Beet Necrotic Yellow Vein Virus in North America

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ABSTRACT

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Beet necrotic yellow vein virus (BNYVV) was isolated from soil samples collected from beneath the canopy of two different cherry trees in the same orchard in the state of Washington. Results of back-inoculation attempts suggested that the virus may have been associated with some weed host rather than with cherry. The Washington BNYVV isolate had a limited host range that differed only slightly from those of isolates found in France and Japan to which the Washington isolate was related serologically. Purified virus preparations aggregated spontaneously at concentrations above 0.2 mg/ml. A purification procedure was developed that yielded enough nonaggregated virus to demonstrate the presence of four nucleoprotein components designated NP 1, NP 2, NP 3, and NP 4 having sedimentation coefficients of 98S, 132S, 154S, and 174S, respectively. Infectivity was associated primarily with NP 4. The virus was serologically unrelated to tobacco mosaic virus, tobacco rattle virus, or wheat soilborne mosaic virus. This appears to be the first report of BNYVV in North America.

During attempts to associate a soilborne virus with the occurrence of stem pitting disease (8) of sweet cherry (*Prunus avium* L.) in Washington (7), we twice encountered a rod-shaped virus, which we will demonstrate was beet necrotic yellow vein virus (BNYVV). The virus was obtained from the roots of *Gomphrena globosa* L. "bait plants" grown in soil samples taken from beneath the canopies of two different diseased sweet cherry trees located in the same orchard. This virus has been associated with the soilborne rhizomania disease of sugar beets (*Beta vulgaris* L.) in France (10), Germany (9), Italy (4), and Japan (12). So far as we are aware BNYVV has not been reported previously from North America and has not been detected in cherry orchards elsewhere but its vector, *Polymyxa betae* Keskin, has recently been found in California (5).

Although BNYVV is known to be a rod-shaped virus with three (12) or four (9) predominant particle lengths and that produces four nucleic acid components upon SDS-phenol extraction, previous workers were unable to resolve its nucleoprotein components because of aggregation problems (9,12).

This report describes procedures whereby we were able to separate four nucleoprotein components with predominant particle lengths similar to those found in leaf dip preparations.

MATERIALS AND METHODS

Virus source. Beet necrotic yellow vein virus (BNYVV) was isolated from the roots of *Gomphrena globosa* plants grown in soil samples collected from beneath the canopies of two different sweet cherry trees. In preliminary studies we could find no differences between the two isolates. All results reported here were obtained with a single isolate increased after two serial single-lesion transfers in *Chenopodium quinoa* Wild.

Host range and symptomology. Various plant species were rub-inoculated with extracts from infected *C. quinoa* leaf tissue obtained by triturating the tissue in 0.01 M borate buffer, pH 9.0. Inoculated plants were observed for symptoms for at least 2 wk after inoculation. Samples from inoculated leaves and from uninoculated tip leaves of all plants were separately back indexed on *C. quinoa*.

Purification procedure. Preliminary experiments indicated that 0.02 M borate, pH 9.0, was the most suitable buffer for extraction. Fresh or frozen infected *C. quinoa* tissue (50 g) harvested 14 days after inoculation was homogenized for 4 min at 4 C in a Waring Blendor with equal volumes of 0.02 M borate buffer, pH 9.0 and chloroform (10 ml of mixture per gram tissue). Igepon T-73 (0.5 g) was added and the extract was stirred with an overhead stirrer for 1 hr. The emulsion was broken by low-speed centrifugation and 25 ml of 30% polyethylene glycol (PEG) containing 6% NaCl was added for each 100 ml of supernatant. After it was mixed vigorously for 1 min, the mixture was stored at 4 C for 1 hr, centrifuged in a Beckman rotor number 30 at 7,000 rpm for 20 min, and the pellets were suspended overnight in 2.5 ml 0.01 M borate buffer, pH 9.0. The suspensions were combined, centrifuged at 3,000 rpm for 5 min and the supernatant was centrifuged at 27,000 rpm for 2.5 hr. The final pellet was suspended in 2 ml 0.01 M neutral phosphate buffer containing 0.5 M urea and 0.1% mercaptoethanol (phosphate-urea buffer). Following low-speed centrifugation the supernatant was layered on 10–40% sucrose gradient columns either unbuffered or buffered with the phosphate-urea buffer and centrifuged 2 hr at 24,000 rpm in a Beckman SW 25.1 rotor. The tubes were fractionated by using an ISCO fractionator.

