Detection of Lily Symptomless Virus by Immunodiffusion

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ABSTRACT

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A simple immunodiffusion test for detecting lily symptomless virus (LSV) has been devised. LSV, a long flexuous rod (640 nm), does not readily diffuse through agar. Repeated freezing and thawing or sonication (4 min, 20 kc/sec) fragmented the virus sufficiently to allow diffusion through agar without destroying its antigenic properties. Because of its low concentration in sap, LSV was concentrated by isoelectric precipitation. LSV was precipitated from clarified sap at pH 4.0–4.4 with 1.0 M acetate buffer, pH 3.1. The precipitate was resuspended in 0.1 M phosphate buffer (pH 7.0) containing 0.05% Igepon T-77 and tested by the Ouchterlony double diffusion method. An LSV concentration of 1.8 μg/ml of clarified sap was detectable with this assay.

Lily symptomless virus (LSV) has been detected by electron microscopy (1) in all lily cultivars examined in this laboratory and by other workers (4,7). LSV reduces plant size and vigor (5) and renders the plants more susceptible to other pathogens such as *Fusarium* and *Botrytis*. The virus can be eliminated from contaminated stocks by means of tissue culture (2,4,6,7). Screening the plantlets derived from tissue culture presents a problem because LSV-infected plants usually bear no obvious visible symptoms of disease (3) and no indicator host of the virus is known. Electron microscopic diagnosis is time consuming and laborious. A reliable and simple LSV detection test would be useful not only for certification programs designed to eliminate LSV but also to plant breeders and virologists.

Several criteria were considered in selecting a suitable immunological test for LSV. Tests requiring pure anti-LSV serum could not be considered because the virus-free plant material necessary for the selective removal of plant specific antibodies was not available in sufficient quantities. It was, therefore, essential that the test would resolve virus-specific and plant-specific reactions. A simple test, which required low quantities of antiserum and with which many samples could be processed simultaneously, was preferable. The Ouchterlony double diffusion test fulfilled these requirements. However, it was necessary to fragment the LSV (a flexuous rod [640 nm]) because it does not readily diffuse through agar. Furthermore, the low levels of LSV in the sap of many lily cultivars necessitated having a simple concentration procedure prior to testing. This report describes a simple and reliable method of screening for LSV.

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MATERIALS AND METHODS

Virus source. LSV-infected foliar tissue was obtained from the *Lilium* cultivar 'Nutmegger', purchased from Oregon Bulb Farms, Gresham, OR, and grown in the greenhouse. Virus-free foliage (assayed by electron microscopy) was obtained from shoots developed from tissue culture of 'Nutmegger' callus tissue (15). 'Nutmegger' callus grew rapidly on Murashige and Skoog's (12) high-salt nutrient medium in agar supplemented with 0.1 mg/L naphthalene acetic acid and 5.0 mg/L 6-benzylamino purine. Root and shoot development was induced by subculturing the callus tissue to an agar medium supplemented with 6-benzylamino purine at 0.1 mg/L.

Purification of LSV. The LSV purification method of Civerolo et al (9) was modified. Leaves were stored at −20 C until used. To clarify the sap, frozen leaves were blended in 0.25 M potassium phophate buffer, pH 7.5, containing 2 mM $MgSO_4$, 0.1% thioglycollic acid, and 0.2% bentonite (1:2 g/ml). The homogenate was expressed through cheesecloth, emulsified in *n*-butanol (8.5% concentration) for 45 min at 4 C, and the precipitate was removed

by centrifugation (1,000 *g* at 22 C for 20 min). The supernatant fluid was stored at 22 C overnight and the precipitate was removed by centrifugation (1,000 *g* at 22 C for 20 min).

To purify LSV, clarified sap was flash evaporated to 80–90% of its original volume in order to remove the dissolved butanol. After centrifugation at 9,600 *g* for 10 min at 4 C, the supernatant fluid was centrifuged at 100,000 *g* for 2.5 hr at 4 C. The pellets were resuspended in 0.1 M potassium phosphate buffer, pH 7.0, (PPB) (1 ml/180 ml clarified sap), and clarified by centrifugation at 9,600 *g* for 10 min, at 4 C. For rabbit immunization, the LSV was subjected to a second cycle of differential centrifugation. The protein content of the supernatant fluid was estimated by the protein-dye binding assay (8).

Electron microscopic detection of LSV. Concentrated samples of LSV were prepared for negative staining by diluting 1:20 (or 1:5 for virus-free extracts) with a solution of 0.25% phosphotungstic acid, pH 7.0, containing 0.002% Bacitracin.

