Identification and Partial Characterization of a Closterovirus Infecting *Nandina domestica*

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ABSTRACT

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Plants of *Nandina domestica* 'Nana-purpurea' exhibit horticultural characteristics (stunting, foliar mosaic, and distortion) frequently associated with virus etiology. Flexuous rod-shaped virus particles with a helical structure and ranging from 696–830 nm were consistently found in negatively stained leaf extracts of this cultivar. Phloem-limited inclusions resembling those described for closteroviruses were seen in ultrathin sections and in tissues stained with azure A. The virus produced distortion and mosaic in leaves of nandina seedlings inoculated by grafting or by slashing stems with a partially purified preparation. Infected plants also developed stem pitting, but were not appreciably stunted. The virus was not transmitted by sap inoculation to nandina seedlings or any herbaceous host tested. The green peach aphid, *Myzus persicae*, did not transmit the virus to nandina seedlings. The virus was purified from nandina tissue by homogenization in 0.1 M sodium phosphate buffer (pH 7.2 and containing 0.5 M Na_2SO_3), concentration with 4% polyethylene glycol, and isopycnic centrifugation in cesium sulfate. The $A_{260/280}$ ratio of purified virus was 1.22–1.24, and a maximum yield was 0.044 mg/g fresh weight. The virus, here designated as nandina stem pitting virus, is distinct from previously described viruses from nandina.

Heavenly bamboo, *Nandina domestica* Thunb. (family Nandinaceae), is an ornamental evergreen shrub native to eastern Asia. It is widely grown in the southern United States as a landscape plant and it can be propagated either vegetatively or by seed. The larger types without varietal designations are referred to as "common nandina" and are typically seed propagated, whereas named dwarf cultivars are vegetatively propagated. Two dwarf cultivars, 'Harbor Dwarf' and 'Nana-purpurea,' are especially popular in the United States. The 'Harbor Dwarf' cultivar closely resembles common nandina in leaf color and form, whereas plants of 'Nana-purpurea' typically have cupped and distorted leaves with variegated patterns resembling virus symptoms (21,24, and M. M. Abo El-Nil, *personal communication*). Three viruses have been reported previously from nandina: cucumber mosaic virus (4), tobacco ringspot virus (25), and a potexvirus nandina mosaic virus (21,24,26).

This study reports a fourth virus from nandina, herein referred to as nandina stem pitting virus (NSPV). Portions of this research have been published previously (1).

MATERIALS AND METHODS

Inoculation experiments. Inocula were prepared by triturating young leaf tissue from 'Nana-purpurea' virus source plants in a mortar in the presence of 0.05 M potassium phosphate buffer, pH 7.2. Different additives were incorporated in the buffer to prevent interference from virus inhibitors reported in nandina (4,9,22,24): 1% Na_2SO_3; 2.5% nicotine in 0.1 M phosphate buffer, pH 7.2; or half saturation with ammonium sulfate. The extracts were applied with a cheesecloth pad on leaves of test plants dusted with 22-μm (600-mesh) Carborundum. Inoculated plants were seedlings of common nandina, *Chenopodium amaranticolor* Coste & Reyn., *C. quinoa* Willd., *Gomphrena globosa* L., *Nicotiana benthamiana* Domin., and cowpea, *Vigna unguiculata* (L.) Walp. In all trials, a minimum of three plants of each species were inoculated, and at least two uninoculated plants were used as controls. All plants were maintained 3–4 wk after inoculation in a greenhouse and observed for symptoms.

Ten 3-mo-old plants of common nandina grown from seed were graft inoculated with side chips taken from stems of 'Nana-purpurea' virus source plants with a sterilized razor blade. Side chips with phloem tissue were inserted under the bark of the receptor plants and wrapped with grafting tape. The grafting tape was removed after 10 days, and the graft-inoculated plants and 10 uninoculated plants of the same age were maintained in a greenhouse at 27–30 C for observation. All plants were fertilized and repotted whenever necessary.

