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**Pathogenic associations with yellows disease of *Dodonaea viscosa*  
in Hawai'i**

**Borth, Wayne Bolton, Ph.D.**

University of Hawaii, 1992

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300 N. Zeeb Rd.  
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PATHOGENIC ASSOCIATIONS WITH YELLOWS DISEASE OF  
*DODONAEA VISCOSA* IN HAWAII

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF  
THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF  
THE REQUIREMENTS FOR THE DEGREE OF

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IN

BOTANICAL SCIENCES (PLANT PATHOLOGY)

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By

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An earlier version of Chapter 2 titled "Association of Double-stranded RNA and Filamentous Viruslike Particles with *Dodonaea* Yellows Disease" was published in *Plant Disease* 74(1990):434-437 by W. B. Borth, Donald E. Gardner, and Thomas L. German and is included here with the permission of the American Phytopathological Society.

## Abstract

*Dodonaea viscosa* (L.) Jacq. in Hawai'i is afflicted with a severe yellowing disorder with symptoms which include the production of pendulous, chlorotic witches' brooms, a decrease in leaf size combined with the distortion of leaf lamina, the suppression of flowering, and progressive defoliation leading to the eventual death of afflicted plants. The disease was first reported in 1984 occurring in Hawai'i Volcanoes National Park on the island of Hawai'i and has since been observed on the islands of Kauai, Maui, and Oahu. Field studies initiated in 1988 and continued over a three year period indicate a slow rate of spread of the disease based upon visual symptom expression. Viruslike particles 16 nm in diameter and 700 nm in length and double-stranded RNA of molecular weight  $3 \times 10^6$  daltons were isolated from diseased plants but were absent from healthy plants. DNA complementary to dsRNA was cloned in *E. coli* and shown to be of non-host origin. Leaves from symptomatic and symptomless plants collected from field sites on Hawai'i, Maui, and Kauai were tested with a probe prepared from the highly conserved 16S ribosomal gene of Western X MLO, which has been shown to reliably detect MLOs from a wide range of hosts. On all the islands sampled, 80% of the symptomatic plants and 33% of the symptomless plants growing near diseased plants tested positively with this probe. Leaves and roots of healthy plants grown from seed collected from symptomless *D. viscosa* did not react with this probe. Pleiomorphic bodies resembling MLOs were observed in phloem tissues of diseased

plants using DAPI staining and transmission electron microscopy. Such structures were not observed in healthy plants grown from seed. Partial alleviation of symptoms on diseased plants was noted following stem injections of oxytetracycline at 100 µg/ml. Attempts to transmit MLOs between *D. viscosa* and *Catharanthus roseus* by *Cuscuta sandwichiana* and *C. campestris* were not successful. The evidence suggests a complex etiology for the yellows disease of *Dodonaea viscosa* in Hawai'i which includes both viral and mycoplasmal agents.



## Table of Contents

Acknowledgments .....	iii
Abstract .....	iv
List of Plates .....	ix
List of Figures .....	x
Chapter 1: Introduction and General Observations .....	1
Chapter 2: Association of dsRNA and Filamentous Viruslike Particles with <i>Dodonaea</i> Yellows Disease .....	5
Materials and Methods .....	7
Isolation of Double-stranded RNA .....	7
PAGE Electrophoresis of dsRNA .....	8
Virus Isolation .....	8
Transmission Electron Microscopy .....	9
Results .....	11
Double-stranded RNA .....	11
Virus Isolation .....	11
Discussion .....	15
Chapter 3: Cloning of dsRNA Associated with <i>Dodonaea</i> Yellows Disease .....	16
Materials and Methods .....	16
dsRNA Isolation .....	16

