Properties of a Strain of Strawberry Latent Ringspot Virus Isolated from Sweet Cherry Growing in Ontario

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ABSTRACT

Strawberry latent ringspot virus (SLRV) was isolated from leaves, flower parts, and embryos of a 16-year-old Bing sweet cherry tree growing in the Niagara Peninsula of Ontario, Canada. It has a dilution end point of 10^{-4}, a thermal inactivation point of 54 C, a particle diam of 29-32 mμ (average 30 mμ), a sedimentation coefficient of 134 S, a buoyant density of 1.274 g/cm^3, and a nucleic acid content of 30%. Seed transmission of SLRV in *Chenopodium quinoa* ranged from 63 to 100%; infection through seed resulted in stunting, deformed leaves, and chlorotic patterns. Peach was infected by approach grafting to *Chenopodium;* no symptoms resulted other than possible stunting. Absence of the vector, *Xiphinema diversicaudatum,* from field soil in Ontario and the failure to detect the virus in other plantings of Bing cherry suggest that SLRV was imported. Phytopathology 60:1262-1265.

Additional key words: Properties, purification, density-gradient centrifugation.

Of the seven "NEPO viruses" (6) reported, only tomato ringspot and tobacco ringspot viruses have been detected in cultivars grown in Canada (9, 10, 19). This paper reports the occurrence and properties of a third NEPO virus, strawberry latent ringspot virus (12), isolated from a sweet cherry tree located in the Niagara Peninsula of Ontario. This is apparently the first report of the occurrence of strawberry latent ringspot virus in established plants in North America.

MATERIALS AND METHODS.—*Viruses, plants, and bioassay.*—Virus isolates used were the *Prunus* isolate of strawberry latent ringspot virus (SLRV-*P*) isolated from *Prunus avium* L. 'Bing' by T. R. Davidson in Lincoln County, Ontario; tomato bushy stunt virus (TBSV) supplied by B. Kassanis of the Rothamsted Experimental Station, England, and maintained at this Station in cucumber since 1966; and tobacco necrosis virus (TNV) isolated by the authors from roots of cucumber grown in Ontario soil.

All viruses were cultured in cucumber (*Cucumis sativus* L. 'Windermoor Wonder'). SLRV-*P* was also cultured in plants of *Chenopodium quinoa* L. that were used in graft transmission tests with peach seedlings (*Prunus persica* L. 'Elberta'). During midsummer and midwinter, SLRV-*P* induced more severe symptoms in cucumber if plants were grown in a growth room with balanced lighting (12,916 lux) on a 16-hr day at 22 C. Light intensity was measured with a Weston Model 756 quartz sunlight meter.

No satisfactory local lesion host for SLRV-*P* was found; therefore, virus concn was assessed by determining the ID_{50} (infective dosage giving 50% infected plants) based on the number of systemically infected cucumber plants at each of four 10-fold dilutions (16); a min of six plants was used to test each dilution.

Centrifugation.—Low- and high-speed centrifugation was done in Sorvall SS-1 (10 min at 13,000 *g*) and Beckman Model L or L-2 (2 hr at 198,425 *g*) centrifuges equipped with angle rotors. Rate-zonal centrifugation in sucrose density-gradients was done in an SW 25.1 rotor (90,137 *g*) at 10 C for virus purification and at 20 C for comparisons of sedimentation velocities.

Continuous density gradients were used for routine virus purification and were prepared with a gradient forming device (3) using 6% and 60% sucrose (w/w) in water; a complete gradient (24 ml) increased from 10 to 38% sucrose. Gradients were fractionated and scanned with ISCO equipment as described elsewhere (2). Virus was recovered from sucrose by high-speed centrifugation. Hand-layered density gradients were used in comparisons of sedimentation velocities and were prepared with 4, 7, 7, and 7 ml of 10, 20, 30, and 40% sucrose (w/w), respectively, buffered with 0.03 M $MgCl_2$ and 0.3 M glycine adjusted to pH 7.2 with NaOH. Each gradient received 1 ml of purified virus. Virus concn was adjusted to give zones, after centrifugation, with similar absorbance peaks that did not exceed 0.07 OD units on the flow analyzer recordings.

