Purification of Lily Symptomless Carlavirus and Detection of the Virus in Lilies

H. T. Hsu, Microbiologist, **J. Y. Kim,** Visiting Scientist, and **R. H. Lawson,** Research Plant Pathologist, U.S. Department of Agriculture, Agricultural Research Service, Floral and Nursery Plants Research Unit, Beltsville Agricultural Research Center, Beltsville, Md. 20705-2350

ABSTRACT

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Lily symptomless carlavirus (LSV) was purified from infected lilies (*Lilium longiflorum*) by employing Nonidet P-40, butanol, and urea during clarification followed by equilibrium centrifugation in cesium chloride. Virus yields averaged 8.5 mg per 100 g of tissues using an extinction coefficient of 2.5. Two of the four rabbits injected three times each with 0.25 mg purified LSV had antiserum dilution endpoints of $10^{-5.4}$ by enzyme-linked immunosorbent assay (ELISA) or tissue-blot immunoassay (TBIA) at the first bleeding; whereas the other two rabbits had serum dilution endpoint of 10^{-6}. An indirect immunological procedure was used to detect LSV antigens in tissue blots on nitrocellulose membranes. A comparison of ELISA and dot-blot immunoassay (DBIA) showed that DBIA was 16 to 32 times as sensitive as ELISA in detection of LSV. LSV was detected in bulb scales by TBIA in samples in which sap extracts from the same scale pieces were negative in both DBIA and ELISA.

Additional keywords: disease indexing method, immunohistochemical detection, immunological assay, serodiagnosis, virus localization, virus purification

There is great versatility in the type of test and format used for serological detection of plant viruses (1,8,9,17,19,20). Both enzyme-linked immunosorbent assays (ELISA) and immunosorbent electron microscopy have sensitivity several orders of magnitude greater than that of double diffusion tests or liquid precipitin tests, but all these procedures require extraction of viral antigens from infected tissues.

An immunological method for detection of plant viruses and a mycoplasma-like organism by direct tissue blotting on nitrocellulose membranes has been described (15). While retaining characteristics of specificity, sensitivity, and rapidity, the tissue-blot immunoassay (TBIA) technique requires neither mechanical maceration nor different buffers for antigen preparation. Therefore, sample application is convenient and a large number of samples can be handled in a short time (14). Furthermore, the technique also provides a means for investigation of virus distribution within the tissue (5,13–16).

Although lilies are commonly infected with lily symptomless virus (LSV), some lilies may show symptoms of the virus but symptoms may not be visible in other lilies unless the plants are also infected with cucumber mosaic virus (2,6,7). In this paper, we describe the purification of LSV from lilies, and TBIA was compared with ELISA and dot-blot immunoassay (DBIA) for detection of LSV infection in lilies. A preliminary report was presented (11).

MATERIALS AND METHODS

Virus and virus purification. Bulbs from field-grown *Lilium longiflorum* Thunb. cv. Nelli White were planted in a greenhouse. Virus was purified from fresh leaves. A partial purification procedure was reported for LSV from symptomless *L. longiflorum* (7). Purification of the virus for antiserum production in current studies was achieved by employing Nonidet P-40, n-butanol, and urea during clarification followed by cesium chloride equilibrium centrifugation. One-centimeter leaf pieces were homogenized in the extraction buffer (0.25 M potassium phosphate, 2 mM magnesium sulfate and 0.1% sodium sulfite, pH 7.5) in a Waring Blender (1 g of tissue per 2 ml of buffer). Homogenates were filtered through two layers of cheesecloth, and then debris re-extracted with one half of the original volume of extraction buffer. Filtrates from both extractions were combined.

