0.006	0.009	0.021	0.006	0.014
HCK	0.234	0.011	0.008	0.056	0.035	0.022

[1] Polyclonal antiserum (PCA) and monoclonal antibody (MCA) obtained from the ATCC; the numbers correspond to their PVAS codes.
[2] The antigens are RTBV = Rembrandt tulip-breaking virus; TBBV = tulip band-breaking virus; TBV-TF = tulip breaking virus Texas Flame isolate; TBV-FA = tulip breaking virus FA isolate; TBV-291 = tulip breaking virus ATCC PV291; TBV-475 = tulip breaking virus ATCC PV475; LiMV = lily mottle virus; TTBV = tulip top-breaking virus; TCBV = tulip chlorotic blotch virus; WaTV = Washington tulip virus; LSV = lily symptomless virus; CMV = cucumber mosaic virus; and HCK = healthy lily sap.
[3] Absorbances were recorded 2 hours after addition of the substrate and represent the averages of duplicate wells.

Table 2-7. Reactivities in ACP-ELISA of five polyclonal antibodies to potyviruses with viruses isolated from lilies or tulips

	Absorbance at 405nm				
Antigen [2]	TBV-lily [1]	TBV-TF RE102	RTBV-RD60	LiMV-UT478	WaTV-AS388
RTBV	0.231 [3]	1.214	2.500	1.179	0.066
TBBV	0.171	1.130	2.404	1.199	0.079
TBV-TF	0.150	0.937	2.785	0.723	0.057
TBV-FA	2.309	1.218	1.096	3.000	0.077
TBV-291	0.223	0.722	0.830	1.896	0.025
TBV-475	0.160	0.602	1.770	1.930	0.025
LiMV	0.090	0.333	0.641	0.941	0.045
TTBV	0.163	1.332	1.391	2.090	0.514
TCBV	0.644	1.584	0.527	2.716	3.000
WaTV	0.215	0.468	0.342	2.848	2.839
LSV	0.057	0.462	1.175	2.112	0.024
CMV	0.046	0.236	0.828	1.005	0.021
HCK	0.059	0.572	0.653	1.086	0.026

[1] TBV-lily = antiserum against a lily strain of TBV provided by Derks in 1989; TBV-RE102, RTBV-RD60 and LiMV-UT478 provided by A.F.L.M. Derks in 1994; WaTV-AS388 = WaTV antiserum of ATCC PVAS 388.
[2] The antigens are RTBV = Rembrandt tulip-breaking virus; TBBV = tulip band-breaking virus; TBV-TF = tulip breaking virus, Texas Flame isolate; TBV-FA = tulip breaking virus, FA isolate; TBV-291 = tulip breaking virus, ATCC PV291 isolate; TBV-475 = tulip breaking virus, ATCC PV475 isolate; LiMV = lily mottle virus; TTBV = tulip top-breaking virus; TCBV = tulip chlorotic blotch virus; WaTV = Washington tulip virus; LSV = lily symptomless virus; CMV = cucumber mosaic virus; and HCK = healthy lily sap.
[3] Absorbances were recorded 30 minutes after addition of substrate and represent the average of duplicate wells.

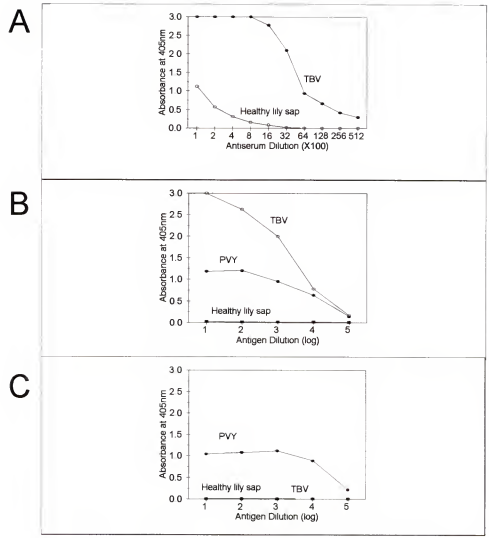

Fig. 2-15. A: Relative absorbance values of TBV-infected and healthy lily leaf tissue at different TBV antiserum dilutions; TBV-lily polyclonal antiserum was used, and the antiserum was serially diluted 1/2 (v:v) with conjugate buffer for ELISA.
B: Relative absorbance values of TBV-infected and healthy lily leaf extracts and PVY-infected tobacco (*Nicotiana tabacum*) leaf extracts against TBV-lily polyclonal antiserum. Antiserum was cross-absorbed with healthy lily leaf sap, and 1/1000 diluted IgG was used. Antigens were serially diluted 1/10 (v:v) with coating buffer for ELISA.
C: Relative absorbance values of PVY-infected and healthy tobacco leaf extracts and TBV-infected lily leaf extracts against PTY1 monoclonal antiserum: Antigens were serially diluted 1/10 (v:v) with coating buffer for ELISA.

Table 2-8. Comparative effectiveness of tulip breaking and lily mottle virus polyclonal and PTY1 monoclonal antisera for detecting tulip breaking virus in lily samples by ELISA

Tissue	Total tested samples	No. of samples reacted with TBV			LiMV			PTY1		
		-	+	++	-	+	++	-	+	++
Leaves[1)]	20	0	0	20	0	0	20	20	0	0
Scales[2)]	86	19	60	7	25	49	12	86	0	0

[1)] Samples collected from TBV-FA-inoculated plants.
[2)] Samples collected from TBV-infected fresh bulbs.
- = ELISA values less than two times that of negative control.
+ = ELISA values at least two times greater than but less than ten times that of negative control.
++ = ELISA values at least ten times greater than that of negative control.

Discussion

Based on host reactions, virus particle morphology, and serological results, the Sir1 isolate of LSV is typical of others that have been described previously. The CMV-K45 isolate from lily is a cucumovirus closely related to the CMV-2100 and WB isolates of CMV used in this investigation. Likewise, the FA isolate of TBV is like other lily-infecting isolates of this virus. Our isolate appears to be identical to TBV-JL, also known as Rembrandt tulip breaking virus and closely related to lily mottle virus (i. e. TBV-L).

Since 1935 when Wellman (168) found lily to be susceptible to CMV, bioassays were used to detect CMV in lilies. The virulence of lily isolates, however, differs from that of typical CMV isolates (35). It is not readily transmitted by sap inoculation directly from lily to other herbaceous hosts. Thus, bioassays for detecting CMV in lilies may be of dubious value.

Civerolo *et al.* (58) purified a lily isolate of CMV in 1969. No information was available about the relationships between isolates infecting lily and other isolates of CMV. In 1987, Maeda and Inouye (109), obtained 17 isolates of CMV from lilies grown in Japan, all of which belonged to the Y serotype. In my work, the results of immunodiffusion tests show that CMV-K45 is serologically related to, but distinct from, all three strains of CMV tested from other hosts i. e. *Commelina diffusa*, cucurbits, and winged bean. Nevertheless, while isolate difference may exist, no evidence exists that polyclonal antiserum from any of these isolates would fail to cross react with lily isolates of CMV, and thus such antisera could be used reliably for indexing purposes.

