

Occurrence of Phytoplasmas in Hawaii

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ABSTRACT

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Using nucleic acid hybridization, polymerase chain reaction (PCR), and transmission electron microscopy (TEM), phytoplasmas were detected in *Dodonaea viscosa* afflicted with Dodonaea yellows disease in Hawaii. In hybridization tests, a phytoplasma-specific probe for conserved 16S ribosomal RNA genomic sequences of phytoplasmas was used to detect phytoplasmas in diseased plants from the field and greenhouse and a few symptomless plants near diseased ones in the field, but not in healthy plants raised from seed in the greenhouse. PCR amplification and direct sequencing of the PCR products were used to show that the phytoplasma from diseased *D. viscosa* is closely related to western X-disease phytoplasmas. Pleomorphic bodies resembling phytoplasmas were observed in diseased but not in healthy plants using TEM. These results, and the witches'-broom symptoms of diseased *D. viscosa*, suggest that phytoplasmas might be involved in the etiology of this disease in Hawaii. This is the first report demonstrating the presence of plant-pathogenic phytoplasmas in the Hawaiian Islands.

Dodonaea viscosa (L.) Jacq., an ecologically important plant in the dry forest and shrublands in Hawaii, is afflicted by a severe yellowing disease characterized primarily by the development and proliferation of usually pendulous, chlorotic witches'-brooms on branches of otherwise healthy-appearing plants (4,10,11). The terminal portions of the broomed stems are often reddened and may be compressed laterally, with the chlorotic leaves crinkled and reduced in size. Afflicted portions of symptomatic plants fail to produce flowers, while flowering is normal on the non-symptomatic parts of the same plants. Characteristic symptoms of this disorder on *D. viscosa* can occur at any stage of plant development; mature individuals may develop symptoms on isolated branches, or young saplings may become entirely symptomatic within a short time after their establishment. The symptoms on mature plants gradually spread to involve the entire plant, which over a period of years becomes progressively defoliated with only a few deformed leaves remaining at the tips of the brooms. These plants eventually die, but their clusters of dried

leafless witches'-brooms remain prominent in the field. Although a relatively small percentage of native *D. viscosa* is afflicted with overt symptoms of this disorder, it is slowly spreading; symptomatic plants can be found on all the major Hawaiian Islands.

Although viruslike particles and associated dsRNA are associated with this disorder in Hawaii (3-5), the possibility that Dodonaea yellows disease in Hawaii may also involve phytoplasmas, formerly known as mycoplasma-like organisms, is indicated by the witches'-broom symptoms. A similar disease of *D. viscosa* in India is associated with sandal spike phytoplasma (14,24). Portions of this research have been presented previously (5).

MATERIALS AND METHODS

To address the possible involvement of phytoplasmas with Dodonaea yellows, we used molecular probes specific for conserved 16S ribosomal RNA genomic sequences of phytoplasmas to screen healthy and diseased *D. viscosa* in both field and greenhouse situations, PCR amplification and direct sequencing of the transfer RNA spacer region between the 16S and 23S ribosomal RNA genes of phytoplasmas, and transmission electron microscopy (TEM). Plant material used was collected either from plants growing in the field or from symptomatic plants transplanted to containers and grown under controlled conditions. Healthy control plants were

raised in the greenhouse from seed collected from nonsymptomatic plants in the field.

Isolation of nucleic acids. Symptomatic and nonsymptomatic *D. viscosa* were sampled in the field on the islands of Hawaii, Maui, and Kauai. Additionally, symptomatic plants transplanted from the field and maintained in pot culture, and nonsymptomatic plants raised from seed in greenhouses were sampled. Total nucleic acids used in hybridization analysis were isolated from leaves or roots of *D. viscosa* using the hot CTAB procedure of Doyle and Doyle (9) with 1% PVP-10 added. Nucleic acid concentrations were determined both spectrophotometrically and in 1% agarose gels in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0) using lambda DNA as standards (25). Nucleic acids isolated using this procedure typically had 260/280 ratios of 1.8 to 2.1 and consisted of both DNA and RNA, as determined by gel electrophoresis.