A "slash" inoculation method, similar to that described by Garnsey et al (12) for citrus tristeza virus (CTV), was used to inoculate 9-mo-old common nandina seedlings with NSPV. For this, a sterilized razor blade was used to make 10–12 wounds on the stem of each of the receptor plants to expose the vascular tissues. The inoculum was freshly purified NSPV, added dropwise with a pasteur pipette to the exposed tissue. The plants were maintained in a greenhouse for 12 wk and monitored for symptoms.

Attempts were made to transmit NSPV from 'Nana-purpurea' inoculum source plants to common nandina seedlings with apterous *Myzus persicae* (Sulz.), reared on seedlings of healthy pepper, *Capsicum annuum* L. Aphids were starved 1–2 hr and, by means of a camel's-hair brush, were placed individually on source plants for a 20–24 hr acquisition access period (23). The aphids were then removed, starved for about 1 hr, and transferred to healthy plants for a 24- to 48-hr test feeding. The test plants were then sprayed with malathion and transferred to a greenhouse for observation. Since mature or nearly mature, fully expanded nandina leaf tissues were unsuitable for aphid probing, special care was taken to transfer aphids exclusively to very young, rapidly expanding leaf tissue.

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Light microscopy. Tissues were prepared for light microscopic examination by the procedures described by Christie and Edwardson (7). Epidermal and subepidermal tissues cleared in ethylene glycolmonomethylether were stained in either calcomine orange-Luxol brilliant green or azure-A and mounted in Flo-Tex X. Vascular tissues cut into 16-μm-thick sections with a cryostat were similarly stained and mounted.

Electron microscopy. Negatively stained leaf extracts were prepared with a Hitachi model 600 electron microscope. Initial procedures with leaf pieces diced in 1–2% aqueous phosphotungstate (pH 6.8) were generally unsatisfactory. More reliable results were obtained when tissues were diced on a glass slide containing several drops of 0.2 M neutral potassium phosphate buffer. A drop of this suspension was transferred to a formvar and carbon-coated 75 × 300-mesh grid for 1–2 min and blotted dry, and grids were washed dropwise with 20 drops of distilled water and then negatively stained for 2–3 sec in solutions (containing 0.1% bacitracin) of either 2% potassium phosphotungstate (pH 5.0 or 6.8), 2% uranyl acetate, or 2% uranyl formate (S. R. Christie, D. E. Purcifull, and N. A. Ahmed, *unpublished*). Particle measurements were made from particles in leaf extracts prepared from 'Nana purpurea' and stained with 2% uranyl acetate. A diffraction grating with a linear spacing of 462.9 nm was used as a measurement standard.

The fine structure of virus particles was studied following sap clarification. Fresh 'Nana-purpurea' nandina leaf tissues were triturated in a mortar in the presence of 0.1 M sodium phosphate buffer (pH 7.2). The extracts were strained through cheesecloth and centrifuged at 5,900 *g* for 10 min. The supernatant was made 4% with polyethylene glycol (PEG-MW 6000) and stirred 1 hr. This step was followed by centrifugation at 12,000 *g* for 10 min. The pellet was resuspended in buffer (0.1 M sodium phosphate buffer, pH 7.2) and a drop of the suspension applied on an electron microscope grid and stained in 2% uranyl formate mixed with 0.1% bacitracin (1:7, v/v). Size determinations for particle ultrastructure were made by comparing projected electron micrographs of particles with micrographs of catalase crystals. The 8.44-nm striation periodicity value reported by Meek (19) was used for our calculations.