LSV was purified in this study without the difficulties described by Civerolo (57). In this study, plantlets regenerated from a bulb which was infected with LSV were used for purification. Propagating the plants from scales allowed sufficient time for the virus to multiply and become distributed more uniformly throughout the plant. As a result,

I was able to obtain high LSV titers. The lily cultivar, 'Sirocco,' was especially suitable as a propagation host for LSV inasmuch as the leaf extracts of this lily were not as viscous as those of *L. formosanum*.

In sharp contrast, repeated attempts to purify TBV-FA did not result in a satisfactory quantity of viral protein for antiserum production. This failure can be attributed to the source plant, *L. formosanum*. Although this host supports high concentration of virus, its viscous extracts interferes with purification. TBV-FA particles aggregated in the extraction buffer even though EDTA or DIECA was added, resulting in an irreversible precipitation of the virus, which was then lost during low speed centrifugation. Moreover, the viral protein was susceptible to isopycnic centrifugation using cesium chloride or cesium sulfate. Based on the high A260/A280 ratios of purified preparations and serological reactions with TBV antiserum, the final product consisted primarily of exposed nucleic acids and degraded protein coat subunits.

Considerable confusion exists regarding the relationships between TBV and other potyviruses from lilies and tulips. The most recent reports in 1993-94 from the Netherlands (23,63,71) provide different names for various isolates of these potyviruses.

There are four viruses comprising the tulip breaking virus complex. One of them, TBBV, apparently does not infect lilies, and hence it is probably not a factor in a lily virus

indexing program. Certain TBV isolates, sometimes referred to as TBV-L, apparently can infect *N. benthamiana* and certain other dicotyledonous hosts. However, none of my isolates was able to infect such plants. Of the remaining two, the Rembrandt tulip breaking virus, or TBV-JL, appears to be most closely related to the viruses encountered in this investigation. Indeed, serological studies confirm that they are more closely related to it than to the TBV-TF serotype, which, according to Dutch workers, is more prevalent in tulips than lilies.

From the data obtained in this investigation, I cannot exclude the possibility that Derks' purified TBV samples, which were from tulip, is mixed with the TTBV, TCBV or WaTV viruses, which are closely related to TuMV and distinct from TBV (87,88). However, before Mowat (126) in 1985 found the TCBV from tulips, TBV was considered the only potyvirus capable of infecting this host. Thus, when Derks *et al.* (73) purified TBV in 1982 from field samples, the presence of any other potyvirus was unsuspected. Yet, the antisera produced by the Dutch workers is still extensively used throughout the world to detect potyviruses in both lilies and tulips. Antiserum is derived from rabbits which receive a booster injections every 6-12 months over a span of 4-6 years (70). If the antiserum was derived from plants infected with both TBV and TuMV, it would inevitably have a correspondingly broader cross-reactivity encompassing both viruses.

According to R. Jordan, the originator of the PTY MCAs (97), TBV is one of the 55 potyviruses that reacts with PTY1 (95,96,98,99). However, in this study, none of the TBV isolates could be detected by this or any of the other cross-reactive potyvirus MCAs. Yet, these and the corresponding polyclonal antisera could readily detect the WaTV, TCBV, and TTBV isolates of turnip mosaic, which do not infect lilies. That being the case, any MCA or PCA derived from Dutch tulip virus isolates prior to 1985 could conceivably have epitopes to either virus, including those MCAs studied by Hsu *et al.* (91). Certain MCAs from these workers reacted to some of our lily samples, but most did not, which could be accounted for if some of the MCA clones were to those tulip-infecting viruses related to turnip mosaic virus. This situation would create confusion in ascertaining virus identification in tulips, and, in relation to detecting TBV in lilies, would compromise indexing results for certification programs if it is assumed that PTY1 or other cross-reactive MCA readily react with viruses of the tulip breaking symptoms. As this study clearly shows, these MCAs do not readily detect any of the potyviruses we found infecting lilies.

CHAPTER 3
VIRUS DISTRIBUTION AND ITS RELATION TO DETECTION

Introduction

For virus-indexing in lilies, especially for certification programs, the irregular distribution of viruses in various parts of lily plants poses a significant problem for their detection by serological or other means (72,92,105,158). Virus titers also can be influenced by the environments in which the plant is grown, especially temperature (60). Likewise, virus titers can vary according to the reaction of different lily cultivars to virus infection (57). It is therefore necessary to take these factors into account when developing a reliable protocol for indexing plants for certification.

Although LSV infects lily systemically, it is not evenly distributed in infected plants. Allen and Lyons (13) used electron microscopy to observe the LSV virions in diseased 'Ace' Easter lily. They found more LSV particles from leaves in the middle of the flower stalk than elsewhere. In contrast, Derks and Vink-Van den Abeele (72) realized that LSV could be detected readily by serological means only during the post-flowering stage, and that the highest concentration of LSV

occurred in the leaves located about one quarter of the distance below the shoot apex. Van Slogteren (162) reported that the highest concentration of LSV is reached shortly after flowering in leaves located about 1/4 of the distance from the top of the plant. In freshly harvested bulbs only the 3 largest outer scales consistently had high LSV titers.

Beijersbergen and Van der Hulst (31) used ELISA to detect LSV in leaves and bulb scales. In their study, LSV was readily detected in leaf samples of all four varieties they tested, but for the bulb scales, it was detected only in the variety 'Enchantment.' To improve the reliability in bulb testing, they suggested adding hemicellulase to reduce the viscosity of extracts for ELISA (32).

After van Schadewijk (158) demonstrated the large differences of virus content in individual scales from lily bulbs, he used this information to improve the reliability of his ELISA indexing procedures. Likewise, scientists in Japan were able to use this information to facilitate their own virus indexing programs (85, 86).

Materials and Methods

Efficiency of LSV Detection in Bulb Derivatives at Different Stages

Scales removed from individual sprouting bulbs of 'Gran Paradiso' lily were indexed for LSV by indirect ELISA. Ten

and 20 weeks after sowing, leaf samples were collected from plants grown in a greenhouse at >30C. Bulbils, bulblets, and daughter bulbs were sampled immediately after harvesting.

Efficiency of LSV Detection at Different Temperatures

Eighty-eight lily plants comprising 11 varieties maintained in greenhouses at <30C and 57 plants comprising 8 varieties at >30C were selected for LSV indexing. Leaves on the lower portions of stems and those on upper portions were collected independently and compared with outer bulb scales for the presence of LSV.

Individual leaves of 'Gran Paradiso' lily plants maintained in greenhouses at <30C and at >30C were indexed for LSV. The distal one-third of leaves were removed for testing at the pre-flowering stage, whereas the proximal portions of each leaf were tested immediately after being collected.

Variety Effects on LSV Detection

Three cultivars of Asiatic lilies ('Gran Paradiso,' 'Nepal,' and 'Pollyanna') and three of Oriental type lilies ('Casablanca,' 'Laura Lee,' and 'Stargazer') maintained in a greenhouse at >30C were tested for LSV distribution. Each leaf of individual plants was collected at the post-flowering stage for ELISA tests.

Patterns of Virus Distribution

The patterns of virus distribution were established with regard to virus content in the different leaves and scales of individual plants. Based on the ELISA values (y axis) and their leaf or scale positions (x axis), it was possible to create a linearized pattern reflecting trends in virus distribution and titer. Most of leaf samples were collected at the flowering stage. In some studies, scales were removed from bulbs at the onset of dormancy and in others, after storage.