Sedimentation velocities also were determined in a Beckman Model E analytical ultracentrifuge using both Schlieren and ultraviolet optics. Centrifugation runs were kindly made by R. G. Douglas, Department of Microbiology, University of Guelph. Viruses received two cycles of density-gradient centrifugation, and virus pellets were suspended in 0.1 M glycine-0.01 M $MgCl_2$-NaOH buffer (pH 7.2) containing 0.1 M KCl. An-D rotor speeds ranged from 24,640 to 24,690 rpm at 20 C, and the sedimenting boundary was photographed at 4 min intervals. Schlieren patterns and ultraviolet absorption profiles were analyzed as reported by Bozarth et al. (3).

Equilibrium centrifugation in buffered sucrose gradients was done in an SW 50 rotor for 16-24 hr at 273,910 *g* and 20 C. Two gradient forming methods were used: (i) hand-layered gradients were prepared the day before their use with 1 ml each of 40, 45, 50, 55, and 60% sucrose (w/w); or (ii) 0.3 ml of buffered virus solution was layered on 4.7 ml of 60% sucrose and centrifuged immediately so that the gradient formed during the run. Virus concn was adjusted in the manner described under comparative rate-zonal centrifugation methods.

Density determinations were made by refractometry. Gradients were divided into 0.25-ml fractions, and sucrose concn (%) of fractions on both sides of the virus fraction was determined with a Zeiss Abbe refractometer and plotted against the fraction number. The location of the virus fraction was determined from flow analyzer recordings, and was checked by refractometer and bioassay. Conversion of per cent sucrose to density (20 C) was made from sucrose tables (11).

Purification.—SLRV-*P* was purified initially from cherry petals. Tissues were blended in a Sorvall Omni-Mixer (2 min at 100 volts) containing 0.02 M sodium diethyl-dithiocarbamate adjusted to pH 8.0 with thioglycollic acid. After low-speed centrifugation, cold chloroform (one-fourth volume) was added and the mixture was stirred for 5 min at 5 C. Sediment and chloroform were removed by centrifugation, and the solution was concnd by dialysis against polyethylene glycol. The virus was then pelleted by centrifugation and suspended in 0.05 M Tris [tris (hydroxymethyl) amino methane]-HCl buffer (pH 7.6) containing 0.15 M NaCl. This preparation was used for antiserum production.

Subsequently, SLRV-*P* was purified routinely from cucumber cotyledons and leaves that were harvested 6 days after inoculation and then frozen for 24 hr. Tissues were blended in 0.05 M boric acid-NaOH buffer (pH 7.2) and chloroform at a ratio of 1 g:1 ml:1 ml, respectively; chloroform and sediment were removed by centrifugation. The supernatant was adjusted to 20% of saturation with $(NH_4)_2SO_4$ and clarified by centrifugation. The supernatant was then adjusted to 35% of saturation with $(NH_4)_2SO_4$ and again centrifuged. The pellet which contained the virus was suspended in 0.03 M $MgCl_2$-0.3 M glycine-NaOH buffer (pH 7.2). The suspending volume was not less than one-twentieth the buffer volume used in virus extraction; the use of smaller volumes considerably increased the rate of virus aggregation.

Electron microscopy.—Micrographs and particle measurements were kindly made by Frances W. Doane, School of Hygiene, University of Toronto. The materials and methods used have been reported (2).

Absorbance spectrophotometry.—Virus that had been centrifuged twice in density gradients was used for spectrophotometric examination. OD measurements were made with a Beckman DK-2A instrument using NIR silica cells at ca. 25 C. Corrections for light scattering were made according to Englander & Epstein (7).