Antiserum production. Two procedures were used for immunizing New Zealand white rabbits:

1. Intravenous injections of 1 ml of the LSV preparation (2.5 mg protein/ml) were administered to four rabbits on days 1, 5, 10, and 15. Serum was collected 7 days after the last injection.
2. For subcutaneous injection, 0.5 ml of the same antigen preparation was homogenized with an equal volume of Freund's incomplete adjuvant and injected on days 1 and 20 into four rabbits. Sera were collected 10 days after the last injection and stored at −20 C in 0.1-ml aliquots.

For the precipitin tests with pyrrolidine, two rabbits were immunized with pyrrolidine-treated LSV (13) by each of the two injection procedures.

Fragmentation of LSV. LSV was fragmented either by freezing and thawing six times or by sonicating on ice for 4 min at 20 kc/sec (Sonic Dismembrator, Quigley-Rochester Inc., Rochester, NY).

Immunoassay. The diffusion medium (18) contained 0.5% agarose, 0.01 Na_2HPO_4-NaH_2PO_4 buffer (pH 7.4), 0.02% sodium dodecyl sulphate, and 0.025% sodium azide. Diffusion occurred for 44 hr, at room temperature. Plates were washed in 0.01 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.4, for 7 hr, and rinsed for 30 min in three changes of double distilled water. When dry, the plates were stained for 30 min in a solution containing 0.5 g light green SF, 0.5 g Buffalo black NBR, 5.0 g $HgCl_2$ and 100 ml 2% acetic acid, and rinsed in several changes of 2% acetic acid.

The antisera either were undiluted or were diluted one-fourth with 0.85% NaCl.

Isoelectric precipitation of LSV. LSV was reversibly insolubilized by lowering the pH of clarified sap to 4.0–4.4 with 1 M HCl, 1 M acetic acid, or 1 M sodium acetate-acetic acid buffer (pH 3.1). For routine processing of many samples, the addition of a known volume of buffer (65% of the clarified sap volume) was found to be the simplest procedure. The precipitate was sedimented (500 *g* 10 min), the supernatant fluid was decanted, and the remaining droplets were blotted off. Precipitated LSV was resuspended in PPB containing 0.05% Igepon T-77 (three drops for each original 5 ml of sap).

RESULTS AND DISCUSSION

Fragmentation of LSV. Degradation of LSV with pyrrolidine as outlined for potato virus S (13) did not yield any virus-specific precipitin bands in the double diffusion test with LSV antiserum or with the pyrrolidine-treated LSV antiserum. The results of the liquid precipitin test agreed with those of Shepard and Secor (14) in that the antigenic properties of the pyrrolidine-treated virus were so altered that reaction with anti-LSV serum produced only a very small amount of precipitate; also, the titer of the antiserum directed against pyrrolidine-treated LSV was too low to be useful. Van Slogteren et al (16,17) used pyrrolidine to degrade LSV, but to conserve antiserum they used a micro-precipitin test, which is much more time-consuming and laborious than a simple double diffusion test.

Gooding's (11) method of immunodiagnosis for potato virus Y in agar, which employs 0.5% SDS, was ineffective for the detection of