LSV. Lilies cvs. 'Sirocco,' 'Yellow Blaze,' and 'Gran Paradiso' determined to be infected with LSV during virus surveys were used for this experiment.

TBV. Plants of 'Sirocco' lily grown from bulbs derived from plants manually inoculated with TBV-FA were maintained in greenhouses at >30C and <30C. Leaves and scales were selected for ELISA tests as described above.

CMV. Plants of 'Sirocco' lily grown from bulbs derived from plants manually inoculated with CMV-K45 were the subjects for this experiment. The plants were separated into two groups: one maintained in a greenhouse at >30C and another one at <30C.

TBV and LSV mixed Infection. A diseased plant of 'Nellie White' Easter lily determined during surveys to be doubly infected with TBV and LSV was selected for this study. Leaves sampled at flowering and at senescence were tested by ELISA

for TBV and LSV. Scales were removed from a freshly harvested bulb and compared with those removed from a bulb stored at 4C for 10 months.

Effectiveness of Virus Indexing

Since all plants of 'Nellie White' Easter lilies tested during surveys were doubly infected with TBV and LSV, these materials were used for ascertaining the effectiveness of the virus indexing methods used in this investigation. Leaves collected from 120 plants at flowering, scales collected from 169 plants at harvest, and scales from 36 sprouting bulbs were included in the tests.

Results

Evaluation of sampling techniques

The various tissues sampled for indexing differed substantially with respect to LSV titers. In general, LSV titers dropped precipitously in above ground tissues subject to high (>30C) growing temperatures (Tables 3-1 and 3-2). Sudden diminutions of LSV titers could be reduced somewhat, however, when plants were grown at lower temperature (<30C). Also, LSV titers were generally higher in the lower leaves of plants than in the newer ones produced at the apex. Differences in LSV titers were also noted between lily cultivars (Figs. 3-1 and 3-2).

Table 3-1. Relative ability to detect LSV by ELISA in different tissues of a 'Grand Paradiso' lily plant grown in greenhouse at >30C

Tissue for virus indexing	No. of samples	% of plants reacted		
		++ [3]	+	-
Mother bulb scale	25	84	12	4
Leaf [1]	25	8	16	76
Leaf [2]	23	4	0	96
Aerial bulbil	15	0	0	100
Bulblet	6	0	0	100
Daughter bulb scale	25	48	20	32

[1] Leaves sampled 10 weeks after planting.
[2] Leaves sampled 20 weeks after planting.
[3] ++ = ELISA values at least ten times greater than that of negative control, + = ELISA values at least twice as high but less than ten times that of negative control, - = ELISA values less than twice that of negative control.

Table 3-2. Relative ability to detect LSV in different lily tissues in relation to temperature

	% of samples reacted with LSV antibody								
	Scales (Mother bulb)			Upper leaves			Lower leaves		
Environment	-[3]	+	++	-	+	++	-	+	++
<30C [1]	25	8	67	33	28	39	33	36	31
>30C [2]	4	11	86	90	10	0	35	56	9

[1] Data from 88 plants comprising 11 varieties.
[2] Data from 57 plants comprising 8 varieties.
[3] - = ELISA values less than twice that of negative control, + = ELISA values at least two times greater but less than ten times that of negative control, ++ = ELISA values at least ten times greater than that of the negative control.

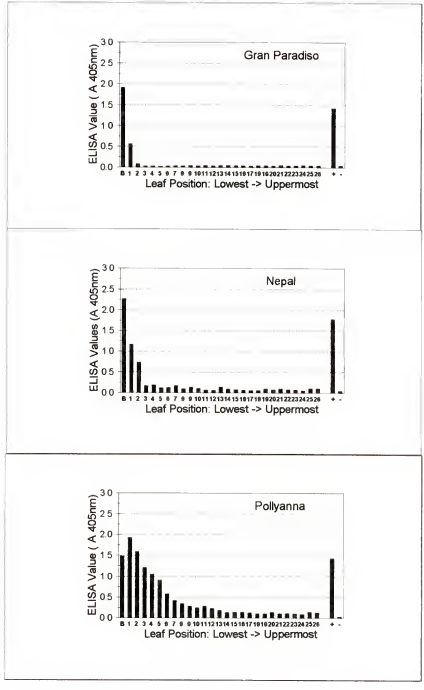

Fig. 3-1. LSV distribution and detection by ELISA in leaves of Asiatic lilies 'Gran Paradiso,' 'Nepal,' and 'Pollyanna' grown at >30C. B = Scales from pre-emergent bulb; + and - = leaves from LSV-infected lilies and healthy control lilies, respectively.

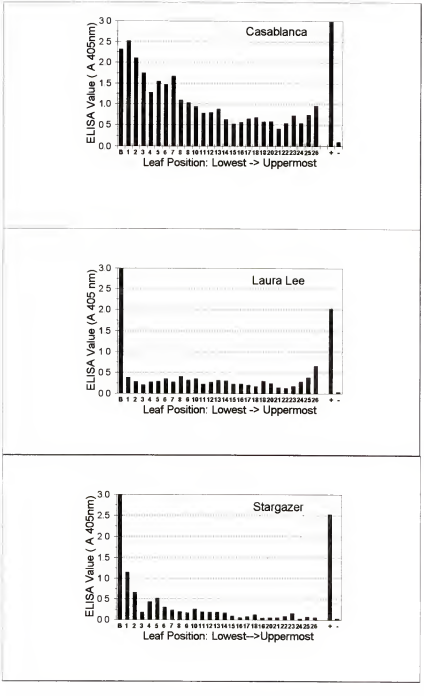

Fig. 3-2. LSV distribution and detection by ELISA in leaves of Oriental lilies 'Casablanca,' 'Laura Lee,' and 'Stargazer' grown at >30C. B = Scales from pre-emergent bulb, + and - = leaves from LSV-infected lilies and healthy control lilies, respectively.

High temperatures inhibited the spread of LSV in the leaves of 'Gran Paradiso' lily. Shortly after sprouting, leaves were produced that contained detectable levels of LSV. Later, however, no virus could be detected from any of the leaves. Yet, when plants were maintained at <30C LSV titers in leaves were still at detectable levels (Fig. 3-3).

The outer lily bulb scales in general had higher LSV titers than inner ones, although in the case of 'Yellow Blaze' lily, differences between inner and outer bulb scales were not statistically significant (Fig. 3-4).

Unlike LSV, TBV-infected plants sustained high titers in every leaf provided plants were maintained at <30C (Fig. 3-5). When plants were maintained at >30C, TBV titers dropped, particularly in the lower leaves (Fig 3-6). TBV titers in freshly harvested bulbs were very low, in contrast to the leaves, especially in plants maintained at relatively high temperatures (>30C) (Figs. 3-5 and 3-6).

CMV distribution patterns differed substantially from those observed in LSV. The highest CMV titers were obtained in leaves near the plant apex and in the innermost bulb scales (Fig. 3-7). Like LSV, however, titers were adversely affected by high temperatures. For example, CMV could not be detected in any of the scales harvested from plants maintained at >30C (Fig.3-8).