Measurement of sedimentation coefficients. Sedimentation coefficients were estimated by using linear-log sucrose gradients in Beckman SW 25.1 rotor as described by Brakke and Van Pelt (2). Three components of tobacco ringspot virus (TRSV) (1 mg/ml) were used as sedimentation standards purified as described by Stace-Smith et al (11). One milliliter of the three TRSV components was layered on one column and 1 ml of BNYVV (0.1–0.2 mg/ml) was layered on a sister column. The samples were centrifuged 2 hr at 24,000 rpm at 10 C.

Serology. Antiserum to BNYVV was prepared in a rabbit given four 2-ml intravenous injections (0.1 mg/ml) at 3-day intervals. Antisera to the Japanese and the French isolates of BNYVV were kindly supplied by T. Tamada and C. Putz, respectively.

Agar gel for double diffusion tests employed 10 ml of 0.45% (w/v) Difco agar dissolved in 0.01 M neutral phosphate buffer containing 0.01% mercaptoethanol, 0.5 M urea, and 0.15% NaN_3. Wells in the agar gels were cut immediately before use. Partially purified BNYVV, suspended in phosphate-urea buffer containing 0.85% NaCl, was used as the antigen in these tests. The effect of urea on the reaction was studied by using partially purified virus and infected *C. quinoa* leaves triturated in 0.01 M phosphate buffer containing 0.01% mercaptoethanol and a range of different concentrations of urea.

Ring-interface tests were done as described by Ball (1). Immune electron microscopy of infected *C. quinoa* leaf tissue was done as

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described by Milne and Luisoni (6).

Electron microscopy. Samples of BNYVV extracted from infected *C. quinoa* and from each visible band in density gradient tubes after two cycles of rate zonal centrifugation were examined by electron microscopy. Preparations were negatively stained with 2% uranyl acetate, pH 7.0, and examined by using a Zeiss EM 9 electron microscope.

RESULTS

Host range and symptomology. The plant species that developed symptoms 5–7 days after rub-inoculation with a triturate from infected *C. quinoa* are listed in Table 1. The virus was recovered from inoculated leaves of *C. quinoa, C. amaranticolor* Caste & Reyne, *C. murale* L., *Beta vulgaris* L., *Nicotiana tabacum* L. H423, *N. glutinosa* L., and *N. tabacum* L. 'Xanthi.' No virus was detected in uninoculated tip leaves of these plants. Other plants including *P. hybrida, Gomphrena globosa, Spinacia oleracea* L., and *N. clevelandii* Gray exhibited systemic symptoms ranging from mild mottle to severe leaf distortion (Table 1). The host range of this isolate is similar to that reported for BNYVV isolates from France (10) but differs from the Japanese isolates (12) by infecting *N. clevelandii* and *N. tabacum* 'Xanthi.' It differs from both in that the Washington isolate invaded *P. hybrida* and *G. globosa* systemically.

Inoculated plant species from which no virus was recovered include: *Apium graveolens*, L. var. *dulce* DC., *Avena sativa* L., *Brassica oleracea* L. var. *capitata* L., *Capsicum frutescens* L., *Citrullus vulgaris* L., *Convolvolus arvensis* L., *Cucumis sativus* L., *Cucurbita maxima* Dcne., *Daucus carota* L. var. *sativa* DC., *Dianthus caryophyllus* L., *Hordeum vulgare* L., *Lathyrus odoratus* L., *Lycopersicon esculentum* Mill., *L. peruvianum* Mill., *Impatiens balsamina* L., *Plantago major* L., *Phaseolus vulgaris* L., *Raphanus sativus* L., *Triticum aestivum* L., *Sesbania exaltate* Cory, *Solanum tuberosum* L., and *Vicia faba* L.