Tissues were prepared for thin sectioning with the procedures described by Karnovsky (14) and Meek (19). The tissues were fixed in Karnovsky's fluid for 3 hr and washed three times for 15 min each in 0.1 sodium phosphate buffer, pH 7.2. They were then postfixed for 3 hr in 1% OsO_4 dissolved in 0.1 M buffer (sodium phosphate, pH 7.2), rinsed three times with buffer, once with distilled water, and stained for 1.5 hr in 1% uranyl acetate prior to ethanol dehydration. Tissues were embedded in Epon Araldite and cut longitudinally or transversely with a diamond knife. Prior to examination with an electron microscope, sections were stained first for 10 min in a 2% aqueous uranyl acetate solution and then for 2 min in an aqueous lead citrate solution.

Serology. Enzyme-linked immunosorbent assay (ELISA) was used when comparing NSPV antigen with the T26 isolate of CTV and its homologous antiserum. The double-antibody sandwich procedure was used, samples were added to microplates as leaf extracts, and results were measured spectrophotometrically at $A_{405\ nm}$ (3,15). The NSPV antigen was tested against apple stem grooving antigen and antiserum (provided by R. M. Lister, Department of Botany and Plant Pathology, Purdue University, West Lafayette, IN 47907) with the protein A and Derrick methods of immunosorbent electron microscopy (20).

Purification. Different procedures were used to purify NSPV. In all instances the starting tissues were symptomatic leaf or bark tissue of 'Nana-purpurea' nandina. Highest yields of virus were obtained as follows. Fresh juvenile stem tissue frozen with liquid nitrogen and powdered in a mortar was added to an extraction buffer (1:2, w/v). The extraction buffer was of 0.1 M sodium phosphate buffer, pH 7.2, containing 0.5 M Na_2SO_3. The resulting homogenate was centrifuged at 12,000 *g* for 10 min. The supernatant was made 0.1 M in NaCl, and PEG was added to a final concentration of 4% (w/v). This mixture was stirred for 1 hr and centrifuged at 12,000 *g* for 10 min. The pellet was resuspended in buffer, centrifuged at 12,000 *g* for 10 min, and the virus was then reprecipitated by adding 0.1 M NaCl and 4% PEG and centrifuging it at 12,000 *g* for 10 min. The resulting pellet was then resuspended in buffer and centrifuged at 12,000 *g* for 10 min. Final fractionation was by isopycnic centrifugation for 17 hr at 140,000 *g* on a Cs_2SO_4 gradient (12 g Cs_2SO_4 in 27 ml 0.1 M potassium phosphate, pH 7.2). Purity of samples was assessed spectrophotometrically and by electron microscopy.

RESULTS

Transmission trials. No symptoms developed on any of the six species of plants that were sap inoculated with extracts from leaves