In plants doubly infected with LSV and TBV, LSV could only be detected in the lower leaves, whereas TBV maintained

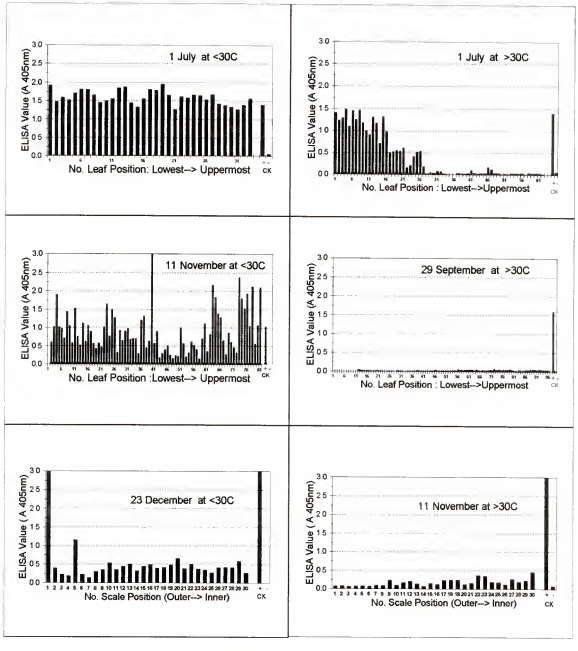

Fig. 3-3. LSV detection in leaves and scales of 'Gran Paradiso' by ELISA on different dates and at different temperatures. On 1 July, there were 33 leaves on the plant grown at <30C and 62 leaves on the one grown at >30C; both bulbs sprouted in March, 1993.

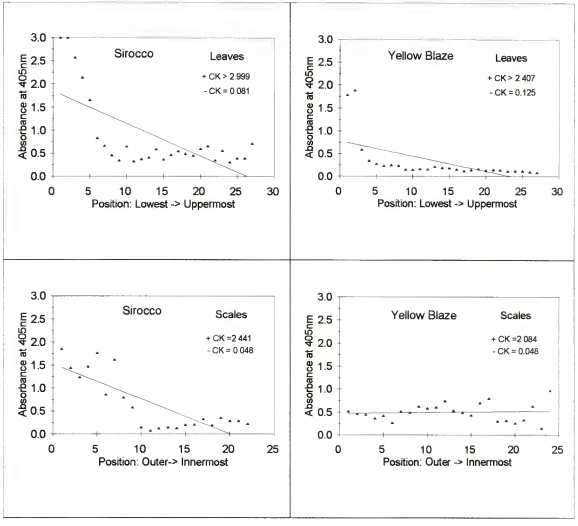

Fig. 3-4. LSV distribution and detection by ELISA in leaves and scales of infected 'Sirocco' or 'Yellow Blaze' lily maintained in a greenhouse at >30C. The orientations of the straight lines were based on the relatedness (slope) between the x and y axes whereby each point was derived from average ELISA values of triplicate wells.

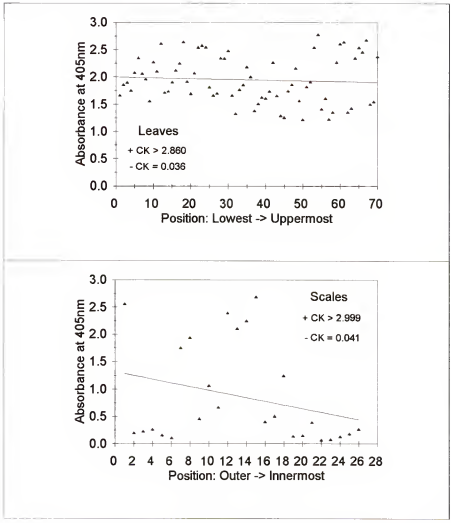

Fig. 3-5. TBV distribution and detection by ELISA in leaves and scales of an infected 'Sirocco' lily maintained in a greenhouse at <30C. The orientations of the straight lines were based on the relatedness (slope) between the x and y axes whereby each point was derived from average ELISA values of triplicate wells.

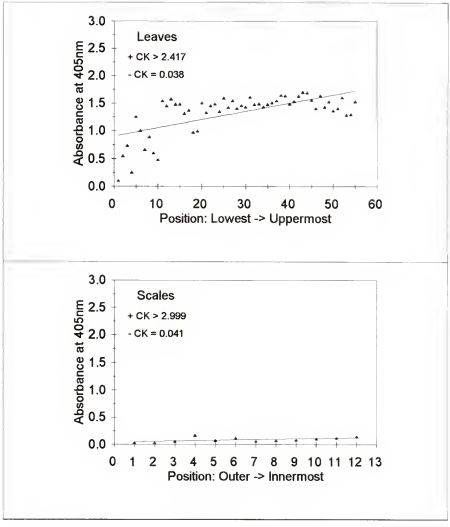

Fig. 3-6. TBV distribution and detection by ELISA in leaves and scales of an infected 'Sirocco' lily maintained in a greenhouse at >30C. The orientations of the straight lines were based on the relatedness (slope) between the x and y axes whereby each point was derived from average ELISA values of triplicate wells.

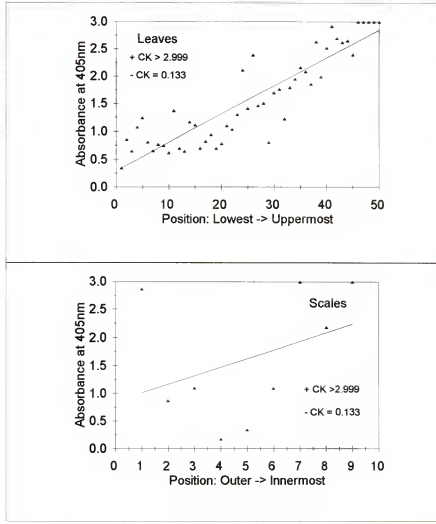

Fig. 3-7. CMV distribution and detection by ELISA in leaves and scales of an infected 'Sirocco' lily maintained in a greenhouse at <30C. The orientations of the straight lines were based on the relatedness (slope) between the x and y axes whereby each point was derived from average ELISA values of triplicate wells.

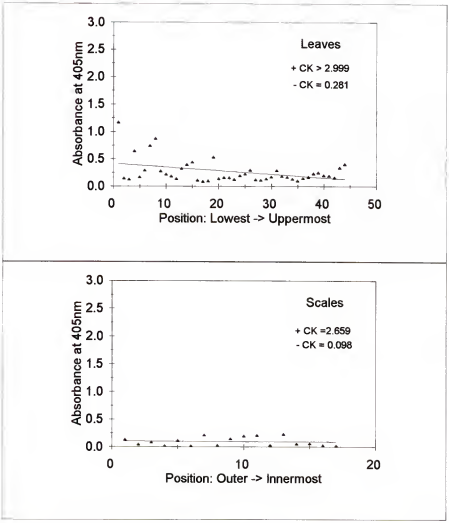

Fig.3-8. CMV distribution and detection by ELISA in leaves and scales of an infected 'Sirocco' lily maintained in a greenhouse at >30C. The orientations of the straight lines were based on the relatedness (slope) between the x and y axes whereby each point was derived from average ELISA values of triplicate wells.

high titers throughout the growing season in newly formed leaves produced near the apex (Fig. 3-9). At the end of the growing season, however, TBV titers dropped considerably, whereas those of LSV remained relatively high (Fig. 3-9). After storage at 4C for 10 months, however, very high titers of both TBV and LSV were recorded (Fig.3-10).

