# Association of Double-stranded RNA and Filamentous Viruslike Particles with *Dodonaea* Yellows Disease

WAYNE B. BORTH, Graduate Research Assistant, Department of Plant Pathology, DONALD E. GARDNER, Research Plant Pathologist, National Park Service, Cooperative Park Studies Unit, Department of Botany, and THOMAS L. GERMAN, Associate Professor, Department of Plant Pathology, University of Hawaii, Honolulu 96822

**ABSTRACT**

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*Dodonaea viscosa* in the Hawaiian Islands is affected by a severe yellowing disease with symptoms resembling those of diseases caused by mycoplasmalike organisms in other woody hosts. Double-stranded RNA with an estimated molecular weight of $3 \times 10^6$ was detected in plants displaying disease symptoms but not in asymptomatic plants. Viruslike particles 16 nm in diameter and 700 nm in modal length were also isolated from diseased plants but were not present in extracts of healthy plants.

Additional keywords: MLO diseases

The genus *Dodonaea* (Sapindaceae) is widely distributed throughout the islands of the Pacific and other tropical and subtropical regions of the world. *D. viscosa* (L.) Jacq. is a highly variable, evergreen species with several forms endemic to the Hawaiian Islands, where they are major components of upland dry forest ecosystems (14,16). The species is ecologically important as an early colonizer of new lava flows and other disturbed sites and occasionally is the dominant woody species in drier habitats (16).

A conspicuous and severe disease affecting *D. viscosa* was reported on the island of Hawaii in 1977 (11) and has since been observed on all of the major Hawaiian Islands. The disease is characterized by the production of abnormally lengthened young branches proliferating from axial buds and forming pendulous, compact witches'-brooms with reflexed tips. Leaves on affected branches are smaller than normal and exhibit a general chlorosis (Fig. 1). Affected shrubs gradually decline over a period of a few years, eventually becoming entirely defoliated. Although most conspicuous on mature specimens, the disease also affects young individuals. Affected individuals have never shown remission of symptoms in the field, and affected tissue is invariably killed by the disease.

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Witches'-brooms, subnormal leaf size, and chlorosis are symptoms of diseases of woody species generally associated with mycoplasmalike organisms (MLOs), and the yellows disease of *D. viscosa* in Hawaii has also been postulated to be caused by MLOs (10). A disease similar in some characteristics to the Hawaiian disease has been reported from India on *D. viscosa* growing in conjunction with the root parasite *Santalum album* L. affected with spike disease (17). Transmission electron microscopy (TEM) of these *D. viscosa* plants by workers in India confirmed the presence of characteristic pleomorphic bodies in diseased tissues. However, details of the location of MLOs within diseased plants were not provided. TEM examination of petioles and peduncles from diseased *D. viscosa* in Hawaii has failed to reveal pleomorphic bodies or viral inclusions in these tissues (W. Sakai, *unpublished*).

Viruslike particles (VLPs) have also been observed in the phloem of some species of plants affected with yellows diseases associated with MLOs (19). We report here the isolation of double-stranded RNA (dsRNA) and flexuous, rod-shaped VLPs from *D. viscosa* affected with yellows disease in Hawaii.

## MATERIALS AND METHODS

**Isolation of dsRNA.** Leaf and stem tissues from symptomatic and healthy *D. viscosa* were subjected to the procedure of Morris and Dodds (15) for the isolation of dsRNA. Samples from field-collected and greenhouse-grown specimens were processed identically. All glassware was baked at 150 C for 16 hr before use. Nucleic acids eluted from Whatman CF-11 cellulose by 16.5% (v/v) ethanol were precipitated by the addition of sodium acetate (pH 5.2, final concentration 150 mM) and 2.5 vol of ethanol. Precipitated nucleic acids were collected by centrifugation, dried under vacuum, and dissolved in sterile TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0). Concentrated stock solutions were added to bring samples to 150 mM in NaCl and 5 mM in $MgCl_2$. Fifty units of RNase-free DNase I (Boeringer-Mannheim) was added, and the solution was incubated for 30 min at 37 C. Following nuclease treatment, sodium dodecyl sulfate was added to a final concentration of 0.1% (w/v), and the mixture was extracted twice with STE-saturated, distilled phenol. The aqueous phase was separated by centrifugation and collected, and the nucleic acids were precipitated as above.