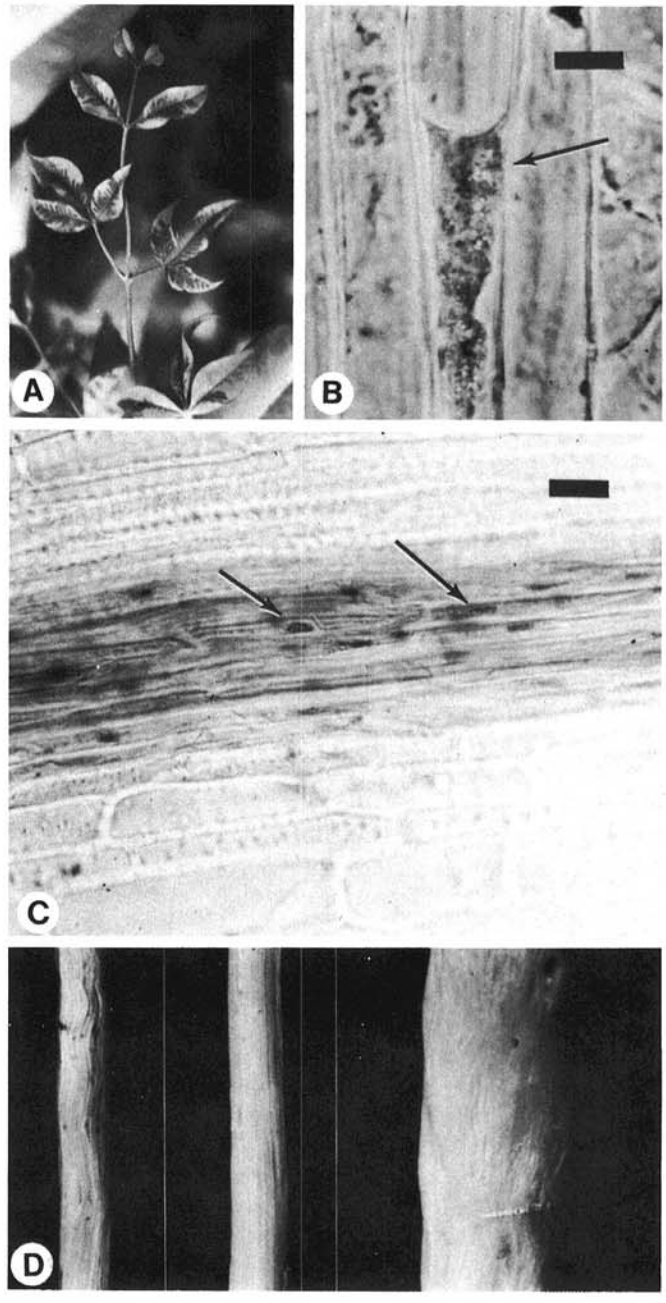

Fig. 1. Symptomatology and cytopathology of nandina stem pitting disease. **A,** Leaf of a graft-inoculated common nandina plant 4–5 wk after inoculation with side chips from 'Nana-purpurea' nandina infected with nandina stem pitting virus. Variegation, curling and distortion of the systemically infected leaflets are shown. **B,** Azure A stained inclusion (arrow) in the cytoplasm of infected nandina phloem cell (bar = 10 μm). **C,** Inclusion bodies (arrows) in the phloem of nandina leaf tissue stained with azure A (bar = 100 μm). **D,** Stem pitting and xylem grooving on a stem of 'Nana-purpurea' nandina infected with NSPV (left) and healthy common nandina (center), and infected common nandina (right).

of cultivar Nana-purpurea, regardless of the diluent. No filamentous virus particles were seen in negatively stained leaf extracts of the sap-inoculated plants. However, nine of the 10 graft-inoculated plants of common nandina developed systemic mosaic and distortion symptoms 3–4 wk after inoculation as did one of the eight slash-inoculated plants. Reddish discoloration and irregular, inward curling of the leaves were the most prominent symptoms (Fig. 1A). The symptoms closely resembled certain foliar mosaic patterns characteristic of the 'Nana-purpurea' nandina source plants. Flexuous rod-shaped virus particles were consistently detected in symptomatic leaves of inoculated plants, but not in uninoculated controls. Whereas the initial leaves to develop after inoculation all had symptoms, symptomless leaves were produced intermittently on all infected plants. Infected plants were not appreciably stunted in relation to their uninoculated counterparts 16 wk after inoculation, and specimens of these infected plants examined by F. Galle (Horticulture Department, Callaway Gardens, Pine Mountain, GA 31822) did not resemble the 'Nana-purpurea' cultivar from which the inoculum was derived (*personal communication*).

Stem grooving, stem pitting, and stem striations similar to those seen in 'Nana-purpurea' nandina source plants (Fig. 1D) were observed when bark tissue was removed from experimentally infected plants. None of the foregoing symptoms were seen in healthy, common nandina plants. We designated the virus nandina stem pitting virus because of these symptoms.

Aphids failed to transmit NSPV from 'Nana-purpurea' source plants to common nandina seedlings. None of 10 test plants exposed to a total of 150 aphids developed symptoms 8 wk after inoculation, during which four to six leaves were produced by each of the test plants subsequent to inoculation.