Discussion

Uneven distribution in plant tissues has been described as a major factor affecting virus indexing. However, the general pattern for virus distribution in lilies is not easy to ascertain within a short period of time. Hagita (85, 86) in 1989 showed that CMV and LSV could be detected within each scale, but nevertheless he recommended that reliable detection was contingent upon homogenizing two or more outer and/or inner bulb scales for ELISA tests to account for erratic virus distributions within individual scales. TBV was not readily detected in his studies. Van Schadewijk (158) in 1986 also showed differences in virus concentrations between individual scales, and he also reported that indexing results were much more reliable when bulbs were indexed after a three week storage period. These titer variations are affected greatly by the temperature conditions as noted in this study. Thus, we conclude that the most reliable indexing results for all three viruses is obtained by sampling stored bulbs. The outermost scale of sprouting bulbs is perhaps the best material for

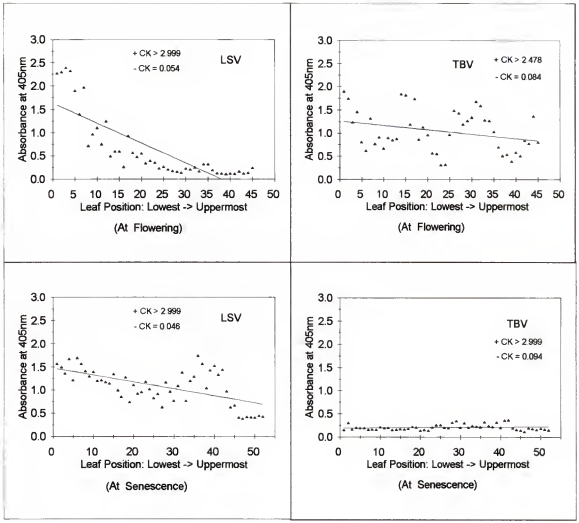

Fig. 3-9. Virus distribution and detection by ELISA in different leaves during and after flowering in a 'Nellie White' plant infected with both LSV and TBV. The orientations of the straight lines were based on the relatedness (slope) between the x and y axes whereby each point was derived from average ELISA values of triplicate wells.

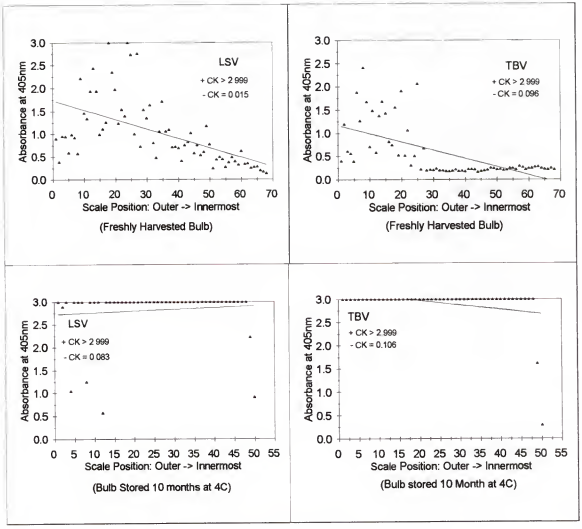

Fig.3-10. Virus distribution and detection by ELISA in 'Nellie White' Easter lily infected with both LSV and TBV in relation to bulb storage time. The orientations of the straight lines were based on the relatedness (slope) between the x and y axes whereby each point was derived from average ELISA values of triplicate wells.

indexing. Inasmuch as high temperatures appear to affect virus titers markedly, great care should be taken to sample only when prospects for high virus titers are at their optima. It is also recommended that mother block plants to be indexed for virus be grown at temperatures less than 30C to maximize prospects for high virus titers.

CHAPTER 4
VIRUS INCIDENCE AND DISEASE CONTROL

Introduction

Epidemiological information is an essential component for developing reliable and practical control strategies for plant viruses. At the very least, the virus identities and their relative prevalences must be ascertained. Viral diseases in lilies have caused problems at least since the late 1800's, and it is in generally acknowledged that LSV is widespread in commercial plantings. Although Brierley (42) in 1940 reported that TBV and CMV are common in commercial Easter lilies, less is known about other important lily viruses. Allen and Fernald (10) found more than 99 percent of the Easter lilies they surveyed in 1972 to contain long rod-shaped virus particles, and they determined that LSV was common in lilies grown in the western U.S. In contrast, Ballantyne *et al.* (29) who conducted a systemic survey in 1979 for viruses of native *Lilium* species detected no LSV even though the plants were collected very close to fields where commercial Easter lilies were grown for many years. A similar field survey was conducted in Japan by Hagita *et al.* (86) in 1989, who found more CMV than either LSV or TBV in the edible Maximowicz's

lily. In Asiatic lilies, however, LSV was the most frequently encountered virus. Yang *et al.* (171) in 1993 studied 17 commercial lily samples from the U.S. and 173 samples from Taiwan, all of which were determined to be infected with LSV.

A better knowledge basis of the relative incidences of different lily viruses has been hampered by the latency of symptoms they induce, especially for viruses such as LSV. Moreover, lily viruses tend to express symptoms intermittently. Indeed, necrotic fleck symptoms tend to be masked at high temperatures (121). Nevertheless, lily viruses cause serious yield losses. As cut flowers, the vase life of lilies is reduced by virus infection (26). Infections with TBV can cause conspicuous symptoms in certain cultivars and can render then unfit for market (26). Since LSV-infected lilies are usually latent this virus cannot be controlled by a program of roguing in cultivation. This could account in part why this virus is so commonly found in commercial plantings on a world-wild basis (5). CMV, in contrast, almost eliminated Bermuda's Easter lily industry 100 years ago but now occurs at low incidence because of a program of roguing being implemented there (121).

Viruses have been eliminated from lilies by meristem-tip culture (166). In the absence of other treatments, only 0.1 - 0.3 mm tissue layer in the shoot apex of CMV-infected lily proved virus-free (125). Thus, unless great care is taken, the use of shoot-tip culture may be only partially successful.

Chemotherapy followed by shoot-tip culture has been suggested as an improvement for obtaining a larger proportion of virus-free lily (38,157). Thermotherapy apparently has not been widely used to obtain virus-free lily planting stock, however, even though titers of both LSV and TBV are reduced precipitously when plants are grown at high temperatures of >30C (60). A certain proportion of bulblets obtained from CMV-infected Easter lily bulbs may be virus-free (44). Likewise, some virus-free planting units can be obtained from LSV-infected Asiatic lilies simply by removing bulblets derived from infected scale explants grown axenically (4,9). Regardless, none of the aforementioned techniques were fail-safe and required subsequent indexing to verify that the propagating units were indexed virus free.

Materials and Methods

Source of Lilies

Bulbs imported from the Netherlands. Nine cultivars of imported Dutch lily bulbs ('Casablanca,' 'Gran Paradiso,' 'Jolanda,' 'Laura Lee,' 'Nepal,' 'Pollyanna,' 'Sirocco,' 'Stargazer,' and 'Yellow Blaze') were provided by J. F. Tammen, in 1993. Another 15 cultivars of imported Dutch lily bulbs ('Beatrix,' 'Berlin,' 'Cascade,' 'Dame Blanche,' 'Dreamland,' 'Geneve,' 'Gypsy Eyes,' 'Montreaux,' 'Nippon,' 'Nove Centro,' 'Royal Dutch,' 'Stargazer Max,' 'Unique,'

'Variant,' and 'Vivaldi') were provided by W. L. Preston, Manatee Fruit Co. (Palmetto, FL.), in 1994.