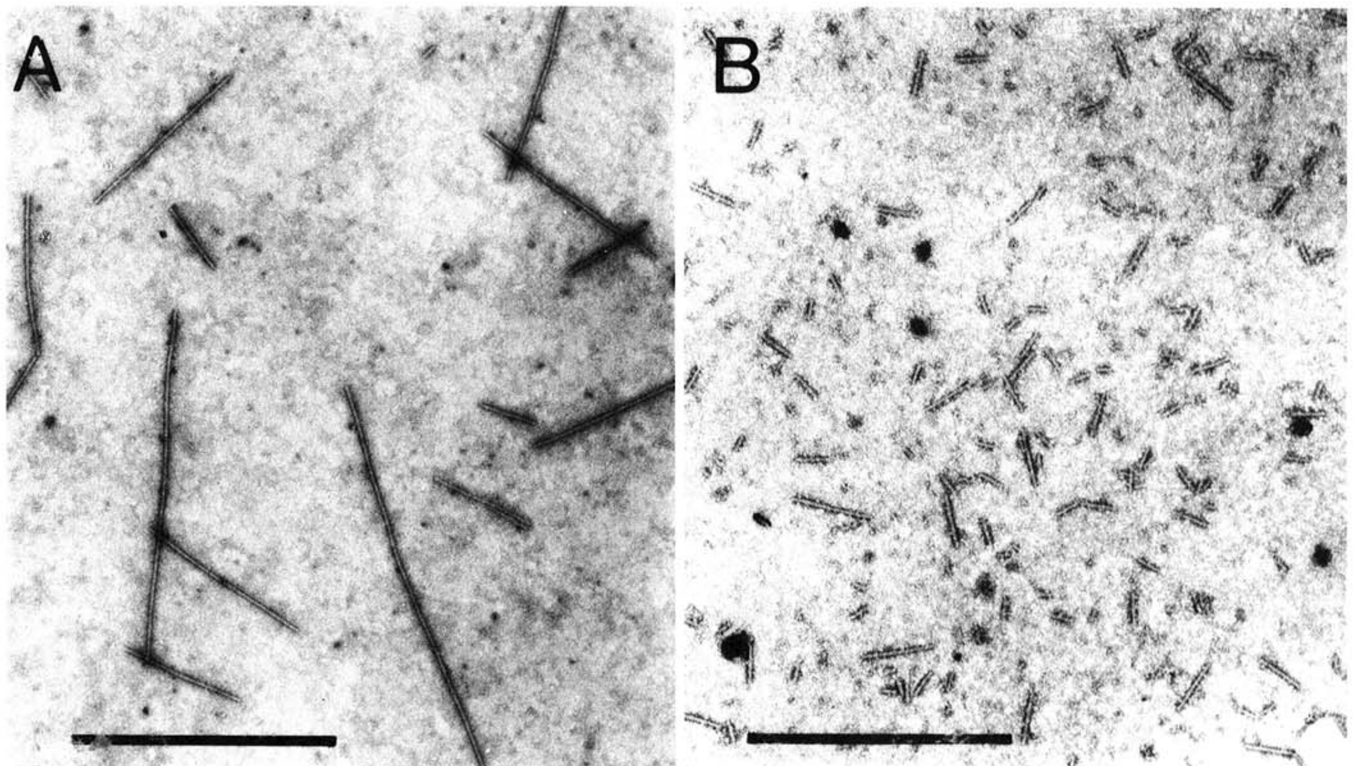

Fig. 1. Electron micrographs of partially purified lily symptomless virus (100 μg protein/ml), negatively stained with 0.25% phosphotungstic acid: **A,** before and **B,** after sonication for 4 min at 20 kc/sec (bar represents 1 μm).