Light microscopy. Phloem cells of 'Nana-purpurea' or symptomatic graft-inoculated common nandina leaves stained in azure-A contained numerous reddish-violet phloem-limited, amorphous inclusions (Fig. 1B and C), which resemble those reported for CTV (7,13). Such inclusions were not seen in mesophyll or epidermal cells, nor were they seen in phloem cells of healthy common nandina plants. No viral inclusions were seen in epidermal or mesophyll tissues stained in calcomine orange-Luxol brilliant green.

Electron microscopy. Flexuous-rod shaped particles were consistently found in negatively stained leaf extracts from 'Nana-purpurea' nandina plants with mosaic and distortion symptoms (Fig. 2A). All plants of this cultivar had these symptoms, and similar particles were found in each of the 50 specimens examined. Of 114 particles measured from one specimen, 84 (74%) were between 696–830 nm with a modal length of 755 nm. Five particles exceeded 830 nm in length and the remainder consisted of various sized fragments ranging from 69 to 622 nm.

Virus particles in clarified leaf extracts appeared to have a helical structure with a pitch of 3.9 nm (Fig. 2A). In some cases, subunits of the particles were resolved (Fig. 2A). The aggregates of virus particles in the phloem tissue closely resembled those described for closteroviruses (2,10,17) (Fig. 2B). They consisted of loosely arranged particles without any apparent order. Viruslike aggregates were found in the nuclei of infected nandina tissues (Fig. 2C). Unusual, branched, tubular structures, comprised of coils of viruslike particles, were also noted consistently in cells that contained virus aggregates (Fig. 3). Neither these structures nor the virus aggregates were found in the sections of healthy common nandina tissues.

P-protein (11) was found in both the healthy and infected nandina phloem tissues. These structures were found either in clusters similar to the virus aggregates or, unlike NSPV virions, distributed in the vacuoles.

Serology. ELISA test results with CTV- and NSPV-infected leaf extracts against CTV antiserum revealed homologous reactions with CTV, but not with NSPV. In ELISA tests, an $A_{405\,nm}$ average value of 0.244 was recorded for CTV antigen, whereas average values of only 0.038, 0.067, 0.033, and 0.037 were noted for NSPV, healthy key lime, phosphate buffer and tris buffer, respectively. Likewise, no relationship between apple stem grooving virus and NSPV was evident in immunosorbent electron microscope studies. Whereas much larger numbers of apple stem grooving virus particles were seen on grids precoated with apple stem grooving antiserum than on untreated grids, no increase in NSPV particles was apparent on antiserum-treated grids.

Purification. The most successful purification attempt resulted in a yield of 0.044 mg/g host tissue. After Cs_2SO_4 gradient centrifugation, a broad zone (~3 mm wide and about 8 mm from the bottom of the tube) was noted, and both intact and fragmented flexuous-rod shaped particles were detected by electron microscopy. Fractions taken from this zone had $A_{260/280}$ values of 1.22–1.24. Foliar mosaic and distortion symptoms, identical to those in graft-inoculated plants, were seen in one of the eight common nandina plants slash-inoculated with this preparation. Virus particles were found in leaves of the plant with symptoms, but not in any of the five uninoculated control plants, or in inoculated plants showing no symptoms.

DISCUSSION

Nandina stem pitting virus is distinct from the other viruses previously reported to occur naturally in nandina (4,21,24–26). Unlike the others, NSPV appears to be phloem limited, and has a modal particle length of about 755 nm. Inoculated common nandina plants developed foliar mosaic and distortion symptoms, but were not appreciably stunted when compared to healthy ones and thus did not resemble the 'Nana-purpurea' cultivar from which the virus was recovered (F. Galle, *personal communication*). Attempts by others to demonstrate a correlation between nandina mosaic virus and the characteristic dwarfing of 'Nana-purpurea' were likewise unsuccessful (24,26).