First-strand Synthesis.....	18
Second-strand Synthesis .....	19
Ligation of cDNA into Bluescript II KS (+) .....	19
Transformation .....	21
Restriction Digests of Plasmid Clone DNA.....	22
Radiolabelling of Inserts for Use as Probes .....	24
Dot Blots and Hybridizations .....	26
Probing of Genomic DNA from <i>D. viscosa</i> .....	27
Results .....	30
Restriction Digests of Plasmid Clones .....	30
Dot Blots and Hybridizations .....	30
Probing of Genomic DNA from <i>D. viscosa</i> .....	35
Discussion.....	35
Chapter 4: Association of Mycoplasma-like Organisms with <i>Dodonaea</i> Yellow's Disease .....	39
Materials and Methods .....	42
Oligomer Probes and Dot Blots of Nucleic Acids .....	42
Dot Blot Detection of MLO Sequences .....	45
Hybridizations .....	45
DAPI Staining .....	46
Transmission Electron Microscopy .....	46
Tetracycline Treatments.....	47
<i>Cuscuta</i> Transmission .....	50
Results .....	51
Detection of Oligomer Sequences .....	51
DAPI Staining .....	52

Transmission Electron Microscopy .....	59
Tetracycline Treatments.....	65
<i>Cuscuta</i> Transmission .....	65
Discussion.....	68
Chapter 5: Epidemiology of <i>Dodonaea</i> Yellow's Disease.....	70
Materials and Methods .....	70
Results .....	78
Discussion.....	78
Chapter 6: Conclusions .....	80
Literature Cited .....	84

## List of Plates

<u>Plate</u>		<u>Page</u>
I.	<i>Dodonaea viscosa</i> with Symptoms of Yellows Disease.....	6
II.	PAGE of dsRNA Isolated from Symptomatic <i>D. viscosa</i> .....	10
III.	TEM of Viruslike Particles Isolated from Diseased <i>D. viscosa</i> .....	12
IV.	High Magnification TEM of VLPs from Diseased <i>D. viscosa</i> .....	13
V.	Agarose Gel of dsRNA Isolated for Use in Cloning.....	17
VI.	Agarose Gel of Clones Digested with <i>Not I</i> .....	31
VII.	Agarose Gel of DNAs Isolated from Clones A1 and A4 .....	32
VIII.	Dot Blot Hybridizations of Inserts from Clones A1 and A4 with dsRNA from Diseased <i>D. viscosa</i> .....	34
IX.	Agarose Gel of Total Genomic DNA Isolated from <i>D. viscosa</i> .....	36
X.	Southern Blot of Total Genomic DNA from <i>D. viscosa</i> .....	37
XI.	Dot Blot of Total Nucleic Acids Isolated from Field-grown <i>D. viscosa</i> .....	53
XII.	Dot Blots of Total Nucleic Acids Isolated from Roots of Pot-cultured <i>D. viscosa</i> .....	54
XIII.	Longitudinal Sections of Stem from Healthy <i>D. viscosa</i> .....	56
XIV.	DAPI-stained Phloem Tissues from Symptomatic <i>D. viscosa</i> .....	57
XV.	TEM of Fine Roots from Symptomatic <i>D. viscosa</i> .....	60
XVI.	TEM of Phloem Tissue from Symptomatic <i>D. viscosa</i> .....	62
XVII.	TEM of Phloem Tissue from Symptomatic <i>D. viscosa</i> .....	63
XVIII.	High Magnification of Portion of Plate XV .....	64

## List of Figures

<u>Figure</u>		<u>Page</u>
1.	Length Distribution of Viruslike Particles Isolated from Diseased <i>Dodonaea viscosa</i> .....	14
2.	Plasmid Bluescript II KS (+).....	20
3.	Molecular Weights of Inserts from Clones A1 and A4 .....	33
4.	Bioassay Regression.....	66
5.	Bioassay for Oxytetracycline in <i>D. viscosa</i> .....	67
6.	Field Plot, SAS Analysis: 1988 .....	72
7.	Field Plot, SAS Analysis: 1990 .....	73
8.	Field Plot, SAS Analysis: 1991 .....	74
9.	Field Plot, Percentage Disease Change: 1988-1990 .....	75
10.	Field Plot, Percentage Disease Change: 1990-1991 .....	76
11.	Field Plot, Percentage Disease Change: 1988-1991 .....	77