**Polyacrylamide gel electrophoresis of dsRNA.** DsRNAs were prepared for electrophoresis by centrifuging precipitated nucleic acids, drying the pellets under vacuum, and dissolving the dsRNA in polyacrylamide gel electrophoresis (PAGE) sample buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, 10% glycerol, 0.002% bromphenol blue; pH 7.8). Samples were heated at 60 C for 2 min and quenched on ice before being applied to 6% acrylamide-2.5% bis-acrylamide slab gels ($0.75 \times 80 \times 80$ mm). Gels were overlaid with buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA; pH 7.8) and run for 4 hr at 25 mA/gel. DsRNAs from *Nicotiana tabacum* L. 'Xanthi' infected with tobacco mosaic virus (TMV) (molecular weight of dsRNA = $4.0 \times 10^6$) and *Hordeum vulgare* L. infected with brome mosaic virus (BMV) (molecular weight of dsRNAs = 2.2, 2.0, and $1.4 \times 10^6$), both prepared as above, were used as molecular weight standards for the gel system.

The method of Bozarth and Harley (5) was used to calculate the molecular weight of the dsRNA from *D. viscosa*. Nucleic acids were visualized by staining with ethidium bromide in buffer and photographed under ultraviolet light using Polaroid type 665 film with a sandwich of 2B and 23A Wratten filters.

**Isolation of VLPs.** The protocol for the isolation of VLPs used differential centrifugation. All steps in the protocol

were done at 4 C. Symptomatic leaves and young twigs were collected from diseased *D. viscosa* in the field and processed without prior freezing. Samples from asymptomatic plants collected from the field were processed identically. Tissues were ground in liquid nitrogen and extracted by stirring in buffer (50–100 g of tissue per liter) containing 300 mM Tris, 400 mM NaCl, 50 mM $Na_2SO_4$, 10 mM $MgCl_2$, and 3% (w/v) Triton X-100 (pH 8.0) for 30 min in a beaker with a magnetic stir bar. The slurry was filtered through four layers of cheesecloth and centrifuged at 8,000 *g* for 10 min. The supernatant was collected and centrifuged at 260,000 *g* for 30 min in a Beckman model L8-70M ultracentrifuge and type 70Ti rotor. Pellets from the high-speed centrifugation were gently resuspended in 100 ml of buffer (100 mM Tris, 400 mM NaCl, 50 mM $Na_2SO_4$; pH 8.0) overnight on a magnetic stirrer. The suspension was centrifuged at 10,000 *g* for 10 min. The resulting supernatant was applied over 30% (w/v) sucrose cushions in resuspension buffer and centrifuged at 260,000 *g* for 30 min as above. Pellets from the final high-speed centrifugation were resuspended in 0.5 ml of resuspension buffer as above and stored at 4 C. The purification protocol was monitored by observing the yields of each step using TEM.

**TEM.** Aliquots of VLPs isolated by the above procedure were applied to carbon-stabilized, Formvar-coated copper grids (Ted Pella, Inc.), washed with 200 μl of bacitracin solution in water (300 μg/ml), and negatively stained in a freshly prepared, saturated solution of uranyl formate in absolute methanol. Grids were air-dried and observed immediately with a Hitachi model HS-8-1 transmission electron microscope at 50 kV. TMV prepared from *N. tabacum* 'Xanthi' was used as a standard for width determinations. To determine the modal length of VLPs isolated from diseased *D. viscosa,* 60 particles were measured using latex spheres 109 nm in diameter (Ted Pella, Inc.) as standards. Electron micrographs of VLPs and spheres were measured with a Kontron IBAS image analyzer.