Nucleic acid probe. The sequence of an 18-base DNA oligomer (WX1) identical to a highly conserved region of the 16S ribosomal gene from the phytoplasma associated with western-X disease of stone fruits has been used to detect complementary RNAs of phytoplasmas infecting a wide range of woody and herbaceous hosts (15). This oligomer (5'-AAACCGCCTACG-CACCCT-3') was synthesized and radiolabeled enzymatically with $\gamma^{32}\text{P}$ -ATP for use as a probe (25). About 300 ng of oligomer DNA in H₂O (0.1% diethylpyrocarbonate-treated) was added to kinase buffer (0.4 M Tris, 0.1 M MgCl₂, 50 mM dithiothreitol, pH 7.6) along with $\gamma^{32}\text{P}$ -ATP (3,000 Ci/mmol), and the reaction was initiated with 2 units of T4 polynucleotide kinase (Promega, Madison, WI). The reaction was incubated for 1 h at 37°C and stopped by heating to 65°C for 10 min. The incorporation of radiolabel into the oligomer was assayed on DE81 filters (Whatman, Inc., Hillsboro, OR) washed in 0.5 M Na-phosphate (pH 7.0) and counted. Incorporation of label was typically 60 to 80% under these reaction conditions.

Dot blot detection of phytoplasma sequences. Two μg of total nucleic acids in TE buffer (10 mM Tris, 1 mM EDTA) were denatured by formalin in 20× SSPE

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(3 M NaCl, 0.2 M Na₂HPO₄, 0.02 M EDTA, pH 7.4) at 65°C for 10 min and quench cooled on ice. Samples were diluted serially fivefold in 10× SSPE and applied with a dot blot apparatus (Millipore Corp., Bedford, MA) to Nytran membranes (MSI, Inc.) pre-equilibrated in 20× SSPE. Samples were washed with 10× SSPE, and nucleic acids were fixed to membranes at 80°C for 1 h under vacuum.

Dot blots were prehybridized at 40°C for 3 to 16 h (6× SSPE, 5× Denhardt's solution, 0.2% SDS, 0.05% sodium pyrophosphate, and 100 µg of denatured sheared salmon sperm DNA per ml) and hybridized at 40°C for 18 h (6× SSPE, 5× Denhardt's, 0.05% sodium pyrophosphate, 0.1% SDS, 100 µg of yeast tRNA per µl, and radiolabeled oligomer at 1 to 5 × 10⁷ cpm). Blots were washed three times for 15 min each at room temperature (6× SSPE, 0.05% sodium pyrophosphate) once at 45°C (6× SSPE, 0.05% sodium pyrophosphate), and once for 20 min at 48°C (2× SSPE, 0.05% sodium pyrophosphate), then exposed to film (Kodak X-OMAT AR) at -80°C with intensifying screens.

Polymerase chain reaction. Two oligomers that allow the PCR-amplification of a 543-bp segment of the phytoplasma genome that spans the tRNA spacer region between the 16S and 23S ribosomal RNA genes (18) were used to amplify DNA iso-

lated from symptomatic *D. viscosa*. PCR reactions were carried out in a Perkin-Elmer 480 Thermal Cycler in a volume of 30 µl. Approximately 20 to 100 ng of DNA was amplified in Tris buffer containing 150 mM MgCl₂, 150 mM each dNTP, 0.5 mM each primer, and 1 unit of *Taq* polymerase (Perkin-Elmer Corp., Norwalk, CT). After initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C (1 min), annealing at 53°C (1 min), and extension at 72°C (1 min), were followed by a final incubation at 72°C (10 min). PCR products were analyzed in 1% agarose gels in 1× TAE buffer containing 0.5 mg of ethidium bromide per ml, and gels were photographed under UV illumination.

Direct sequencing. The PCR products amplified using the above primer set were sequenced with the dsDNA Cycle Sequencing System (Gibco, BRL, Gaithersburg, MD) according to the manufacturer's instructions. Primers for the sequencing were prepared by end-labeling of oligos with T4 polynucleotide kinase and γ³²P ATP (25). Template DNA was purified from PCR reactions by column chromatography using the Wizard PCR Preps DNA purification system (Promega) according to the manufacturer's instructions. Approximately 200 ng of purified DNA was sequenced in each reaction. Sequencing products were separated on 6% polyacrylamide (19:1 acrylamide:bis-acrylamide) gels containing 7 M urea in 1× TBE buffer, dried onto Whatman 3MM

paper, and visualized by exposure to Kodak X-OMAT AR film at -80°C.