While stirring at room temperature, the filtrate was adjusted to 0.5% Nonidet P-40, 8.5% n-butanol, and 1 M urea. After 20 min, the mixture was clarified by low-speed centrifugation at 6,000 × *g*. The aqueous phase was centrifuged at 20,000 × *g*, 4°C for 60 min in a Beckman Ultracentrifuge Model L8-70M (Beckman Instruments, Inc., Pal Alto, Calif.). Pellets were resuspended in two-tenths volume of the original tissue weight with one-tenth the concentration of the original extraction buffer. Following clarification again by low-speed centrifugation, 4 ml of the virus suspension was layered on 7 ml of 31% cesium chloride solution prepared in one-tenth strength extraction buffer and centrifuged in a Beckman SW41 Ti rotor at 120,000 × *g*, 10°C for 18 h. Viruses containing fractions were collected, pooled, and dialyzed against three changes of a larger volume of phosphate buffered saline solution (PBS, 0.01M potassium phosphate, 0.15 M sodium chloride, pH 7.4). Yields of purified virus were estimated using an extinction coefficient of 2.5 at 260 nm. Purified virus preparations were adjusted to 1 mg per 0.5 ml and stored at –70°C. Virus density was calculated from the refractive index of the cesium chloride solution associated with the virus-containing fraction.

A drop of virus, after dialysis in 0.01M phosphate buffer, pH 7.4, was mixed with a drop of 2% phosphotungstic acid, pH 6.5, on a carbon-stabilized formvar coated grid. Grids were examined in a JOEL 100CX electron microscope, and particles were measured using a grating replica as an internal standard.

Rabbit anti-LSV serum. Polyclonal rabbit antisera for LSV were produced by Cocalico Biologicals, Inc. (Reamstown, Pa.). Three intramuscular injections of 250 µg each in 50% Freund's complete adjuvant were made on days 0, 21, and 28, and first bleedings were made on day 35. Serum titers were determined by ELISA using antigen-coated plates, or by TBIA.

ELISA. Antiserum titers were determined using an indirect ELISA procedure previously described using alkaline phosphatase-labeled goat anti-rabbit immunogobulins (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) (12). An avidin-biotin mediated ELISA (AB-ELISA) procedure was employed for detection of LSV infection in lilies (13). Rabbit immunoglobulins were purified by affinity chromatography through a protein A-sepharose column (10). Biotin-antibody conjugates were prepared by reaction with biotinyl-*N*-

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hydroxysuccinimide ester as described for tomato spotted wilt virus (15) and were used at a 1 ng antibody per ml concentration. Antigen solutions were used at 100 µl per well of polyvinyl chloride plates in triplicates, the other reagents at 150 µl per well. Wells that contained no plant extracts but were similarly treated were used to adjust the base line of an ELISA reader.

DBIA. The procedure for DBIA was similar to the one previously described (14) except that alkaline phosphatase-labeled goat anti-rabbit immunoglobulins were used. Samples (50 µl) were applied in duplicates with the aid of a Minifold apparatus (Scheicher & Schuel, Inc., Keene, N.H.) followed by a rinsing/washing step with 100 µl of PBS. Rabbit anti-LSV serum was used at $10^{-3.3}$ (1/2,000) dilutions (rabbit number 19).

TBIA. An indirect immunological method using alkaline phosphatase-labeled goat anti-rabbit immunoglobulins and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate was used to detect LSV in tissue blots. Preparation of plant tissue blots and detection of viral antigens in blots on nitrocellulose membranes was previously described (15).

Tissue samplings for LSV assays. Each tissue cut was made separately with a new razor blade. Tissue blots were made as described (15). Scale tissues about 2 mm adjacent to the blotted surfaces were excised, weighed, and triturated in 19 parts of PBS in a mortar with a pestle. The whole extracts were clarified by low speed centrifugation and supernatants were assayed for LSV by AB-ELISA at 1/20 dilutions and by DBIA at 1/200 dilutions. Use of tissue extracts at 1/20 dilution in DBIA resulted in incomplete application of samples caused by the presence of particulates that interfered with sample application. Buffer without plant materials was used as a control to adjust the baseline of an ELISA reader. In addition, three virus-free lily seedlings grown from seed propagation were used as controls A, B, and C.

RESULTS AND DISCUSSION

Yields of purified LSV ranged from 6.5 to 12.8 mg per 100 g of lily leaves with an average of 8.5 mg from 12 separate purifications. The average density of the virus in cesium chloride solution was 1.2970 g per cm^3, derived from measurements ranging from 1.2950 to 1.2991 of 6 purifications. Negatively stained virions had an average length of 645 nm based on the measurement of 100 particles recovered from the cesium chloride gradient. No isometric particles were found in purified preparations.