General properties. General properties of our BNYVV isolates from Washington were: dilution end point, 10^{-5}; thermal inactivation point, 70 C; and in vitro longevity, 3 days.

Purification. Four visible bands located 20, 25, 28, and 30 mm below the meniscus were observed following rate zonal sucrose density gradient centrifugation of partially purified virus. No comparable bands were found in tubes layered with preparations from similarly treated healthy plants. The four bands were designated NP 1, NP 2, NP 3, and NP 4 according to their increasing sedimentation velocities. An ISCO scanning pattern of a typical gradient containing purified BNYVV is shown in Fig. 1. Component NP 2 usually appeared as a faint but discrete visible band in density gradient tubes. However, it was resolved only as a shoulder on the trailing edge of NP 3 in most ISCO patterns.

Association of infectivity with visible density gradient zones. When rate zonal sucrose density gradient tubes containing purified BNYVV were fractionated into 2-ml fractions and those were assayed on *C. quinoa* following differential ultracentrifugation, most of the infectivity was found to be associated with fractions that corresponded to components NP 3 and NP 4. Although the fractions that corresponded to components NP 1 and NP 2 reacted with the homologous antiserum, they exhibited essentially no infectivity.

To achieve better separation, the four visible bands of BNYVV were separated by two cycles of rate zonal centrifugation. Fractions corresponding to individual components were removed, dialyzed overnight against 10% sucrose solution (containing phosphate-urea buffer), layered individually on 15–40% density gradient tubes, and centrifuged at 24,000 rpm for 2 hr in a Beckman SW 25.1 rotor. Fractions containing the separated components were removed (Fig. 2), adjusted to the same A_{260}, and assayed on *C. quinoa*. Each preparation also was tested serologically against homologous antiserum and examined with the electron microscope. Assay results (Table 2) indicate that nearly all infectivity was associated with NP 4. Components NP 1 and NP 2 were noninfectious, while NP 3 showed traces of infectivity, possibly because it was still contaminated with small amounts of NP 4. Mixing any of the other three components with NP 4 did not enhance its infectivity (Table 2).

All four nucleoprotein components reacted with the homologous antiserum, and fractions containing each component were found by electron microscopy to contain elongated particles (Fig. 3). Normal lengths for components NP 1, NP 2, NP 3, and NP 4 were found to be about 85, 115, 260, and 385 nm, respectively. The width of all particles was ~20 nm.

Ultraviolet light absorption spectra. Each component suspended in sucrose exhibited a similar ultraviolet light absorption spectrum with minimum absorbance at 250 nm and a broad maximum between 264 and 272 nm. The A_{260} A_{280} (uncorrected for light scattering) for each component was 1.11, suggesting a nucleic acid content of about 4%.

Virus aggregation. The procedure described above provided adequate amounts of virus to resolve the four nucleoprotein components. Yields estimated spectrophotometrically varied from 0.1 to slightly over 0.2 mg/ml prior to density gradient centrifugation. However, all attempts to increase the virus concentration much above 0.2 mg/ml (either before or after density gradient centrifugation) resulted in the formation of virus

TABLE 1. Symptoms produced on plant species infected with beet necrotic yellow vein virus

Plant species	Symptoms
Petunia hybrida	Mild systemic mottle and leaf puckering.
Gomphrena globosa	Severe distortion and rugosity of systemically infected leaves.
Chenopodium quinoa	Chlorotic local lesions that occasionally turn necrotic. Lesions rarely spread to the leaf veins.
Chenopodium murale	Chlorotic local lesions.
Beta vulgaris	Chlorotic local lesions that tend to diffuse along the veins. No systemic symptoms.
Spinacia oleracea	Systemic mottle and distortion.
Nicotiana clevelandii	Mild systemic mottle.
Nicotiana glutinosa	Chlorotic local lesions on inoculated leaves.
Nicotiana tabacum 'Xanthi'	Few mild chlorotic lesions.
Nicotiana tabacum H423	Symptomless but the virus could be recovered from inoculated leaves.