Nandina stem pitting virus appears to be a closterovirus. While both potyviruses and certain closteroviruses (17) have particle lengths of ~750 nm, NSPV differs from the former in being phloem limited and not being readily sap transmitted. Moreover, the helix pitch of NSPV (3.9 nm) is in closer agreement with values reported for closteroviruses than potyviruses (3.7–3.9 nm and 3.4 nm, respectively) (18). The cytological properties of this virus are more like closteroviruses than potyviruses, as no cylindrical inclusions typical of potyviruses (7,8) were seen in infected nandina plants. In contrast, the infected tissues examined by light (Fig. 1B and C) and electron microscopy (Fig. 2B) had virus particle aggregates in the phloem cells closely resembling those described for CTV and other closteroviruses (2,7,12,17). The viruslike aggregates found in the nuclei of NSPV-infected phloem cells (Fig. 2C) were similar to those described for sugar beet yellows virus (10). The unusual tubular-coil structures found in infected phloem cells (Fig. 3) are distinct from the vesicles associated with subgroup I closteroviruses and apparently have not been described for any virus, although they superficially resemble the "proliferated and dilated endoplasm reticulum" induced by lilac chlorotic leaf spot virus, a subgroup I closterovirus (5,6,17). However, the structures associated with the latter virus appear to be membranous rather than particulate in structure. On the basis of their size and staining characteristics, the tubular-coil structures associated with NSPV appear to be composed of virus particles.

The closteroviruses are not a uniform assemblage of viruses and have been subdivided into groups either according to vector transmission and coat protein molecular weight (2) or particle length (17). Both classification schemes are arbitrary, however, and additional study is needed to ascertain the taxonomic validity of these criteria. The short particle length of NSPV is clearly suggestive of a "subgroup II" affiliation. This group consists of the following four members: apple stem grooving virus, apple chlorotic leaf spot virus, heracleum latent virus, and potato virus T (17). Nandina stem pitting virus is probably a distinctive subgroup II closterovirus, but this interpretation should be viewed cautiously until more detailed comparative studies are completed. The $A_{260/280}$ ratios recorded for NSPV (1.22–1.24) are closer to those reported for potato virus T (1.17–1.19) and apple stem grooving (1.18) virus than for either heracleum latent (1.5) or apple chlorotic leaf spot (1.55–1.85) virus (17). The immunosorbent electron microscopy