Production of high quality antisera to LSV does not require as many injections of purified virus in rabbits as have been reported for several plant viruses (19). Three intramuscular injections of 0.25 mg each of Freund's complete adjuvant emul-

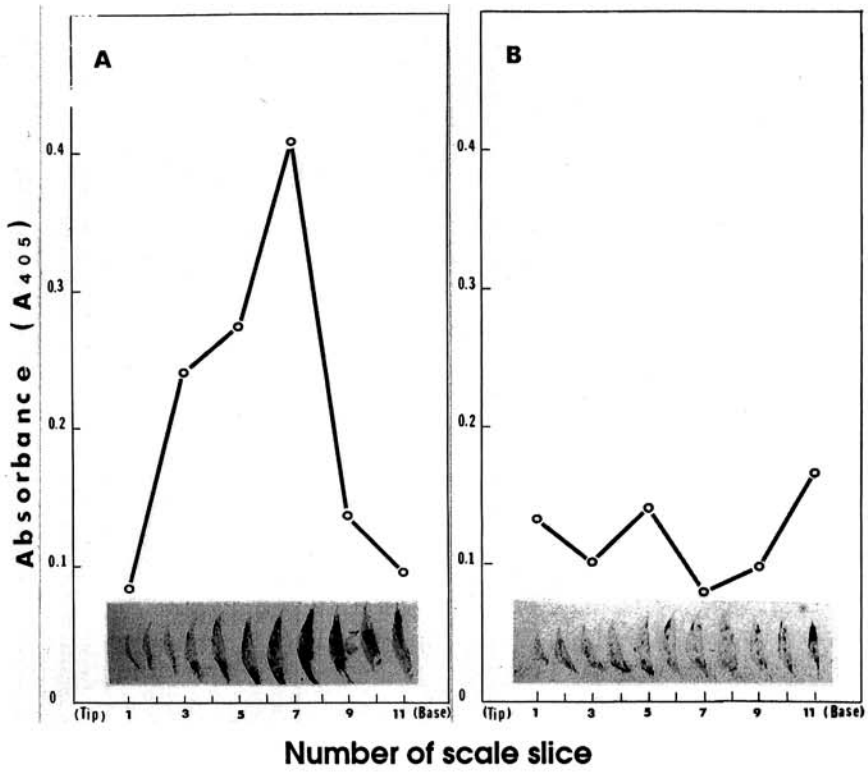

Fig. 1. Distribution of lily symptomless virus (LSV) antigen in scale tissues of infected lily bulbs from **(A)** plant number S- 24 and **(B)** plant number 79. Scales were consecutively cut every 2 mm from the tip to the base and blotted onto a nitrocellulose membrane. Relative concentrations of LSV antigens in each tissue slice were determined by avidin-biotin mediated enzyme-linked immunosorbent assay and the presence of LSV in tissue blots were analyzed by an indirect serological procedure.