**Transmission studies.** Leaf and twig tissues from field-collected and greenhouse-grown symptomatic *D. viscosa* were used in mechanical transmission studies. Tissues were ground in 50 mM sodium phosphate buffer (pH 7.0) with or without the addition of 50 mM $Na_2SO_3$. Extracts were then immediately rubbed on leaves of herbaceous indicator plants which had been dusted with fine Carborundum (600 grit). All plants were returned to the greenhouse and monitored for symptom development. The following indicator species were used: *Chenopodium amaranticolor* Coste & Reyn., *C. quinoa* Willd., *Datura stramonium* L., *Emilia sonchifolia* (L.) DC., *N. tabacum* L., *N. glutinosa* L., *Petunia* × *hybrida, Phaseolus vulgaris* L., and *Physalis* sp.

In addition, attempts were made to infect healthy *D. viscosa* with extracts from diseased plants. Healthy *D. viscosa* grown from seed were mechanically inoculated with freshly prepared extract (as above), and plants were monitored for development of symptoms in the greenhouse.

## RESULTS

**DsRNA.** PAGE analysis of RNAs isolated from diseased *D. viscosa* revealed the presence of a dsRNA in symptomatic tissues that was not present

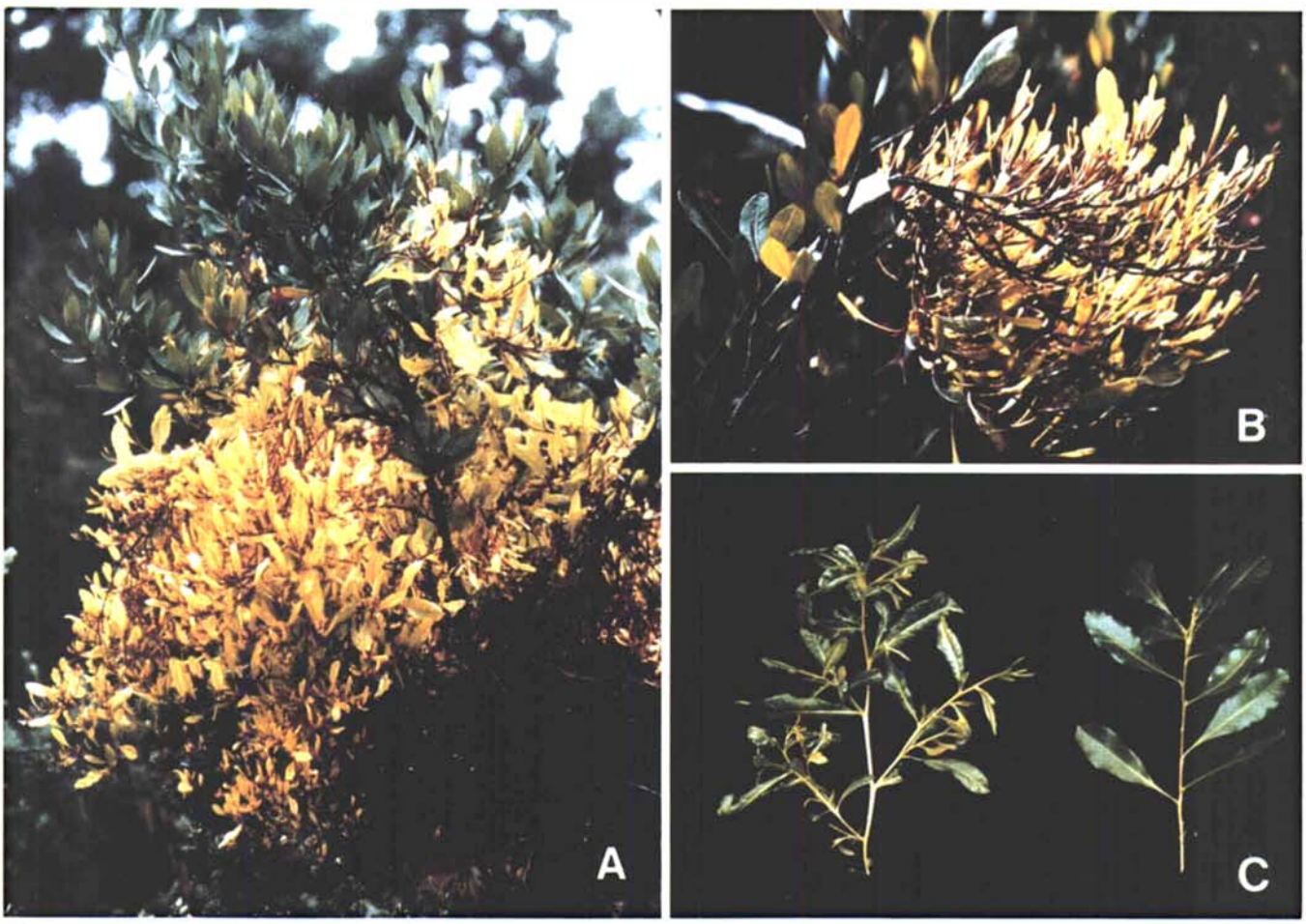


**Fig. 1.** *Dodonaea viscosa* with symptoms of yellows disease. **(A)** Chlorosis and brooming of lower branches with normal growth on upper branches. **(B)** Individual witches'-broom showing epinastic development, reflexed tips, reduced leaf area, and reddening of stems. **(C)** Young stems from well-fertilized plants grown in the greenhouse, showing proliferation of axial buds and crinkling of leaves (left) and healthy growth (right).

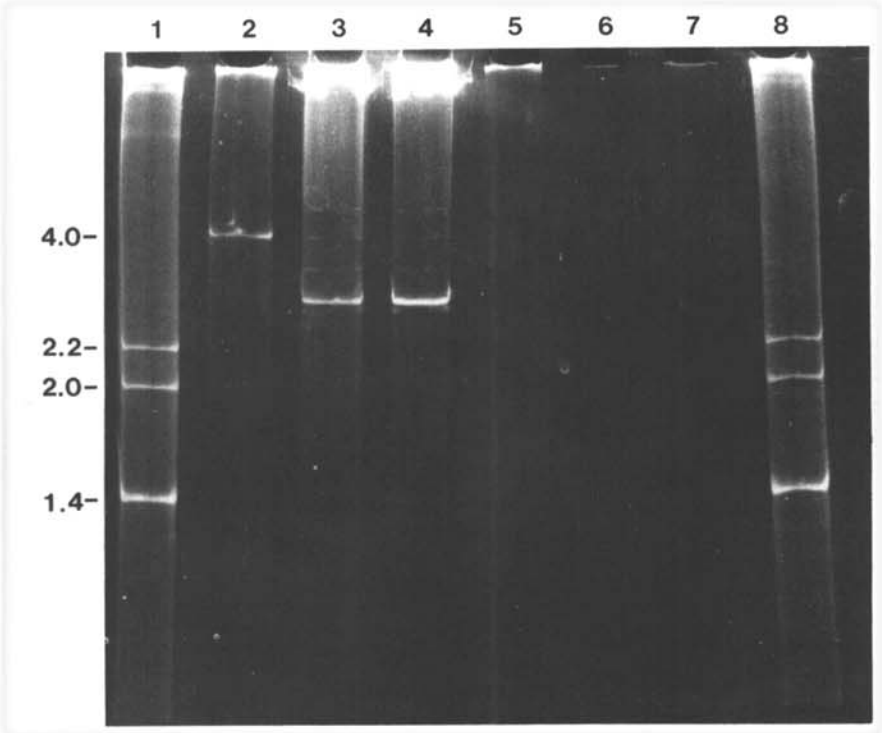


**Fig. 2.** Polyacrylamide gel electrophoresis of ethidium bromide-stained double-stranded RNA (dsRNA) isolated from symptomatic *Dodonaea viscosa.* Lanes 1 and 8, brome mosaic virus; lane 2, tobacco mosaic virus; lanes 3 and 4, diseased *D. viscosa;* lanes 5–7, healthy *D. viscosa* grown from seed collected from asymptomatic plants. Molecular weights of dsRNA standards are shown at left ($\times 10^{-6}$).