## CHAPTER I

### Introduction and General Observations

*Dodonaea viscosa* (L.) Jacq. is an important plant in the dry forest and shrublands in many tropical and subtropical regions worldwide. In Hawai'i, it can be found from coastal dunes, through low elevation shrublands and dry, mesic, or sometimes wet forests to the subalpine shrubland near 2500 m on all the main islands except Kahoolawe. It is an evergreen dicot with simple, alternate, more or less spatulate, narrow leaves 2-15 cm long and 0.5-6 cm wide which are usually covered by a rather sticky epicuticular exudate. The inconspicuous flowers are borne in small clusters at the ends of the branches or leaf axils. The flowers are usually unisexual and plants are dioecious or sometimes andromonecious, although perfect flowers may also be present. Fruit is an indehiscent capsule, straw colored to dark reddish purple about 1-2 cm long with 2-4 lobed wings. Each capsule of 2-4 cells contains 1-3 seeds which are small, compressed-ovoid, dark-colored about 2 mm in diameter, which when germinated produce individuals which may flower in just 2 years.

Although sometimes assigned to its own family Dodonaceae, *Dodonaea* is usually placed in the family Sapindaceae(47,78). The species is extremely polymorphic throughout its range which has led to many different classifications. Presently, the many different forms of *Dodonaea* in Hawai'i are considered as ecotypes or varieties of *Dodonaea viscosa* (L.) Jacq.(78). With growth habits ranging from low shrubs less than a meter high, to trees of more than 10 m, this Hawaiian indigenous plant is important

in Hawaiian ecosystems as a successful early colonizer of disturbed sites. It can often be found growing on relatively new lava flows on the island of Hawai'i (47,63), where it is drought tolerant and able to provide a rich litter layer beneath its canopy which contributes to the formation of new soils on sites poor in organic material.

*Dodonaea viscosa*, known as a'ali'i to native Hawaiians, was highly valued for use of the colorful fruit and attractive leaves in traditional ceremonies and in dye making, the strong wood for tool handles and buildings, and various parts of the plant for medicine (47,53). Today *D. viscosa* is used in lei making and is also becoming available for use in drought-tolerant landscaping.

Gardner and Kaegler first reported in 1984 that *D. viscosa* plants on the island of Hawai'i were developing symptoms of a severe yellowing disorder characterized by the proliferation of usually pendulous, chlorotic, "witches' brooms" on sections of otherwise healthy plants (31). Terminal portions of these stems are often reddened and may be compressed laterally. Afflicted portions of plants fail to produce either male or female flowers while flowering appears normal on the nonsymptomatic portions of the same plant. The syndrome gradually spreads to involve the entire plant which eventually over a period of years becomes progressively defoliated with only a few deformed leaves remaining at the tips of the brooms. These plants ultimately wither but remain standing in the field where their clusters of dried leafless witches' brooms are prominent. Recovery of symptomatic plants has never been recorded in field situations.

Although at present only a small percentage of native *Dodonaea* is afflicted with overt symptoms of this disorder, it is slowly spreading to healthy *D. viscosa* in areas where it occurs; symptomatic plants have been found on all the major Hawaiian islands. Individual *D. viscosa* plants may display symptoms of this disorder at any point in their development. Mature individuals may develop symptoms on isolated branches,

or young saplings may become entirely symptomatic within a short time after their establishment.