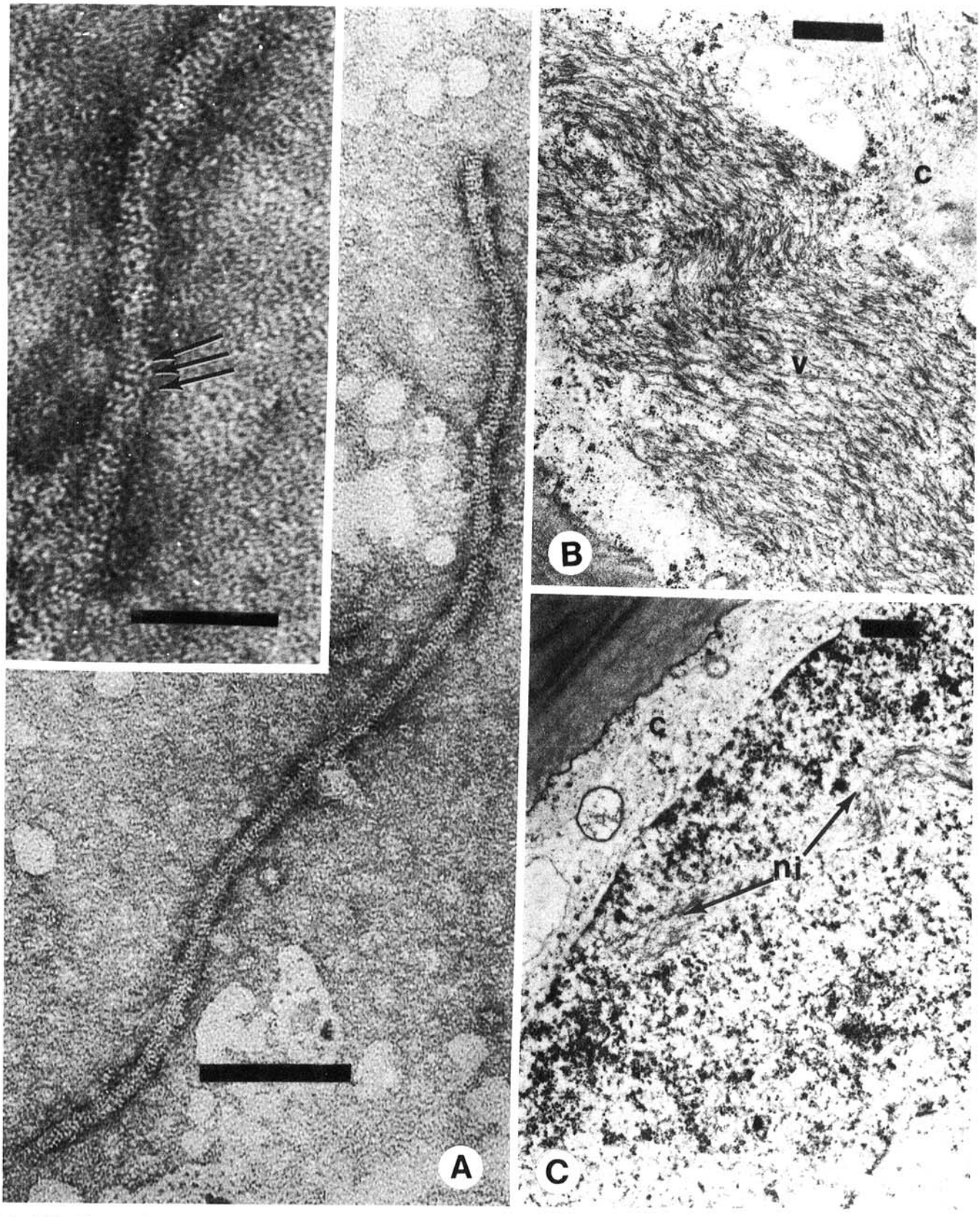

Fig. 2. A, Nandina stem pitting virus particle from stem tissue of 'Nana-purpurea.' Particle was in clarified extract and stained with 2% uranyl formate, pH 7.2 (bar = 100 nm); inset, fragment of nandina stem pitting virus particle stained with 2% uranyl formate, pH 7.2. Arrows show the helical arrangement of subunits (bar = 50 nm). **B,** Section through intracellular inclusion of NSPV-infected 'Nana-purpurea' nandina phloem cell showing virus aggregate (v) in the cytoplasm (c) (bar = 1 μm). **C,** Section through fibrous nuclear inclusions (ni) in infected phloem cell (bar = 1 μm, c = cytoplasm).