TEM. Root tissues from potted plants that had tested positive for phytoplasmas in dot blots and from healthy potted plants were examined using TEM. Fixation in 6% glutaraldehyde in 0.05 M phosphate buffer (pH 7.8) at room temperature for 16 h was followed by postfixation in 2.0% OsO₄ in 0.1 M phosphate buffer (pH 6.8) for 4 h. The samples were then dehydrated in a graded ethanol series followed by substitution with propylene oxide and infiltration with Spurr's resin in pressure vessels (Kontes, Inc., Vineland, NJ) under continuous rotation. Tissues were polymerized in fresh Spurr's at 70°C. Silver sections were cut with a DDK diamond knife on an RMC M7 ultramicrotome, stained in 2% uranyl acetate in 50% ethanol, counterstained in lead citrate, and examined in a Philips model 200 transmission electron microscope at 80 kV.

RESULTS

Detection of phytoplasma sequences. Using the WX1 probe, phytoplasma sequences were detected in *D. viscosa* collected from the field on the islands of Kauai, Maui, and Hawaii, and in symptomatic plants grown in pots (Fig. 1). In samples collected from the area of Hawaii Volcanoes National Park on the island of Hawaii, nucleic acids that were isolated from 12 of 15 plants with visual symptoms of Dodonaea yellows hybridized with the

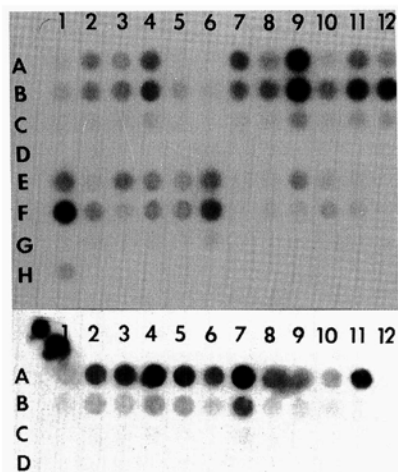


Fig. 1. Representative dot blot detection of MLO sequences using radiolabeled WX1 oligomer as probe. Fivefold dilutions of nucleic acids were applied vertically in rows ABCD and EFGH of each column. Nucleic acids were extracted from plants growing in pot culture (upper panel) or in field situations (lower panel). In the upper panel, nucleic acids in lanes 1 to 12 (ABCD) and 1 to 6 (EFGH) were obtained from symptomatic pot-cultured plants; nucleic acids in lanes 7 to 12 (EFGH) were isolated from greenhouse-grown, nonsymptomatic, pot-cultured plants. Nucleic acids isolated from field-grown plants collected from the island of Kauai are shown in the lower panel (orientation marks appear at upper left); lanes 1 to 7 (ABCD) were from symptomatic plants and lanes 8 to 12 (ABCD) were from nonsymptomatic plants.

<i>Dodonaea</i> :	CCTCCTTCT	AAGGAAAAA	TAAAATTATC	ATCTTCAGTT	TTGAAAGACT
WX-PYLR:C.....G.....
Walwb:
Vacwb:C.....
Pd:	-----	-----	---T.....C..GC.A.
TAGAAAATTC	ATAAGTTTTT	C-TTTTTTAA	GGCATT-AGG	GCCTATAGCT	CAGCTGGTTA
.....T.....A.....
.....T.....A.....
..A.....C.....A.....
GGA.....T.....A.....
GAGCACACTC	CTGATAAGCG	TGAGGTCGGT	GGTTCAAGTC	CATTTAGGCC	CACCAGTTTA
.....G.....
.....G.....
.....G.....
.....G.....
TTTTAAAAGT	TATAAGCACT	GTCGGAAAAA	AAAGTCTTT	GAAAAGTAGA	T-----
.....TT.....	C.....	..AAACATAGT
.....TT.....AAACATAGT
.....TT.....AAACATAGT
.....CTT.....	..AG.....AAACATAGT
--AAATCAAA	AAAATGGAAG	GAATTAAGAG	CGCACAGTGG	ATGCCTTGCA	CCAAG
-A.....
-A.....
-A.....
AA.....C.....

Fig. 2. 16S-tRNA spacer-23S gene sequences of PCR amplified products from *Dodonaea viscosa* afflicted with yellows disease in Hawaii, compared to sequences from phytoplasmas causing disease in peach (WX-PYLR: western X-peach yellow leaf roll), walnut (Walwb: walnut witches'-broom), *Vaccinium* spp. (Vacwb: *Vaccinium* witches'-broom), and pear (Pd: pear decline). Individual nucleotide identities between phytoplasmas are indicated by periods, and deletions are indicated by dashes.