Two characteristics of this disorder suggest a pathogenic etiology. First, symptomatic plants can be found growing in different environments of rainfall, temperature, and substrate composition. Second, when plants with symptoms are transplanted into controlled environments, kept free from insects, and given regular applications of macro- and micro-nutrients, they retain their original symptoms. Thus, symptoms on afflicted plants are not caused by environmental factors, toxic substances associated with insect feeding, or poor soil or nutritional factors. Preliminary attempts to culture fungal or bacterial pathogens from symptomatic plants did not reveal any association between these agents and the disease syndrome. These data and the symptomatology associated with the syndrome led to the initiation of this dissertation research which focused on the identification and characterization of an association between viral and mycoplasmal agents with diseased *D. viscosa*. These agents are absent in healthy *D. viscosa* and the evidence provided implicates these agents in the disease etiology. Throughout the study, the source of plant material used was either collected directly from plants growing in field situations or from plants grown in containers under controlled conditions. Isolations of viruslike particles, dsRNA isolation and analysis, surveys of MLO infection in field situations and epidemiological studies were all carried out on plants growing in the field. Some dsRNA analysis, tetracycline injections, DAPI and TEM investigations, and *Cuscuta* transmission experiments were done on plants growing in pots in controlled environments. The symptomatic plants maintained in pot cultures were established from field collected, symptomatic individuals, which were young seedlings when transplanted. These plants, which were usually less than 50 cm tall, were dug from the field and brought to the Manoa campus where they were planted into separate containers. During the course



of the work, eight collections were made. All of them were from the Mauna Loa strip road area of Hawai'i Volcanoes National Park on the island of Hawai'i at elevations of 1000-2000 m above sea level. Healthy control plants grown from seed collected from non-symptomatic individuals growing in field situations, were used as negative controls for the work presented with the exception of the field surveys for MLO incidence and epidemiology.

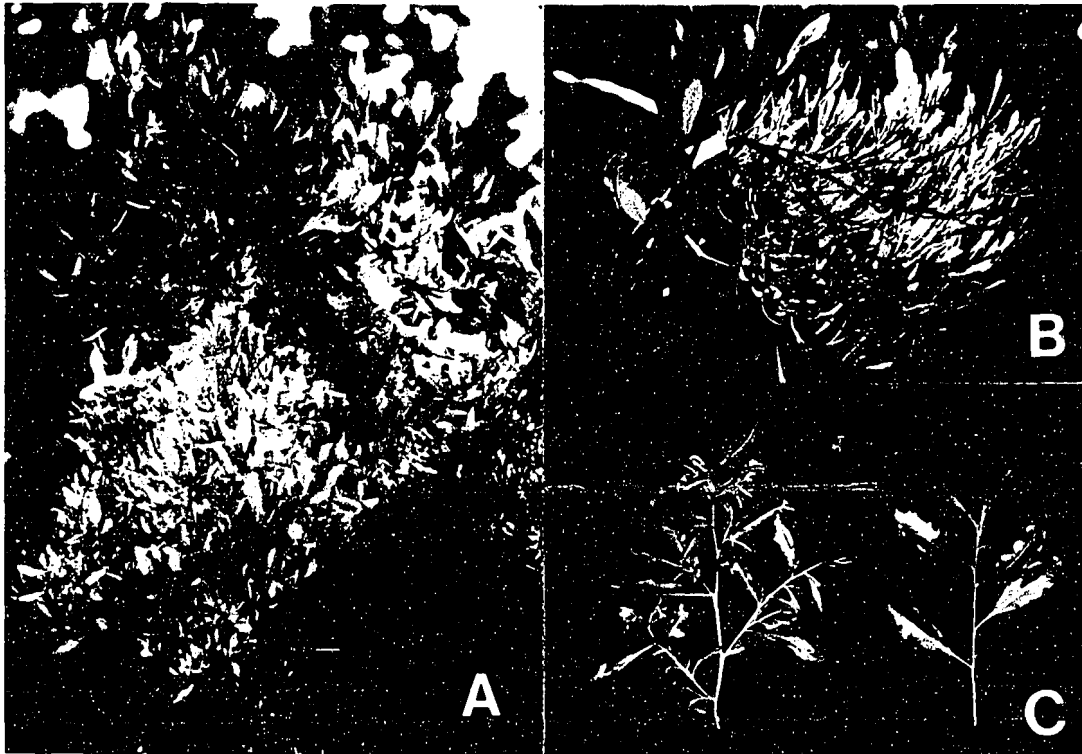
## CHAPTER 2

# Association of dsRNA and Filamentous Viruslike Particles with *Dodonaea* Yellow's Disease

The genus *Dodonaea* (Sapindaceae) is widely distributed throughout the islands of the Pacific and other tropical and subtropical regions of the world. *Dodonaea 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 (47,63). 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 (63).