Bulbs purchased from various retailers. A collection of lily bulbs was obtained through various mail-order catalogues and local markets for the retail market. They included 6 Asiatic lilies ('Connecticut King,' 'Enchantment,' 'Gran Paradiso,' 'Red Lilium,' 'Sirocco,' and 'Yellow Lilium') and 2 Oriental lilies ('Casablanca' and 'Stargazer'). In addition, 'Golden Splendor' and 'Pink Perfection,' and the Aurelian lilies, 'Red Tiger' and 'Album' were included, as were the Trumpet lilies not designated by cultivar. Six miniature lilies ('Golden Pixie,' 'Minicharisma,' 'Minicream,' 'Minicream Brushmark,' 'Orange Pixie,' and 'Orange Pixie New') were obtained from plant breeders in Oregon in 1993 through J. F. Tammen. Most of the Easter lilies were purchased from local retailers as pot plants during the Easter holidays. The bulbs were field grown in northern California or southern Oregon and sprouted for the retail market in Florida, North Carolina, or Canada.

Virus Incidence Survey

Antisera against CMV, LSV, and TBV were adopted for indirect ELISA as described in chapter 2. For routine indexing, the lower leaves or the outer scales were selected.

Virus Incidence in Plantlets through Micropropagation

Meristem-tip culture. Lily plantlets propagated according the meristem shoot tip procedure recommended by Van Aartrijk *et al.* (157) to eliminate virus were supplied by J. F. Tammen and indexed by ELISA for LSV, TBV, and CMV. These plantlets were divided into two groups for comparison. In the first group, 13 varieties comprising 96 plantlets were derived from bulbs that indexed virus-free by the Bulb Inspection Service, Lisse, the Netherlands (Van Schadewijk, personal communication). In the second group, 5 clones consisting of 17 plants originated from bulbs determined to be infected with LSV in the previous indexing.

Performance of Virus-Free Lilies

A 'Sirocco' lily plant determined to be virus free and an LSV-infected counterpart were selected for comparison. The bulbs of each plant were allowed to sprout and were maintained in the same greenhouse at <30C for one growing season. The bulbs were then harvested and used for scale propagation. After the second season, the synchronized scale-derived plantlets were harvested and stored at 4C for 2 months. For yield trials, 15 virus-free bulbs and 15 LSV-infected bulbs were selected and planted in a greenhouse at <30C. Similar bulbs were also field grown in Alachua County, FL, near an experimental planting containing non-indexed lily germ plasm. At the end of the growing season, leaf samples representing

each treatment in the experiment were tested by indirect ELISA for LSV, TBV, and CMV.

Results

Virus Incidence

Commercial materials

Surveys conducted in 1993-95 show that LSV is the most predominant virus infecting lilies. Of 1001 samples tested, 55.6, 13.7, and 1.2 per cent were infected with LSV, TBV, and CMV, respectively (Table 4-1).

Table 4-1. Virus incidence in lilies surveyed in 1993-95 [1]

Lily types	Source	No. of samples	Virus [2]			% Healthy
			LSV	TBV	CMV	
Asiatic	Holland	354	154 [3]	1	1	56.2
	USA	90	37	0	2	54.3
Oriental	Holland	234	140	5	0	40.2
	USA	63	36	0	0	42.9
Easter	USA	117	117	117	0	0
Others	USA	152	73	14	9	41.4
Totals		1001	557	137	12	42.7

[1] Summary of Tables 4-2, 4-3, 4-4, 4-5 and 4-6.
[2] LSV = lily symptomless virus, TBV = tulip breaking virus, CMV = cucumber mosaic virus.
[3] Number of samples reacting positively with antiserum in indirect ELISA had A_{405nm} values at least two times greater than those of negative controls.

The relative incidences of certain viruses varied with *Lilium* species. However, in commercial Asiatic and Oriental varieties, infection rates were similar, regardless whether the bulbs were directly imported from Holland for commercial cut-flower production or were for the retail market. For instance, of the 81 bulbs obtained from domestic retailers, only two (both 'Connecticut King') were doubly infected with LSV and CMV. Other bulbs were either singly infected with LSV (43.2%) or virus free (54.3%) (Table 4-2). Likewise, of 354 imported Asiatic bulbs, 56.2% were virus free. The remaining plants were all infected with LSV (Table 4-3), including one 'Vivaldi' lily plant which was doubly infected with TBV and LSV and one 'Yellow Blaze' which was doubly infected with CMV and LSV. Slightly lower percentages of healthy bulbs were found in the Oriental varieties, either from the domestic markets or from the imported materials. Among the diseased bulbs sold for the retail market, only LSV infection was found, whereas 5 TBV-infected bulbs were found among bulbs intended to be grown for cut flower production (Tables 4-4 and 4-5).

In contrast to Asiatic and Oriental varieties, a relatively large number of Aurelians and Trumpet lilies were infected with TBV, and the incidence of TBV exceeded that of LSV (Table 4-6). In addition, relatively high frequencies of CMV were found in among the six miniature varieties surveyed (Table 4-6).

Table 4-2. Virus incidence in Asiatic lilies obtained from U. S. retailers

Variety	Retailer [1]	No. of samples	Virus [2]			% Healthy
			LSV	TBV	CMV	
Connecticut King	LM	6	6 [3]	0	2	0
	VB	12	3	0	0	75.0
Enchantment	LM	5	5	0	0	0
	VB	12	10	0	0	16.7
Gran Paradiso	DG	3	1	0	0	66.7
Red *Lilium*	VZ	20	4	0	0	80.0
Sirocco	DG	3	0	0	0	100.0
Yellow *Lilium*	VZ	20	8	0	0	60.0
Totals		81	37	0	2	54.3

[1] LM = Lilies & More Inc., Vancouver, WA; VB = Van Bourgondien Inc., Babylon, NY; DG = Dutch Garden, Inc., Adelphia NJ; VZ = Van Zyverden Bros., Inc., Meridian, MS.

[2] LSV = lily symptomless virus, TBV = tulip breaking virus, CMV = cucumber mosaic virus.

[3] Number of samples reacting positively with antiserum in indirect ELISA had A_{405nm} values at least two times greater than those of negative controls.

Table 4-3. Virus incidence in Asiatic lilies imported from the Netherlands

Variety	No. of samples	Virus [1]			% Healthy
		LSV	TBV	CMV	
Beatrix	27	1 [2]	0	0	96.3
Dreamland	25	25	0	0	0
Geneve	25	1	0	0	96.0
Gran Paradiso	25	21	0	0	16.0
Jolanda	26	0	0	0	100.0
Nepal	25	21	0	0	16.0
Nove Cento	25	3	0	0	88.0
Pollyanna	20	19	0	0	5.0
Royal Dutch	25	20	0	0	20.0
Sirocco	26	6	0	0	76.9
Unique	25	8	0	0	68.0
Variant	26	4	0	0	84.6
Vivaldi	30	1	1	0	93.3
Yellow Blaze	24	24	0	1	0
Totals	354	154	1	1	56.2

[1] LSV = lily symptomless virus, TBV = tulip breaking virus, CMV = cucumber mosaic virus.