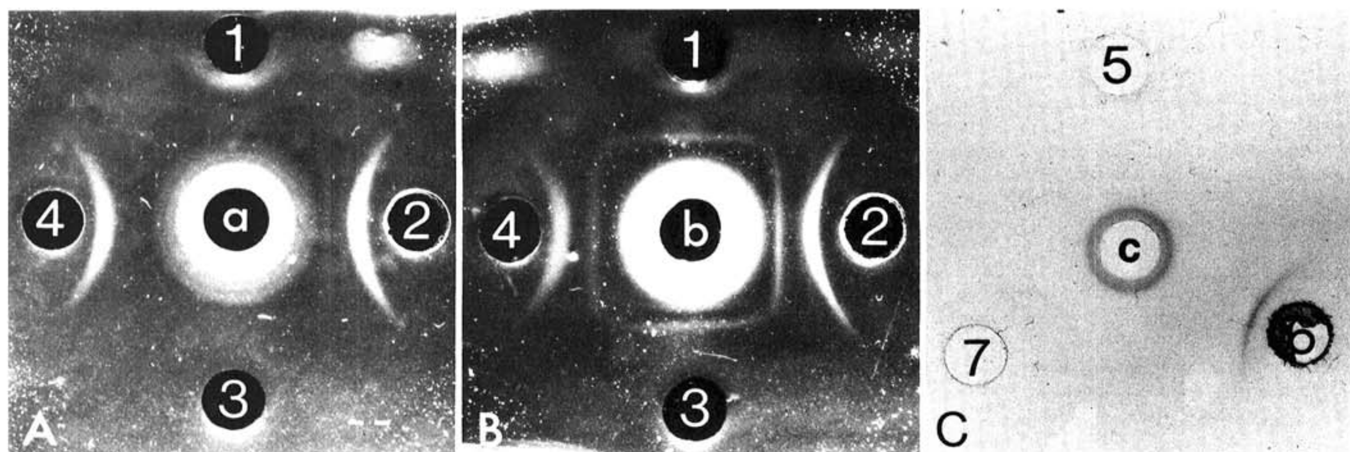

Fig. 2. (A to C) Immunodiffusion reactions of lily symptomless virus (LSV). The central wells of each plate contained anti-LSV serum: **a,** intravenous route of injection; **b,** subcutaneous route of injection with adjuvant; **c,** intravenous route of injection, serum diluted one-fourth with 0.85% NaCl. The reactants in the outer wells of plates A and B were ultracentrifuged to concentrate the LSV: ① LSV, not fragmented; ② sonicated LSV; ③ sonicated virus-free sample; and ④ sonicated LSV, 4 wk old preparation. Sonication was conducted for 4 min at 20 kc/sec. The LSV in plate C was concentrated by isoelectric precipitation: ⑤ LSV, not fragmented; ⑥ LSV, frozen and thawed, six times; ⑦ virus free preparation, frozen and thawed six times. The immunodiffusion was in 0.5% agarose, 0.01 M sodium phosphate buffer, pH 7.4, and 0.02% sodium dodecyl sulphate at 22 C for 36 hr (plates A and B) and 44 hr (plate C). Plate C was stained with light green SF and Buffalo black NBR.

LSV. Similarly, other detergents (0.2, 0.5, and 1% SDS; 1% Tween-20, 40, 60, 80, and 85; and 0.1 and 0.4% sodium deoxycholate) added to the LSV isolate, to the immunodiffusion medium, or to both, were ineffective. Sonication for 4 min at 20 kc/sec fragmented LSV (Fig. 1). Immunodiffusion tests with LSV antisera (Figs. 2A and B) showed a specific reaction band with sonicated purified LSV which was not present with nonsonicated LSV or a virus-free sample. A specific reaction obtained with sonicated LSV stored at 4 C for 4 wk, indicated that the LSV fragments remained dispersed on storage. These results were confirmed by electron microscopy. Freezing and thawing, six times, although not as effective as sonication for the disruption of purified LSV, was the superior method of fragmentation for LSV precipitated isoelectrically, and more samples could be handled simultaneously.

Antiserum specificity. Both routes of injection resulted in the production of high-titer antisera to the LSV antigens (1/2,048 in the precipitin test and 1/32 in the double diffusion test). Subcutaneous injection with virus and adjuvant, however, resulted in a greater production of plant antibodies than did intravenous injection (Figs. 2A and B). This is not unique because antiserum titers of dilute antigens are generally enhanced by subcutaneous or intramuscular routes of injection combined with adjuvants (10).

Isoelectric precipitation of LSV. Twofold serial dilutions with PPB, of purified and sonicated LSV (2 mg protein per milliliter) were tested by immunodiffusion. The highest dilution of purified LSV giving a positive reaction was 1/32 or 63.5 μg protein per milliliter. LSV in clarified sap could not be detected; its concentration was equivalent to a dilution of 1/180 or about 11 μg protein per milliliter.

To find a simple method of concentrating LSV, various methods of reversible precipitation of the virus were tried. Although LSV could not be reversibly precipitated with increasing concentrations of alcohol (5, 10, 20, 30, 40, and 50% of sap) or salted out with ammonium sulphate (5, 10, 20, 33.3, and 50% saturated sap), it did precipitate reversibly at its isoelectric point. The greatest amount of LSV was precipitated between pH 4.0 and 4.4 with 1.0 M sodium acetate-acetic acid buffer, pH 3.1. Immunodiffusion of the precipitates, resuspended in PPB containing 0.05% Igepon T-77, showed (Fig. 2C) a positive reaction with the LSV-infected sample fragmented by freezing and thawing and no reaction with a virus-free sample or a nonfragmented LSV-infected sample. These results were confirmed by electron microscopy of resuspended precipitates. The highest dilution of the resuspended LSV precipitate detectable was 1/6. This is equivalent to an LSV concentration of 1.8 μg/ml of clarified sap.

LSV concentrated from equal volumes of clarified sap by purification or isoelectric precipitation and resuspended in equal volumes of buffer had the same dilution endpoints in the double diffusion test. However, the protein concentration of the LSV precipitated isoelectrically was approximately twice that of the purified LSV. Evidently, plant protein was coprecipitated, but it did not interfere with the assay. Van Slogteren et al (17) reported that with pyrrolidine-fragmented LSV, a protein concentration of 4.9 μg/ml could be detected with the single immunodiffusion drop test. However, a direct comparison of the two immunoassay systems is not possible because the limit of detection of virus in sap, in the immunodiffusion drop test, has not been defined. Concentrations of LSV of 1.8 μg/ml in sap can be detected with our assay. Our method relies on the total content of LSV in sap rather than its concentration. To detect concentrations of virus lower than 1.8 μg/ml, larger sap volumes could be employed. This immunoassay should be of particular value when screening for LSV is required for evaluating breeding or clonal stocks and when tissue culture is used to eliminate LSV from infected stocks.

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