Immunization and serology.—Antisera to SLRV-*P* were induced in white New Zealand rabbits with preparations from cherry petals or cucumber leaves. Virus that was purified from cucumber by density-gradient centrifugation was cross-absorbed with unfractionated anti-cucumber serum (freeze-dried powder). Virus was emulsified (1:1) in Freund complete Bacto-Difco adjuvant and injected intramuscularly as described earlier (1). Cardiac bleedings were taken weekly, and secondary injections with virus in adjuvant were given 40 days after the primary stimulation. Serological tests were done in petri dishes by the double-diffusion technique (1).

Antiserum to the type strain of SLRV (Hampshire isolate T39) was kindly provided by B.D. Harrison, Scottish Horticultural Research Institute, Dundee.

Physicochemical tests.—The thermal inactivation point of SLRV-*P* was determined with virus that had been clarified with chloroform and precipitated with $(NH_4)_2SO_4$ at 35% of saturation; pellets were suspended in 0.03 M MgCl-0.3 M glycine-NaOH buffer (pH 7.2). Thin-walled glass tubes were used, and 10 min heating periods were started when water in control tubes reached bath temp. Virus solutions were immediately cooled, diluted, and assayed on cucumber.

The effect of salt concn on the stability of SLRV-*P* during extraction from cucumber was studied using boric acid-NaOH buffer (pH 6.0) at various molarities and an equal volume of chloroform. After low-speed centrifugation, the pH was adjusted to 6.0 with HCl and the supernatant was bioassayed. Equal portions of the supernatant were then adjusted to 35% of saturation with $(NH_4)_2SO_4$ and centrifuged. The pellets were suspended in the glycine buffer and the relative virus concn determined serologically (2).

The effect of pH on the stability of SLRV-*P* was studied with extracts from cucumber prepared by blending tissues in 0.05 M boric acid-NaOH buffer (pH 6.0) and an equal volume of chloroform. Portions of the low-speed supernatant were adjusted with NaOH or HCl to give a range in pH (steps of 0.5 pH units) from 3 to 12. After standing for 30 min, each portion was centrifuged at low speed and the pellets were suspended in the original volume of boric acid buffer (pH 6.0). Supernatants and suspended pellets were then bioassayed.

Seed transmission.—Transmission of SLRV-*P* through seeds to seedlings was studied with *Chenopodium quinoa* L. Seed plants were selected at random from groups inoculated during November and December. Seed clusters were removed when pericarps were brown, and the seeds were then dried for 1 month. Seeds of each plant were planted in separate flats and widely spaced to prevent root contact. Assessment of infection was based largely on symptoms: stunt and misshapen leaves with occasional chlorotic patterns. These symptoms were shown to be correlated with virus infection as determined by indexing on cucumber.

RESULTS.—*Occurrence and transmission.*—SLRV-*P* was accidently isolated in 1964 from a 16-year-old Bing cherry located in the Niagara Peninsula and expressing shot-hole symptoms and chlorotic line patterns usually associated with infections by the necrotic ringspot virus (NRSV). The grower had propagated the tree from a local bud-wood source using *Prunus mahaleb* L. understock imported apparently from the United States. Both SLRV-*P* and NRSV were manually transmitted to cucumber from embryos, flower petals, and leaves of this tree. A number of plants were singly infected with each virus; therefore, pure cultures of SLRV-*P* were readily established.

It is doubtful that SLRV-*P* occurs widely in the Niagara Peninsula because extensive manual indexing

of mahaleb seed trees and most commercial sweet cherry cultivars over the past 15 years has failed to detect the virus. In view of the limited knowledge of the history of the parent stock, there is little value in speculating on the probable initial source of SLRV-*P*.

In vitro properties.—1) *Dilution end point.*—Virus preparations retained infectivity when diluted to 10^{-4} but not 10^{-5}. Similar results are reported for other isolates of SLRV (12, 21).

2) *Thermal inactivation point.(TIP).*—Plots of ID_{50} values indicated that the rate of thermal inactivation was exponential from about 49 to 56 C, at which temp inactivation was complete. The 54 TIP for SLRV-*P* agrees with that reported for other isolates of SLRV (12, 21).