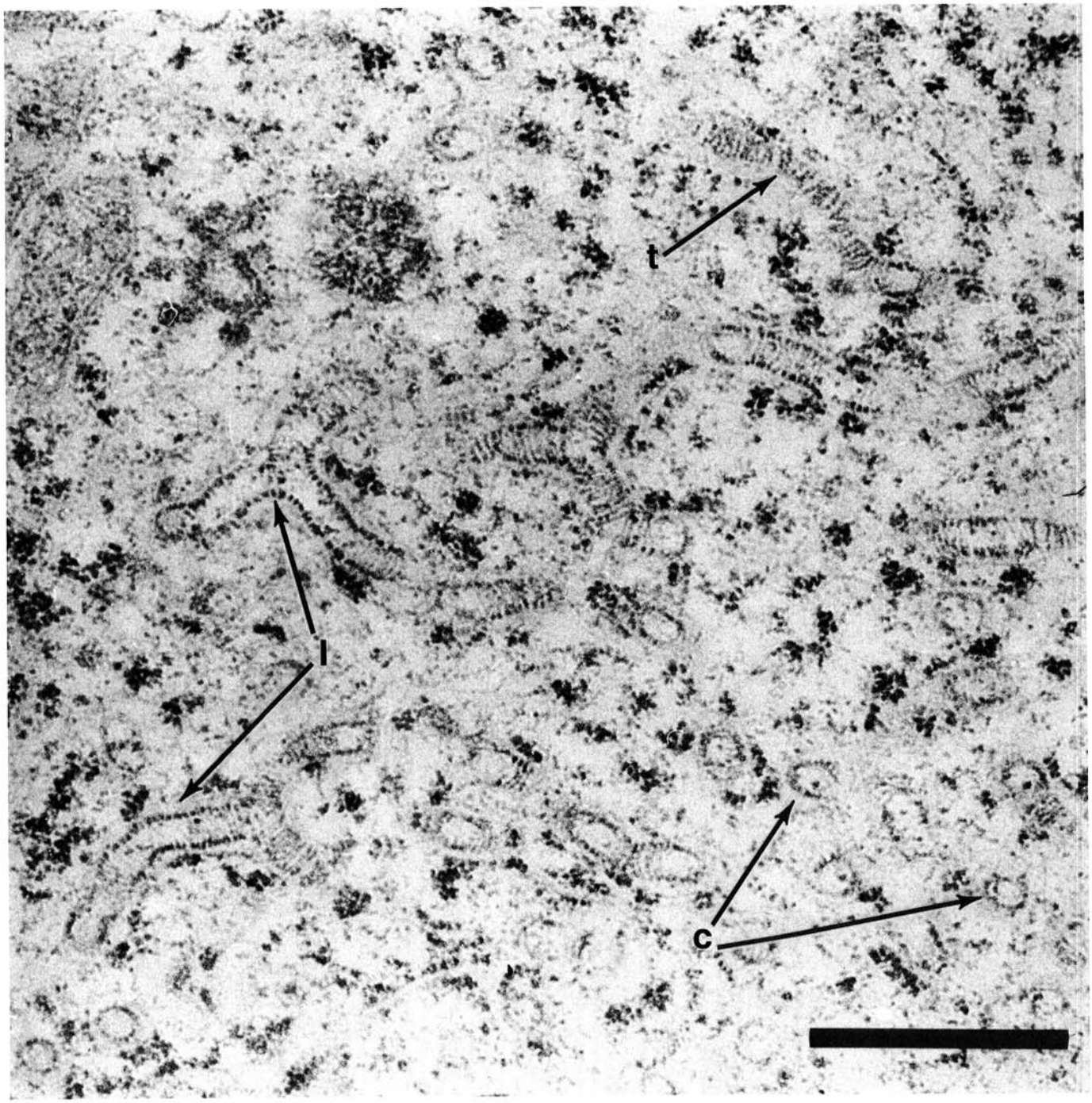

Fig. 3. Section through the cytoplasm of phloem cell of NSPV-infected 'Nana-purpurea.' Tubular-coil structures, often branched, are seen in cross (c) and longitudinal (l) section. Note tangential planes (t) that clearly show the architecture (bar = 1 μm).

studies with apple stem grooving virus reveal that it differs from NSPV. However, this test was limited inasmuch as NSPV antiserum was not available for reciprocal tests. Finally, although all four subgroup II members have been shown to be sap transmissible to *C. quinoa* and other herbaceous indicator plants (17), this difference with NSPV could be attributed to the relative difficulty of manual transmission with most closteroviruses and to the presence of virus inhibitors in nandina sap (22).

The natural vectors of most closteroviruses are aphids that transmit the virus in the semipersistent manner. *Myzus persicae* is the only species reported to colonize nandina (16, and V. F. Eastop, Department of Entomology, British Museum of Natural History, London, *personal communication*).

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