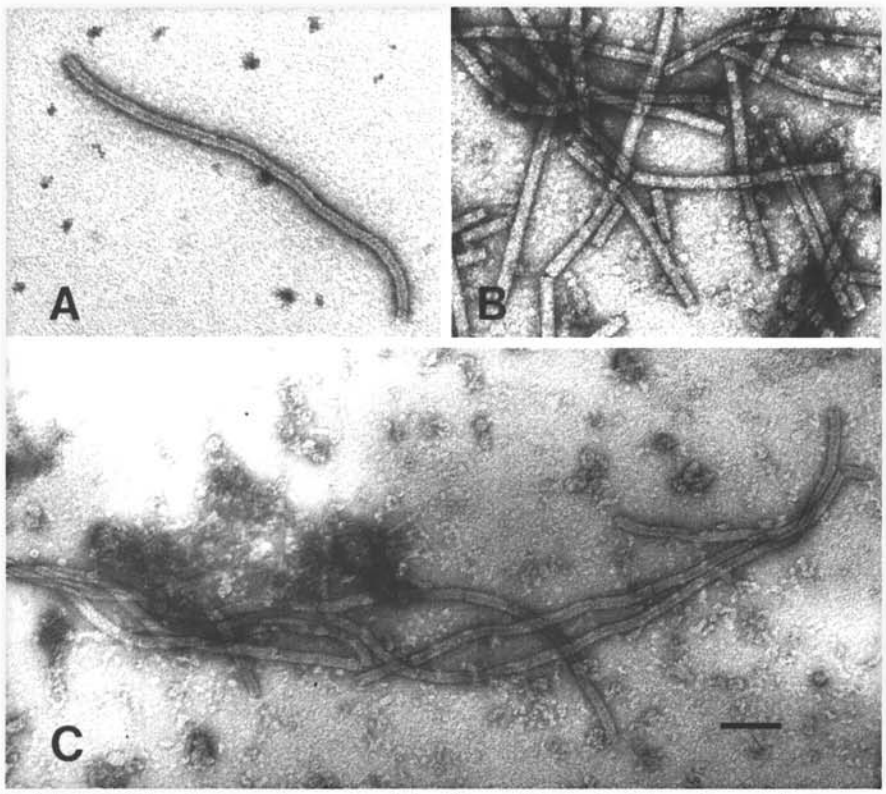


**Fig. 3.** Transmission electron micrographs of viruslike particles isolated from diseased *Dodonaea viscosa.* **(A)** Particle about 700 nm long, showing central core and coat protein subunit texture. **(B)** Tobacco mosaic virus standard isolated from infected *Nicotiana tabacum.* **(C)** Group of particles from *D. viscosa,* showing aggregation and breakage commonly observed in partially purified preparations. Particles were negatively stained with uranyl formate in methanol. All micrographs are at the same magnification; the scale bar represents 100 nm.

in healthy tissues (Fig. 2). The double-stranded nature of the nucleic acid was confirmed by its sensitivity to digestion with RNase A under low-salt (10 mM NaCl) but not under high-salt (300 mM NaCl) conditions (*data not shown*). This dsRNA was consistently associated with diseased plants collected from different locations. Interestingly, the levels of this dsRNA in diseased plants varied during the year; the lowest levels occurred in the warm summer months and higher levels in the cooler winter months (*data not shown*). Using the dsRNAs isolated from TMV- and BMV-infected plants as standards, the molecular weight for the unique dsRNA from diseased *D. viscosa* was estimated to be $3 \times 10^6$.

**Isolation of VLPs.** The isolation of VLPs from diseased *D. viscosa* was difficult because of the inherent tendency of homogenized tissues to form gelatinous suspensions under widely different extraction conditions, salt concentration, buffer composition, and pH. The procedures described in the Materials and Methods section gave the most reproducible yields of particles from symptomatic leaf tissues. Yields were maximized by using only fresh tissues for all extractions. It was not possible to obtain sufficient quantities of these particles to analyze either coat protein subunit molecular weight or virion nucleic acids. No particles were isolated from healthy *D. viscosa.*

Uranyl formate negative staining of VLPs associated with diseased *D. viscosa* suggested a symmetric helical arrangement of coat protein subunits around a hollow central core (Fig. 3). Based on a diameter for TMV virions of 18 nm, the width of the flexuous, rod-shaped VLPs from diseased *D. viscosa* was estimated to be 16 nm. Using latex spheres 109 nm in diameter as standards, the modal length of the particles was estimated to be about 700 nm (Fig. 4).