WX1 probe. Similar results were obtained when this probe was used to screen symptomatic material from Maui (four of five positive), and Kauai (six of seven positive). Nucleic acids isolated from some symptomless plants growing near diseased plants also hybridized with WX1 probe (Fig. 1). Overall, about 80% of symptomatic plants and about 30% of nonsymptomatic plants growing near symptomatic plants contained nucleic acid sequences recognized by the WX1 probe. Nucleic acids from healthy *D. viscosa* seedlings grown for two successive generations in pot culture did not hybridize with the probe.

PCR and direct sequencing of PCR products. An approximately 543-bp sequence was amplified using total nucleic acid extracted from *D. viscosa* with *Dodonaea* yellows symptoms as the template in PCR. This fragment was similar in size to that amplified from peaches afflicted with peach yellow leaf roll disease, which is caused by infection with the western X-disease phytoplasma (not shown). Direct sequencing of this product and comparison of gene sequences within the tRNA spacer and flanking 16S and 23S regions of other phytoplasmas that cause disease in woody plants, including western X-disease, walnut witches'-broom, *Vaccinium* witches'-broom, and pear decline, indicate that the phytoplasma associated with *Dodonaea* yellows disease is closely related to these other phytoplasmas (18) (Fig. 2).

TEM. Pleomorphic bodies 50 to 300 nm in diameter containing what appear to be ribosomes and fine fibrillar material were observed in collapsed metaphloem elements within the vascular cylinder of roots from diseased *D. viscosa* that had brooms (Fig. 3). Similar structures were not found in roots of healthy potted plants. These structures were bounded by a single unit membrane and were occasionally found with appendages or constrictions (Fig 3B). Their size, shape, contents, and ultrastructure, together with their location in living and degenerating phloem elements, are similar to characteristics of phytoplasmas known to occur in phloem of other plants (8,13,21,28).

DISCUSSION

Plant diseases associated with phytoplasmas have not previously been reported from Hawaii. Although *Dodonaea* afflicted with yellows disease in Hawaii exhibits witches'-brooms that are pendulous, in contrast to those of many other yellows diseases of woody plants caused by phytoplasmas in which the brooms are oriented more vertically, this symptom suggests that phytoplasmas are involved in this disease. A similar disease of *D. viscosa* in India has been attributed to phytoplasma infection (14,24).

Dot blot detection of ribosomal RNA sequences similar to those of western X-disease phytoplasma and the PCR ampli-

cation of phytoplasma-specific sequences from total nucleic acids extracted from symptomatic *D. viscosa* clearly show that phytoplasmas are present in Hawaii. Comparison of genomic ribosomal RNA sequences of the PCR-amplified products from *D. viscosa* with other known phytoplasmas reveals similarity between the phytoplasma associated with *Dodonaea* yellows and walnut witches'-broom, *Vaccinium* witches'-broom, pear decline, and western X-disease phytoplasmas. TEM has provided additional evidence that phytoplasmas are associated with this disease in Hawaii. The morphology of the pleomorphic bodies observed is similar to that of phytoplasmas found in many plants infected with these organisms (8,13,21,28).

The occurrence of WX1-positive plants that were nonsymptomatic in the field indicates (i) that overt symptoms may develop over a relatively long time, as indicated by the slow spread documented in field surveys (3); (ii) that individual susceptibility may be variable, with only certain populations of the highly variable *D. viscosa* capable of developing the characteristic symptoms of *Dodonaea* yellows following infection; or (iii) that symptoms of *Dodonaea* yellows occur only when both phytoplasma and virus are present in the infected plants. As noted, not every symptomatic plant tested positively with the WX1 probe, suggesting that perhaps the phytoplasma is not uniformly distrib-