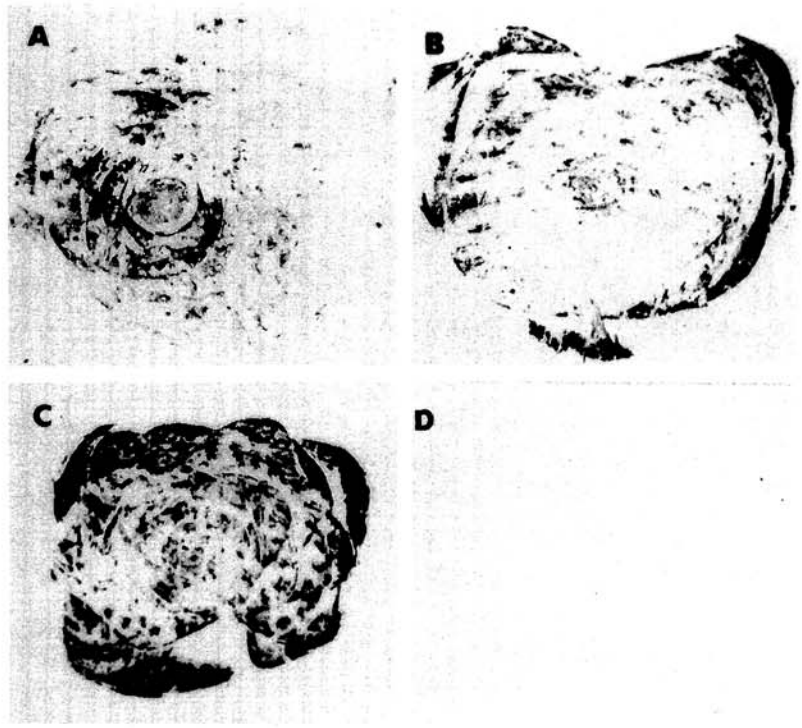

Fig. 2. Distribution of lily symptomless virus (LSV) antigens in **(A, B, C)** infected, and **(D)** healthy lily bulbs by direct tissue-blot immunoassay on nitrocellulose membranes. LSV antigens were detected on incubation of tissue blots with virus-specific rabbit antibodies followed by alkaline phosphatase-labeled goat anti- rabbit immunoglobulin antibodies. The image of healthy bulbs (D) on the membrane was so light that it could not be registered on the print.

sified with purified LSV elicited satisfactory immune responses in all four rabbits tested. ELISA titers from the first bleeding (1 week after the third injection) were $10^{-5.4}$ for rabbits number 19 and 20 and $10^{-6.0}$ for rabbits number 21 and 22. Similar titers were also attained by TBIA. Additional injections on day 42 and 63 with 0.5 mg each of purified LSV in 50% Freund's complete adjuvant did not increase titers of sera collected on days 49, 56, or 70. The LSV immunogen used in serum production contained little host plant materials; thus, results of tests using sera from all four rabbits revealed no serological reaction to healthy lilies by either ELISA or by TBIA. Pre-immune sera tested negatively to both LSV and healthy lily tissues.

Studies showed that DBIA was more sensitive than ELISA (4,13,14). Current investigations with LSV also indicate that DBIA is not only more sensitive but also more reliable than ELISA for detecting virus and virus infection in lilies. In a sample size of 100 µl, as little as 62 ng per ml of purified LSV was detected by AB-ELISA, whereas 4 ng per ml of LSV was detected by DBIA in 50-µl samples. The detection of purified LSV diluted in the extract of healthy lily leaves did not, however, affect the sensitivities of either assay (data not shown). The dilution endpoints of LSV in the extract of infected *L. longiflorum* leaves was $10^{-3.1}$ by AB-ELISA and $10^{-4.6}$ by DBIA; and those of *L.* × *elegans* Thunb. (obtained from M. Roh, USDA, ARS, Beltsville, Md.) were $10^{-3.7}$ and $10^{-5.2}$, respectively.

Analysis of bulbs by TBIA revealed that diverse patterns of localization of LSV antigens occurred not only in different tissues within a single scale (Fig. 1) but also in different scales within a single bulb (Fig. 2). In a scale taken from bulb number S-24, a higher concentration of LSV antigens was detected in tissue between one-third and one-half the distance from the base to the tip of the scale by TBIA or AB-ELISA (Fig. 1A). Although tissue extracts from both ends of the scale gave ELISA readings (A_{405}) of <0.1, the TBIA analysis revealed the presence of localized LSV antigens in small areas within the scale (Fig. 1A). Analysis of a scale taken from another bulb, number 79, showed variable distribution of LSV antigens within the scale (Fig. 1B). AB-ELISA A_{405} were <0.2 for extracts from various portions of the scale and, in some instances, <0.1. TBIA analysis, again, revealed the localization of LSV antigens in numerous small areas. Uneven distribution of the LSV antigen was commonly found in different tissue positions of a scale taken from infected bulbs (data not shown). Analysis of various positions of a healthy bulb scale showed that the ELISA A_{405} ranged from 0 to 0.05, and no LSV antigen was detected in tissue blots.