3) *Effect of salt concn.*—Bio- and seroassays indicated that the stability of SLRV-*P* was affected little by salt concn ranging from 0 to 1 M.

4) *Effect of pH.*—Only slight variability in the infectivity of SLRV-*P* preparations was noted over the range pH 5 to 8. All infectivity was lost between pH 4 and 4.5 and between pH 9 and 9.5. On the average, ID_{50} values indicated that the optimum for extraction was near pH 6. Although different extraction procedures and buffers were used, these results agree with those obtained with other isolates of SLRV (12).

5) *Particle size and shape.*—SLRV-*P* particles ranged in diam from 29 to 32 mμ (30 mμ average), and were generally hexagonal in profile. Particle diam of other isolates of SLRV have been reported to be 26 mμ (12), 28 mμ (5), and 25-29 mμ (21).

6) *Sedimentation velocity.*—The sedimentation coefficient of SLRV-*P* was determined initially by the Brakke method (4) in sucrose density gradient columns using both TNV and TBSV as marker viruses. With assumed $S_{20,w} = 132$ and 118 for TBSV and TNV (8), respectively; values of 134-137 S were calculated for SLRV-*P*. Analytical centrifuge data indicated that the sedimentation velocity of SLRV-*P* was concn-dependent, and sedimentation coefficients extrapolated to $S_{20,w}$ = ca. 134 at zero concn. This value was calculated largely from ultraviolet absorption profiles. Brunt (5) has reported sedimentation coefficients of 128-130 S for a narcissus isolate of SLRV; the conditions under which the determinations were made were not reported.

7) *Buoyant density.*—Values obtained for TNV, TBSV, and SLRV-*P* remained unchanged after 19 and 24 hr of centrifugation in sucrose gradients. Single peaks were usually obtained with TNV and TBSV, but SLRV-*P* peaks often had high-density shoulders. Density (g/cm^3) values obtained for these viruses are as follows: TNV = 1.252, TBSV = 1.257, and SLRV-*P* = 1.274.

8) *Ultraviolet absorption spectra.*—SLRV-*P* possessed uncorrected absorption min and max at 241 and 259 mμ, respectively. The average uncorrected 280:260 ratio was 0.60. Corrections for light scattering gave an average 280:260 ratio of 0.57 that would indicate a nucleic acid content of ca. 30% according to the determinations of Paul (15).

Transmission through seed.—Based largely on visual inspection, the transmission of SLRV-*P* through seeds of *Chenopodium quinoa* L. averaged 87%. Transmission ranged from 63-100% in 580 seedlings from eight plants. One flat of 70 seedlings was indexed on cucumber and found to be 97% infected. About 3% of the infected plants appeared normal, but were nevertheless slightly smaller than healthy plants. Stunting, deformed leaves, and chlorotic patterns were the usual symptoms resulting from infection through seed. Extremely stunted plants attained only about 30% of the height of healthy plants at maturity.

Emergence time, percentage germination (98-100%), and seedling survival (98-100%) were the same for healthy and for infected seed lots. Healthy seedlings grew and expanded much faster than diseased plants, so that within 2 weeks from emergence it was apparent which plants had escaped infection. Also, healthy seedlings were quite uniform in their rate of development, whereas infected plants varied greatly.

The only other report of seed transmission of SLRV was with *Mentha arvensis* in which ca. 6% of the seedlings were infected (20). Neither parents nor progeny expressed symptoms.

Serology.—SLRV-*P* was positively identified with antiserum to the type strain that was isolated by Lister (12) from strawberry. The dilution end point of this serum for SLRV-*P* was within two 2-fold dilutions of the end point indicated for the homologous virus.

Host range.—The identity of SLRV-*P* was not known initially; therefore, a host range study was made to obtain information on its probable identity and on the most suitable hosts for bioassay and virus purification. Since only small groups of each species were inoculated and later checked for systemic infection, a number of potential hosts of low susceptibility was possibly missed.