**Transmission studies.** None of the herbaceous hosts tested developed any symptoms associated with virus infection as a result of mechanical inoculation with extracts from symptomatic *D. viscosa.* All attempts to mechanically transmit the disease to healthy *D. viscosa* were unsuccessful.

## DISCUSSION

The symptoms of the yellows disease of *D. viscosa* in Hawaii resemble those caused by MLOs in other woody hosts (19,20). However, no evidence has been presented that conclusively demonstrates the etiology of this disease. We have demonstrated here the presence of flexuous, rod-shaped VLPs in diseased tissues and the presence of dsRNA in diseased plants, both of which are absent in healthy plants. Although the dimensions of these particles do not allow the certain assignment of the isolated particles to any known group

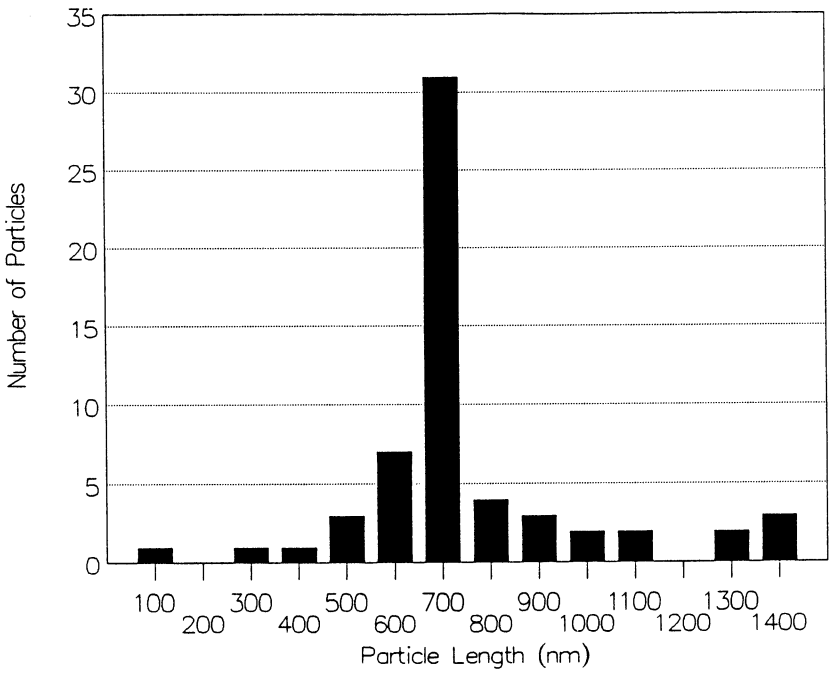


**Fig. 4.** Length distribution of viruslike particles ($n$ = 60) isolated from diseased *Dodonaea viscosa,* showing modal length of 700 nm. Latex spheres of defined diameter were used as size standards.

of viruses, they closely resemble potato virus X or potato virus Y type virions.

The analysis of plants for the presence of dsRNA is a useful tool in the diagnosis of many plant diseases of viral origin (6,7). Suitable controls must be included in any such analysis to show that endogenous dsRNA, which has been found in tissues of certain plant species not infected with RNA viruses (18,21), is not present in tissues from healthy plants. We do not consider the dsRNA present in diseased *D. viscosa* to be endogenous, because asymptomatic plants from the field, in addition to those propagated from seed under controlled environmental conditions, have been shown to be free of the dsRNA present in diseased individuals.

The presence of VLPs in diseased plants does not preclude the possibility that MLOs or spiroplasmas are also present in these tissues or that these pathogens may be involved in the etiology of *Dodonaea* yellows disease. Examples of dicots and monocots infected with both viruses and mollicutes (1,2,4,8,9,12,13,22) are widely known. Such dual infections may alter the disease symptoms of susceptible hosts infected with either agent alone (3). The report of a similar disease of *Dodonaea* spp. in India, possibly associated with MLOs (17), makes speculation about dual infections in *Dodonaea* attractive. Further work is in progress in our laboratory to address this aspect of *Dodonaea* yellows disease in Hawaii.

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