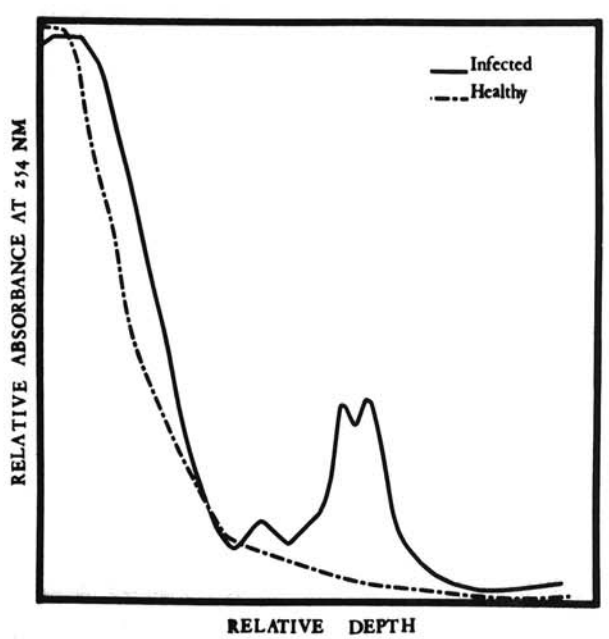

Fig. 1. Ultraviolet absorbance scan pattern of a typical rate zonal sucrose density gradient tube on which partially purified beet necrotic yellow vein virus had been centrifuged for 2 hr at 24,000 rpm.

aggregates large enough to be removed by low-speed centrifugation. We were unable to resuspend these aggregates in any of several buffers tested.

Sedimentation coefficients. Sedimentation coefficients of 98S, 154S, 174S for components NP 1, NP 3, and NP 4, respectively, were estimated in linear-log sucrose gradient using three components of tobacco ringspot virus as markers. A sedimentation coefficient of 132S was estimted for NP 2 by its relative distance from the meniscus.

Effect of urea on BNYVV infectivity and serological reaction. Since urea is known to prevent aggregation of viruses caused by hydrophobic interactions (3) its effect on infectivity and on agar gel immune diffusion was tested using crude sap or partially purified preparations. The results (Table 3) indicate that 0.5 M urea enhanced the serological reaction between BNYVV and its homologous antiserum probably by preventing hydrophobic aggregation of virus particles. No serological reaction was detected at 2.0 M urea and above, possibly because urea at these concentrations prevented hydrophobic interactions between the virus and its specific antibodies.

Infectivity seemed either not to be affected or was slightly enhanced with 0.5 M urea present in the grinding buffer. However, infectivity decreased gradually as the urea concentration was increased.