[2] Number of samples reacting positively with antiserum in indirect ELISA had A_{405nm} values at least two times greater than those of negative controls.

Table 4-4. Virus incidence in Oriental lilies obtained from U. S. retailers

Variety	Retailer [1]	No. of samples	Virus [2]			% Healthy
			LSV	TBV	CMV	
Casablanca	VB	6	2[3]	0	0	66.7
	DG	3	3	0	0	0
	PS	20	20	0	0	0
Stargazer	VB	6	6	0	0	0
	DG	3	3	0	0	0
	PS	25	2	0	0	92.0
Totals		63	36	0	0	42.9

[1] VB = Van Bourgondien Inc., Babylon, NY; DG = Dutch Garden, Inc., Adelphia NJ; PS = Park Seed, Inc. Greenwood, SC.

[2] LSV = lily symptomless virus, TBV = tulip breaking virus, CMV = cucumber mosaic virus.

[3] Number of samples reacting positively with antiserum in indirect ELISA had A_{405nm} values at least two times greater than those of negative controls.

Table 4-5. Virus incidence in Oriental lilies imported from the Netherlands

Variety	No. of samples	Virus [1] LSV	TBV	CMV	% Healthy
Berlin	26	11 [2]	0	0	57.7
Casablanca	21	20	3	0	4.8
Cascade	26	14	0	0	46.2
Dame Blanche	24	13	0	0	45.8
Gypsy Eyes	25	15	1	0	40.0
Laura Lee	21	21	1	0	0
Nippon	25	2	0	0	92.0
Stargazer	22	4	0	0	81.8
Stargazer Max	44	40	0	0	9.1
Totals	234	140	5	0	40.2

[1] LSV = lily symptomless virus, TBV = tulip breaking virus, CMV = cucumber mosaic virus.

[2] Number of samples reacting positively with antiserum in indirect ELISA had A_{405nm} values at least two times greater than those of negative controls.

Table 4-6. Virus incidence in miscellaneous lilies obtained from retailers or plant breeders

Types/Variety	Retailer [1]	No. of samples	Virus [2] LSV	TBV	CMV	% Healthy
Aurelian lilies:						
Golden Splendor	VB	12	0[3]	0	0	100.0
Pink Perfection	VB	12	2	7	0	41.6
Trumpet lilies	DG	8	1	5	0	37.5
Black Beauty Tetra	LM	3	3	2	0	0
Red Tiger	VB	12	12	0	0	0
Album *L. speciosum*	VB	12	0	0	0	100.0
Miniatures:	Breeder					
Golden Pixie		15	10	0	6	0
Minicharisma		14	9	0	2	21.4
Minicream		15	6	0	0	60.0
Minicream Brushmark		15	3	0	0	80.0
Orange Pixie		18	18	0	0	0
Orange Pixie New		16	9	0	1	43.8
Totals		152	73	14	9	41.4

[1] VB = Van Bourgondien Inc., Babylon, NY; DG = Dutch Garden, Inc., Adelphia NJ; LM = Lilies & More Inc., Vancouver, WA; Breeder = bulbs collected in 1993 from unspecified breeders in Oregon.

[2] LSV = lily symptomless virus, TBV = tulip breaking virus, CMV = cucumber mosaic virus.

[3] Number of samples reacting positively with antiserum in indirect ELISA had A405nm values at least two times greater than those of negative controls.

All 117 Easter lily plants purchased from local retailers were infected with both LSV and TBV (Table 4-7).

Virus-free, commercially available bulbs, representing all lily cultivars except 'Connecticut King,' 'Dreamland,' 'Yellow Blaze,' 'Laura Lee,' 'Black Beauty Tetra,' 'Red Tiger,' 'Golden Pixie,' and 'Orange Pixie' were found. Some varieties or species with potential virus resistance to these three viruses were also found. Indeed, all the 'Jolanda,' 'Golden Splendor,' and 'Album' plants indexed were virus-free, regardless of their source (Tables 4-3 and 4-6).

Shoot-tip cultured materials.

The indexing results for plantlets derived from Dutch virus-certified bulbs demonstrate their capacity to commercially produce plants free of LSV on a commercial scale. Only two samples had any virus, both TBV (Table 4-8). In contrast, 17 plantlets originating from LSV-infected plants through shoot-tip culture remained infected with LSV (Table 4-9).

Performance of Virus-free lilies

Yield Trial. Despite the lack of overt foliar symptoms, LSV had a significant effect on yield and quality (Table 4-10). The most pronounced difference between healthy an diseased plants was in bulb weight, with infected ones

Table 4-7. Virus incidence in *Lilium longiflorum* 'Nellie White' field-grown in western North America

Year	Location of [1] bulb forcer	No. of samples	Virus [2]			% Healthy
			LSV	TBV	CMV	
1994	Florida	5	5[3]	5	0	0
	Florida	7	7	7	0	0
	Florida	24	24	24	0	0
1995	Canada	12	12	12	0	0
	North Carolina	7	7	7	0	0
	Florida	22	22	22	0	0
	Florida	36	36	36	0	0
	Florida	4	4	4	0	0
Totals		117	117	117	0	0

[1] Plants purchased from different retailers at Gainesville, FL. Field-grown bulbs originated from California and Oregon but were shipped for forcing as pot plants to Florida, North Carolina, or Canada, after which they were sold in Florida for the Easter market.

[2] LSV = lily symptomless virus, TBV = tulip breaking virus, CMV = cucumber mosaic virus.

[3] Number of samples reacting positively with antiserum in indirect ELISA had A_{405nm} values at least two times greater than those of negative controls.

Table 4-8. Virus incidence of plantlets derived from virus-certified lily bulbs imported from the Netherlands in 1994

Variety	Virus [1] status	No. of samples	Virus [2] LSV	TBV	CMV	% Healthy
Casablanca	-	5	0	0	0	100
Corsica	-	6	0	0	0	100
Laura Lee	-	9	0	1	0	89
Le Reve	-	9	0	0	0	100
Menton	-	6	0	0	0	100
Mona Lisa	-	7	0	0	0	100
Nepal	-	4	0	0	0	100
Odeon	-	8	0	0	0	100
Paris	-	12	0	0	0	100
Pollyanna	-	5	0	0	0	100
Snowstar	-	10	0	0	0	100
Toscane	-	10	0	1	0	90
Unique	-	5	0	0	0	100
Yellow Blaze	+LSV	6	6	0	0	0
Totals		102	6	2	0	

[1] Virus indexed against mother bulbs by Bulb Inspection Service, Lisse, the Netherlands. - = indexed free of LSV, LVX and TBV. + LSV = indexed free of TBV and LVX, but not LSV.

[2] LSV = lily symptomless virus, TBV = tulip breaking virus, LVX = lily virus X, CMV = cucumber mosaic virus.

[3] Number of samples reacting positively with antiserum in indirect ELISA had A_{405nm} values at least two times greater than those of negative controls.