Distribution of LSV antigens in lily bulbs differed among bulbs. In some bulbs, higher concentrations of LSV were observed in center scales than in outer scales (Fig. 2A); in others the distribution pattern was just the opposite in that less LSV antigens were observed in scales in the center of the bulb (Fig. 2B). In a few cases, uneven distribution of LSV antigens was observed in infected bulbs in which no LSV antigens were detected by TBIA in some of its scales (Fig. 2B). Antigens were frequently found throughout the cross-sections of infected bulbs (Fig. 2C). LSV antigens were, however, not detected in tissue blots made from healthy bulbs although their images on the nitrocellulose membranes were too light in color to be photographed. (Fig. 2D).

Uneven distribution of LSV in lily bulbs has been recognized for some time (4). Current studies also showed diverse patterns of LSV localization both in scales and bulbs. Therefore, more than one scale from each bulb should be selected for accurate indexing. The actual compartmentalization of LSV in lily bulbs can only be demonstrated by TBIA. One of the applications in which TBIA can be used is the elimination of viruses from infected plants. Careful excisions of LSV-free tissue from infected plants potentially may provide a source of LSV-free tissue cultures.

One scale each from 26 lily bulbs was tested for the presence of LSV in the tissues. AB-ELISA analysis revealed 13 samples tested negatively and eight samples tested positively for LSV infection (Figs. 3 and 4). The remaining five samples (plants D-123, D-24, D-20, D-42, and 79) gave A_{405} <0.1 and could not be rated because the healthy controls A and B grown from seeds gave A_{405} 0.023 and 0.009, respectively, using a buffer control adjusted ELISA reader (Fig. 3). Tests by DBIA showed that all eight samples that tested positively by AB-ELISA were infected with LSV (Fig. 3). In addition, the presence of LSV antigen was identified by DBIA in four (plants D-123, D-24, D-20, and D-42) of the five samples that could not be rated by AB-ELISA. Furthermore, DBIA identified LSV antigen in seven additional samples from the 13 samples that gave A_{405} 0 by AB-ELISA (Fig. 4). Analysis of 26 samples by TBIA revealed the presence of LSV antigens in tissue blots of 20 samples including two samples (plant 79 of Fig. 3 and the arrow of plant

Plant	ELISA	Tissue blots	Dot-blots
D-40	2.000		++++
63	1.367		+++
122	0.964		+++
D-57	0.689		++
D-111	0.623		++
17	0.479		++
67	0.356		++
B	0.163		+
D-123	0.095		+
D-24	0.090		+
Control A	0.023		-
D-20	0.019		++
D-42	0.019		++
79	0.011		-
Control B	0.009		-

Fig. 3. Detection of lily symptomless virus (LSV) in lily scales by enzyme-linked immunosorbent assay (ELISA), dot-blot immunoassay (DBIA) and tissue-blot immunoassay (TBIA). One scale from each bulb was cut twice near middle portions, and each slice was blotted onto nitrocellulose membranes. A 1/20 dilution tissue extract was prepared from the slice by triturating 1 part of tissue in 19 parts of phosphate buffered saline solution. The extracts were assayed for LSV by avidin-biotin mediated ELISA at 1/20 dilutions and by DBIA at 1/200 dilutions. Use of tissue extracts at 1/20 dilution in DBIA resulted in incomplete application of samples caused by the presence of particulates that interfere with sample application.

D-94 of Fig. 4) that tested negatively by DBIA. The presence of LSV antigen was detected by AB-ELISA and DBIA in one sample (sample B) that tested negatively by TBIA (Fig. 3). Samples J, W, U, 130, and T were, however, negative by all three procedures (Fig. 4). All three healthy controls were tested negatively by TBIA and DBIA and their ELISA A_{405} values were below 0.03.

Comparative examinations of sections of LSV-infected bulb scale tissues by the three methods, however, revealed the superior reliability of TBIA over ELISA or DBIA for detection of LSV. Similar results were also obtained for detection of Cymbidium mosaic virus in orchids (14). Detection of peanut stunt, bean yellow mosaic, and clover yellow vein viruses showed that sensitivity and specificity of direct tissue immunoblotting were equal or superior to indirect ELISA (18). In addition, it was also found that direct tissue blotting was less laborious and more economical than ELISA (14,18). TBIA examination of samples tested by ELISA that gave A_{405} values close or similar to healthy controls revealed the presence of LSV antigens that were confined to a very small area of only a few infected cells in tissue blots. Extraction of such tissues dilute LSV antigens in extracted solutions below the limit that can be detected by ELISA. Analysis by DBIA may, however, show the presence of LSV antigens in extracts from some of the tissues in which the virus is very localized. Although quantitative analysis of an unknown sample cannot be done by DBIA with the degree of accuracy of ELISA, dilution endpoint titers obtained by DBIA were several times higher than those obtained by ELISA. The application of DBIA in the detection of plant virus infection has not been used as extensively.