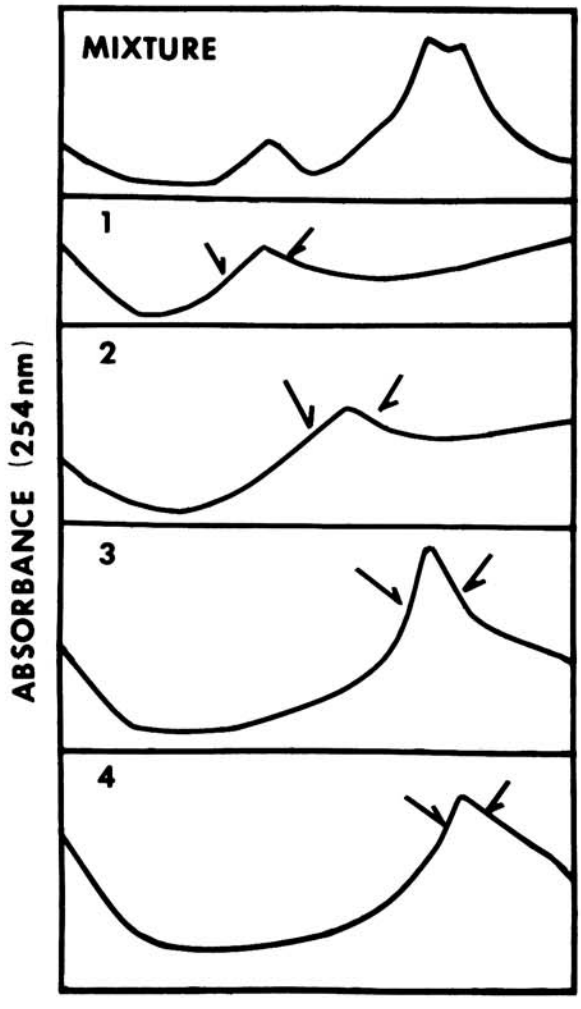

Fig. 2. Ultraviolet absorption scan patterns of 25–40% sucrose density gradient tubes containing a mixture of all components of beet necrotic yellow vein virus (top pattern) or separated components **1,** NP 1, **2,** NP 2, **3,** NP 3, and **4,** NP 4. The preparations were suspended in 10% sucrose prepared with phosphate-urea buffer and centrifuged 2 hr at 24,000 rpm. Fractions between arrows were removed for assay.

Serology. In agar gel double diffusion, ring interface, and immune electron microscopy tests the Washington isolate of BNYVV reacted with its homologous antiserum as well as with antisera prepared against the Japanese and the French BNYVV isolates. The virus did not react with antisera prepared against

TABLE 2. Relative infectivity of beet necrotic yellow vein virus components, separated by two cycles of rate zonal density gradient centrifugation, and of their mixtures

Centrifugation component	Average number of local lesions[a] Exp 1	Exp 2	Serological reaction[b]
NP 1	0.0[c]	0.0	+
NP 2	0.0	0.0	+
NP 3	0.1	0.1	+
NP 4	7.9	13.9	+
NP 3 + 4	8.3	11.3	+
NP 1 + 2 + 3 + 4	7.8	7.9	+

[a] All components were adjusted to A_{260} = 0.06 and assayed on opposite half leaves of *Chenopodium quinoa* randomized to minimize the effects of leaf position.
[b] Ring interface test using homologous antiserum prepared against a mixture of components.
[c] Average number of lesions per half leaf.

TABLE 3. Effect of urea on beet necrotic yellow vein virus infectivity and on serologial reactions in agar gel double diffusion tests

Urea conc. (moles per liter)	Serological reaction[a] Exp. 1[c]	Exp. 2[c]	Exp. 3[d]	Infectivity[b] Exp. 1[c]	Exp. 2[c]
0.0 (control)	+	+	++	12	25
0.5	+++	+	+++	16	24
1.0	−	+	+	13	16
2.0	−	−	−	12	5
4.0	−	−	−	8	3
6.0	−	−	−	6	1
8.0	−	−	−	0	0

[a] +++ = Strong reaction, lines appearing in 2 days; ++ = intermediate reactions in 2–3 days; + = faint reactions requiring more than 3 days; and − = no detectable reaction.
[b] Average number of lesions on *Chenopodium quinoa* half leaves.
[c] Each treatment consisted of 2 g of infected *C. quinoa* leaves triturated in 0.01 neutral phosphate buffer containing 0.1% mercaptoethanol and the desired concentration of urea. Each treatment was tested in a separate plate.
[d] Partially purified virus suspended in 0.01 M neutral phosphate buffer containing the desired concentration of urea.