Twenty-seven species were tested, including peach and mahaleb cherry. In general, the symptoms induced by SLRV-*P* were similar to those associated with the mild strains isolated by Lister (12). Plants not previously tested (12, 21) that developed symptoms were *Daubertonia punicea* DC. and *Trichosanthes anguina* L.; latent infection was detected in *Torenia fournieri* Lind. No infection was induced in *Beta vulgaris* L., *Capsicum annuum* L., *Cassia tora* L., *Cucumis dipsaceus* Ehrh., *Helianthus annuus* L., *Luffa cylindrica* Roem., *Mormordica* spp., *Tithonia speciosa* Hook, or *Raphanus sativus* L.

Peach and mahaleb cherry apparently were not infected by manual inoculation. Peach was easily infected, however, by approach-grafting to systemically infected *Chenopodium quinoa* L. SLRV-*P* was recovered from the forced leaves of peach, but no definite symptoms were noted with the possible exception of slight stunting and a faint transient mottle.

DISCUSSION.—Strawberry latent ringspot virus is reported to occur in asparagus, sweet cherry, black and red currant, elder, euonymus, black locust, narcissus, peach, plum, raspberry, rhubarb, and strawberry, and in the following countries: England, Germany, Ireland, Italy, Scotland, and Switzerland (13, 17, 18). In 1967,

A. Vuittenez (Station de Pathologie végétale, Colmar) sent to the senior author antisera to two virus isolates associated with stunting or rosette of peach growing in the Lyon district of France; the antisera reacted with SLRV-*P*. These peach isolates were subsequently shown to be similar to a SLRV isolate obtained from the Scottish Horticultural Research Institute, Dundee (22). Consequently, because of the wide host range and generally latent nature of SLRV, its distribution is probably far wider than is now known.

We were not able to investigate spread of SLRV-*P* because the source orchard was removed. The only known vector (12) of SLRV, *Xiphinema diversicaudatum,* has not been found in Ontario soils except in commercial glasshouses, and it is believed that the nematode cannot survive here naturally. Pollen transmission of SLRV-*P* is a possibility, as the virus was isolated from cherry flower parts as well as from embryos. Experiences with other NEPO viruses, however, indicate that although embryos and the resultant seedlings may become infected through pollination, the seed parent may escape infection (14). Therefore, because a vector for SLRV-*P* has not been found in Ontario, the virus may be presumed to be an importation, and significant spread will most likely be through the use of infected budwood or rootstocks.

NEPO viruses generally induce slight or no obvious symptoms in progeny infected through seed (14). Exceptions have been noted (14), however, and to these must be added SLRV-infected *C. quinoa*. Nevertheless, it is quite likely that SLRV is latent in some hosts and that recovery occurs in others. Infected progeny from these hosts would be expected to be symptomless, although growth effects might be demonstrable. This latent condition may be one that exists generally in many SLRV-infected *Prunus* species.

Indexing of *Prunus* spp. for SLRV is currently limited to the use of herbaceous plants, of which cucumber and *Chenopodium* species are probably the most diagnostic. Lister's work indicates that *Tetragonia expansa* (New Zealand spinach) may also be a useful indicator (12). As pointed out by Lister (12), many tree fruit viruses, including the NEPO group, induce symptoms in cucumber and *Chenopodium* species, and their association with SLRV probably would mask symptoms induced by the latter, assuming systemic movement of each virus (12). Symptoms induced by pure cultures of SLRV-*P*, however, were sufficiently distinct to prevent confusion with those induced by manually transmitted tree fruit viruses with which the authors have had some experience, viz., necrotic ringspot, prune dwarf, line pattern, tobacco ringspot, tomato ringspot, tomato bushy stunt, arabis mosaic, apple chlorotic leaf spot, and peach rosette mosaic (a new NEPO virus to be described by H. F. Dias of this Station) viruses. This may not be true, however, for all strains of SLRV. As demonstrated by Lister, isolates from various regions may differ so widely in their symptomatology that serology must be relied upon for their identification (12).

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