Detection of LSV in lilies is essential in the production and certification of virus-free lily bulbs (3,4). The procedures used to detect LSV infection in lilies has shifted from detection by gel diffusion tests to detection by ELISA (4,20–22). Diluted extracts used in ELISA gave higher A_{405} readings than the same extracts at lower dilutions, indicating the presence of certain inhibitory materials in the antigen preparations (4). Incorporation of cellulase or hemicellulose into extraction buffer solutions further improved the detection of LSV antigens by ELISA (3,4). Current studies showed that better detection of LSV infection was achieved by DBIAs in which LSV antigens were identified from samples that were negative or marginally positive by ELISA. Analysis by TBIA further identified many infected samples that were not identified by either ELISA or DBIA.

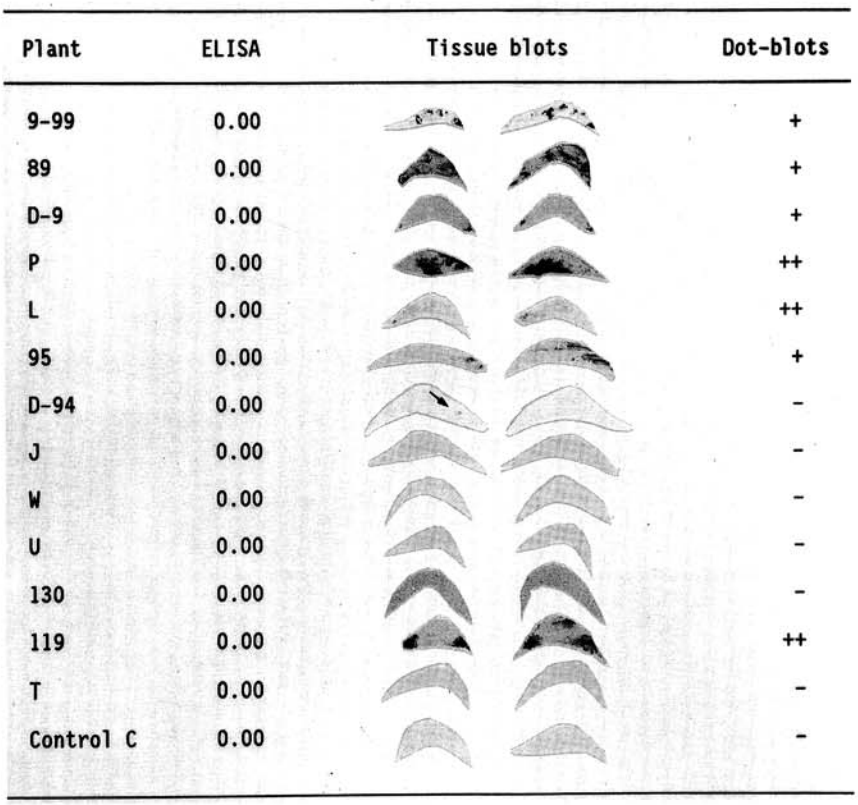

Fig. 4. Identification of lily symptomless virus (LSV) by dot- blot immunoassay (DBIA) and tissue-blot immunoassay (TBIA) in lily bulb scales that tested negatively by avidin-biotin mediated enzyme-linked immunosorbent assay (ELISA). One scale from each bulb was cut twice near middle portions and blotted onto nitrocellulose membranes. A 1/20 dilution tissue extract was prepared from the slice by triturating 1 part of tissue in 19 parts of phosphate buffered saline solution. The extracts were assayed for LSV by avidin-biotin mediated ELISA at 1/20 dilutions and by DBIA at 1/200 dilutions. The arrow indicates LSV antigen that was confined to a tiny dot.

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