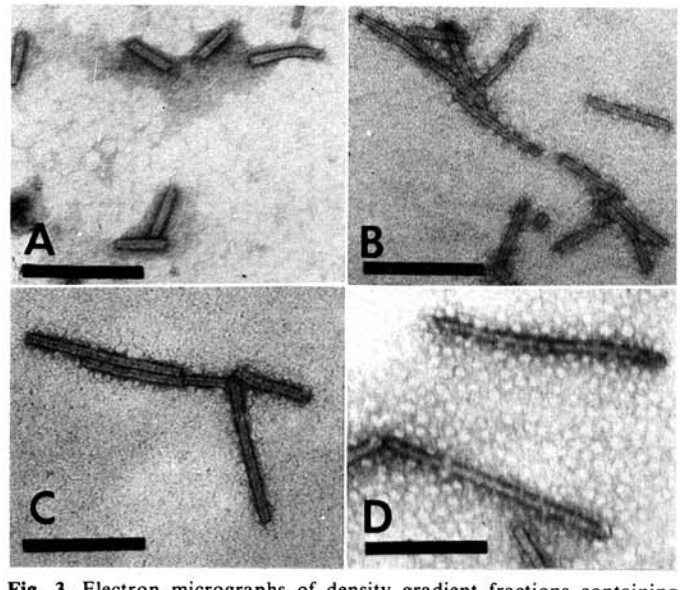

Fig. 3. Electron micrographs of density gradient fractions containing nucleoprotein components **A,** NP 1, **B,** NP 2, **C,** NP 3, and **D,** NP 4 of beet necrotic yellow vein virus (bar = 200 nm).

tobacco mosaic virus, tobacco rattle virus, or wheat soilborne mosaic virus, all of which resemble BNYVV in morphology.

Back-transmission attempts. Forty-eight *Prunus tomentosa* Thunb. and 10 *Prunus mahaleb* L. seedlings were grafted with various tissues from BNYVV-infected *G. globosa, C. quinoa*, and petunia and observed for 3 yr in the greenhouse and lathhouse. Newly emerging roots of 15 *P. tomentosa* and 10 *P. mahaleb* seedlings were rub-inoculated with purified BNYVV (~0.2 mg/ml) and the plants were observed for 2 yr. In addition, four *P. tomentosa* and four *P. mahaleb* seedlings were grown for 3 yr in soil samples collected from the locations where the original BNYVV isolates were obtained. None of the above plants developed visible symptoms. Furthermore, we were unable to transmit BNYVV from leaves or roots by rub-inoculation to *C. quinoa* and were unable to detect BNYVV in leaf triturates by serological tests.

DISCUSSION

Putz (9) demonstrated that particles of BNYVV observed in *C. quinoa* sap had four predominant particle lengths similar to those reported here. He also obtained four nucleic acid components from purified virus that ranged in molecular weight from 0.6×10^{-6} to 2.3×10^{-6}. However, Putz was unable to resolve the nucleoprotein components because of severe end-to-end aggregation of the purified virus (9). The use of borate buffer in the initial grinding medium, the inclusion of urea and mercaptoethanol in the suspending buffers, and the avoidance of high-speed centrifugation as much as possible partially resolved the aggregation problem. By using these modifications we were able to produce enough non-aggregated virus to demonstrate the existence in rate zonal surcose density gradients of four nucleoproteins with low nucleic acid content and particle lengths similar to those found in leaf dip preparations. However, we were unable to prevent aggregation of BNYVV in suspensions containing more than about 0.2 mg/ml purified virus. Consequently, we have been unable to determine relationships between the four nucleoproteins and the four RNAs described by Putz.

Although our isolates of BNYVV were obtained from a sweet cherry orchard that had not been previously cropped to sugar beets, our inability to infect either *P. mahaleb* (the rootstock present in the orchard) or *P. tomentosa* seedlings suggests that BNYVV may have been associated with a weed host rather than the cherry trees.

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