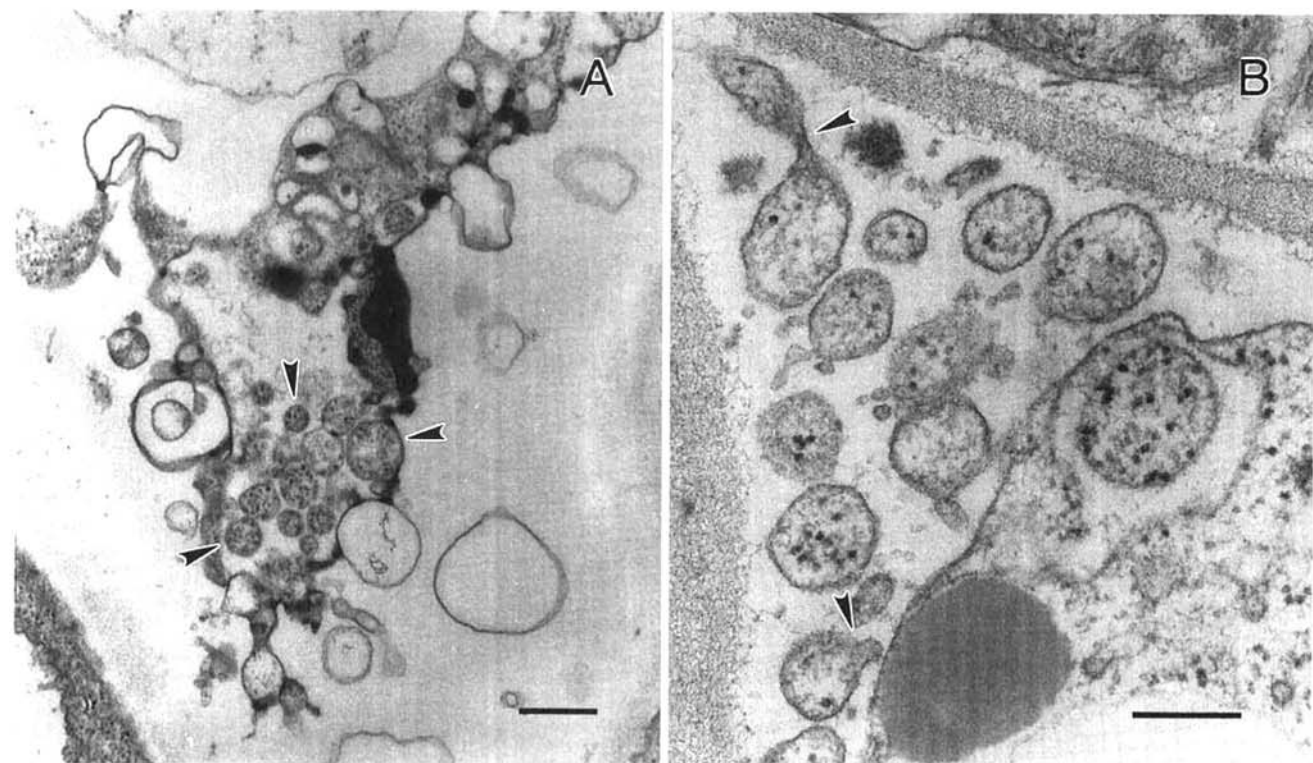


Fig. 3. (A) Transmission electron micrograph of collapsed metaphloem element in tissues of symptomatic *Dodonaea viscosa* showing pleomorphic, membrane-bound bodies (arrows). Scale bar = 100 nm. (B) Higher magnification of pleomorphic bodies associated with phloem elements of diseased *D. viscosa* showing ribosomes and fine fibrillar material contained within the single-membrane bound bodies. Constrictions and appendages are indicated by arrows. Scale bar = 200 nm.

uted within infected plants or that both phytoplasma and virus infection are necessary for symptom expression. Previously, viruslike particles resembling type 3 closteroviruses and a unique dsRNA were detected from diseased *D. viscosa* plants in the Hawaiian Islands (3–5). The relative importance of the viruslike particles and co-occurring dsRNA has not yet been determined.

This is the first report offering direct evidence of plant-pathogenic phytoplasmas in Hawaii, and it confirms the earlier report of Gardner and Kageler (11) that phytoplasmas are implicated in Dodonaea yellows disease. Because *D. viscosa* is important in the island ecosystem (22), Dodonaea yellows may one day cause serious harm. Insects capable of transmitting phytoplasmas are present in the Hawaiian Islands. Because many of these species are polyphagous, and diseased *D. viscosa* is found near established tropical fruit and nut orchards in Hawaii, the phytoplasma associated with Dodonaea yellows might be spread from diseased *D. viscosa* to other ecologically or agriculturally important plants in Hawaii.

Recent classifications of plant-pathogenic phytoplasmas are based on chromosomal and extrachromosomal DNA similarities (1,2,6,7,12,15–17,19,20,23,26,27). Further work using such an approach to characterize the relationship of the phytoplasma affecting *D. viscosa* in Hawaii to other important phytoplasmas, including the spike disease of sandal and the associated yellowing disease of *D. viscosa* in India, is now underway.

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