Table 4-9. Virus incidence of lily plantlets derived from LSV-infected donor plants in shoot-tip culture [1]

Variety	No. of samples	Virus [2]			% Healthy
		LSV	TBV	CMV	
Minicream Brushmark	1	1[3]	0	0	0
Minicharisma	2	2	0	0	0
Nepal	3	3	0	0	0
Orange Pixie	2	2	0	0	0
Yellow Blaze	9	9	0	0	0
Totals	17	17	0	0	0

[1] Shoot tips of bulblets taken as described by Van Aartrijk et al. (157) to obtain virus-free plantlets from infected bulbs.

[2] LSV = lily symptomless virus, TBV = tulip breaking virus, CMV = cucumber mosaic virus.

[3] Number of samples reacting positively with antiserum in indirect ELISA had A_{405nm} values at least two times greater than those of negative controls.

Table 4-10. Effects of lily symptomless virus (LSV) on yields of lily cv. 'Sirocco'[1)]

Treatment	Plant height (cm)	No. of leaves	Length of leaves[2)] (cm)	Width of leaves[2)] (cm)	No. of flowers	Days of anthesis	Bulb yield (g)	Circumference of main bulb (cm)
LSV-infected	39.51	33.27	8.14	1.62	1.27	5.53	16.93	11.05
LSV-free	50.00*	34.60	10.35*	2.19*	1.53	7.07	26.80*	12.52*

[1)] Data are means of 15 plants grown from LSV-infected or 15 plants from LSV-free bulb scales.
[2)] Data based on measurements of the largest leaves on each plant.
* indicates the significant differences at the P=0.01 level between LSV-infected and their LSV-free counterparts.

weighing only 63.2% of those that were virus-free. That LSV affects plant vigor is also apparent from their reduced plant weight (79.0% as tall as their healthy counterparts), leaf length (78.6%), leaf width (73.9%) and bulb circumference (88.3%). While similar differences were also noted in the number of leaves and flowers produced and in length of anthesis, these differences were not statistically significant.

Virus reinfection. During the yield trial, all virus-free propagating units remained healthy for at least two crop cycles. Thirty samples collected from each crop cycle were all free of LSV, TBV, and CMV. In contrast, LSV persisted in propagating units throughout the two-year duration of the experiment. Thirty samples representing the plants in each crop cycle were all singly infected with LSV. However, TBV and CMV were both transmitted to virus-free lilies under field conditions in Florida. About 30% of the virus-free 'Sirocco' lilies planted in the field became infected with CMV at the end of first season of growth. By the end of the second season, however, 44% were infected with CMV and 11% with TBV. None of these plants contracted LSV, however, by the end of the second year.

Discussion

The survey results confirm reports by others (10,86,171) that LSV is the most prevalent lily virus. Despite high incidences of LSV, many plants free of LSV, TBV, and CMV.

These results indicated that viruses are not ubiquitous and that, at least in Holland, these viruses are being actively controlled. The low virus levels in commercial Asiatic and Oriental varieties in particular, reflects the effectiveness of virus certification programs conducted in the Netherlands. Nevertheless, low levels of virus were detected in most of the imported Dutch bulbs, thereby indicating that their health status cannot necessarily be taken for granted.

In marked contrast, the ubiquitously high incidences of LSV and TBV were found in Western-grown Easter lily 'Nellie White' pot plants. Apparently mixed infections with these two viruses in this variety of Easter lily do not preclude its being acceptable for marketing. Indeed, even though the diseased Easter lilies in our study showed strong yellow mottle symptoms when young, they looked relatively healthy at flowering. This situation contrasts sharply with the demise the Easter lily production in Florida when LSV-infected plants became infected with CMV. It is possible that I did not find any CMV in Easter lily because the plants with necrotic fleck are clearly seen and thus rogued as it is for gladiolus in Florida, California and elsewhere. Prompt attention to roguing would be important inasmuch as field infection can occur readily, as shown in this study.

Neither Asjes *et al.* (26), in the Netherlands or Mowat and Stefanac in Great Britain (127) detected any CMV from their tested lily samples. CMV thus was disregarded in the Dutch

lily certification program. Indeed, our studies reveal very low incidences of this virus. However, one 'Yellow Blaze' bulb from Holland was determined to be doubly infected with LSV and CMV in this study. Although Allen and Fernald (10) indicated that CMV was not common in their surveys of plants in western North America, the virus nevertheless remains a potential threat, especially in the southeastern U. S.

The plants determined to be virus-free during the surveys were treated as potential mother block materials to be used to propagating stock for field production in Florida. For some varieties, however, no virus free plants could be found. Meristem tip culture as recommended by Van Aartrijk *et al.* (157) failed to obtain virus free lilies in this study, when shoot tips were 1 mm or more in length. Apparently, as shown in this study and others (125) only shoot tips less than that in size are likely to be virus free.

In some instances, LSV-free Asiatic hybrid lilies could also be obtained from infected plants by removing bulblets regenerated *in vitro* (4). Cohen *et al.* (60) reported that during *in vitro* culture, LSV became undetectable in most bulblets if maintained at 30C or higher. However, since the optimum temperatures for bulblet initiation or growth from scale pieces are 15-25C, prolonged maintenance at high temperatures could impair the efficiency of *in vitro* propagation rates.

Under the circumstances presented in this study, the most practical prospects for establishing virus free planting material would be to identify virus-free plants from commercial sources without resorting to thermotherapy or meristem shoot tip culture. As shown in this study, prospects for obtaining such stock are good, but the effort must include a reliable program of indexing candidate plants.

Virus-free lilies were more robust and taller than virus-infected ones and produced many more bulbils (5). Under most circumstances, differences between the infected and healthy lilies are difficult to discern due to lack of overt symptoms. In this study, the physiological situations of healthy and diseased plants in the yield trial were synchronized by scale propagation. All of the plants produced their first flower during the trial, and thus the full potential of healthy bulbs compared to diseased ones could be compared objectively. Based on these data, it appears that greater production of lilies can be realized through the use of virus-free stocks, despite the high degree of latency in lily virus symptom expression, and that virus diseases are worth controlling.

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BIOGRAPHICAL SKETCH

Ting-Chin Deng was born in Tainan, Taiwan, on May 1955. He graduated from the Provincial Tainan First High School in 1973. He attended National Chung-Hsing University at Taichung, Taiwan, majoring in Plant Pathology, and received a Bachelor of Science degree from that institute in June, 1977. He continued his studies there and received a Master of Science degree in June 1979.

After two years of military service, he worked as a Research Assistant at the Asian Vegetable Research and Development Center (AVRDC) located in Shanhua, Taiwan. In February of 1984, he was appointed as an Assistant Plant Pathologist by the Taiwan Agricultural Research Institute (TARI) in Wufeng, Taiwan. In August of 1992, he was sponsored by National Science Council of the Republic of China in Taiwan and began his studies at the University of Florida. He received the degree of Doctor of Philosophy in December 1995, and returned to TARI continuing his research on plant diseases.

Ting-Chin Deng is currently a member of American Phytopathological Society, North American Lily Society, Taiwan Plant Pathology Society, and the Taiwan Plant Protection Society.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

F. W. Zettler, Chairman
Professor of Plant Pathology

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E. Hiebert, Cochair
Professor of Plant Pathology

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D. E. Purcifull
Professor of Plant Pathology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

M. E. Kane
Associate Professor of
Horticultural Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

R. D. Hartman
President and Chief Executive
Officer
Twyford International, Inc.

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1995

Jack L. Fry

Dean, College of Agriculture

Dean, Graduate School