In 1977 a conspicuous and severe disease affecting *D. viscosa* was observed on the island of Hawai'i (31). It has since been observed on all the major islands of the 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 reduced in size and exhibit a general chlorosis (Plate I). Affected trees or shrubs gradually decline over a period of years, eventually becoming entirely defoliated. Although most conspicuous on mature specimens, young individuals can be similarly affected.

Witches' brooms, subnormal leaf size, and chlorosis are symptoms of diseases of woody species generally associated with mycoplasma-like organisms (MLOs), and the yellows disease of *D. viscosa* in Hawai'i has also been postulated to be caused by MLOs (30). A disease similar in some characteristics to the Hawaiian disease has been



**Plate I. *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).

reported from India affecting *D. viscosa* growing in conjunction with the root parasite *Santalum album* L. affected with spike disease (64). Transmission electron microscopy (TEM) of these *D. viscosa* plants by workers in India confirmed the presence of characteristic, pleiomorphic 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 Hawai'i have failed to reveal any pleiomorphic bodies or viral inclusions in these tissues (W. Sakai, unpublished).

The presence of viruslike particles in the phloem of plants affected with yellows diseases associated with MLOs have also been shown for some species (2,76). The isolation of double-stranded RNA and flexuous, rod-shaped virus particles from *D. viscosa* affected with yellows disease in Hawai'i is reported here.

## MATERIALS AND METHODS

### Isolation of Double-stranded RNA

Symptomatic and symptomless *D. viscosa* growing in the field or in greenhouse pot cultures were assayed for the presence of double-stranded RNA by the method of Morris and Dodds (62). Leaf and twig tissues were sampled from 20 symptomatic and 10 non-symptomatic plants from field populations and 15 symptomatic and 7 non-symptomatic plants from greenhouse populations. Only those portions of diseased plants with well-developed symptoms were sampled. 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 Na-acetate (pH=5.2, final concentration of 150 mM) and 2.5 volumes 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). Samples were made to 150 mM in NaCl and

5 mM in  $MgCl_2$  by the addition of concentrated stock solutions. Fifty units of RNase-free DNase 1 (Boeringer-Mannheim) was added and the solution was incubated for 30 min at 37°C. Following nuclease treatment, SDS 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, collected, and the nucleic acids were precipitated as above.

### **PAGE Electrophoresis of dsRNA**

Double-stranded RNAs were prepared for electrophoresis by centrifuging precipitated nucleic acids, drying the pellets under vacuum, and dissolving the dsRNA in PAGE sample buffer (40 mM Tris; 20 mM Na-acetate; 1mM 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 x 80 x 80 mm). Gels were overlayed with buffer (40 mM Tris; 20 mM Na-acetate; 1 mM EDTA; pH 7.8) and run for 4 hr at 25 mA. Double-stranded RNAs from *Nicotiana tabacum* L. cv. Xanthi infected with tobacco mosaic virus (TMV; dsRNA=4.0 x 10<sup>6</sup> daltons), and *Hordeum vulgare* L. infected with brome mosaic virus (BMV; dsRNAs=2.2 x 10<sup>6</sup>, 2.0 x 10<sup>6</sup>, and 1.4 x 10<sup>6</sup> daltons), both prepared as above, were used as molecular weight standards for the gel system. For the calculation of the molecular weight for the dsRNA from *D. viscosa*, the method of Bozarth and Harley was utilized (8). 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.

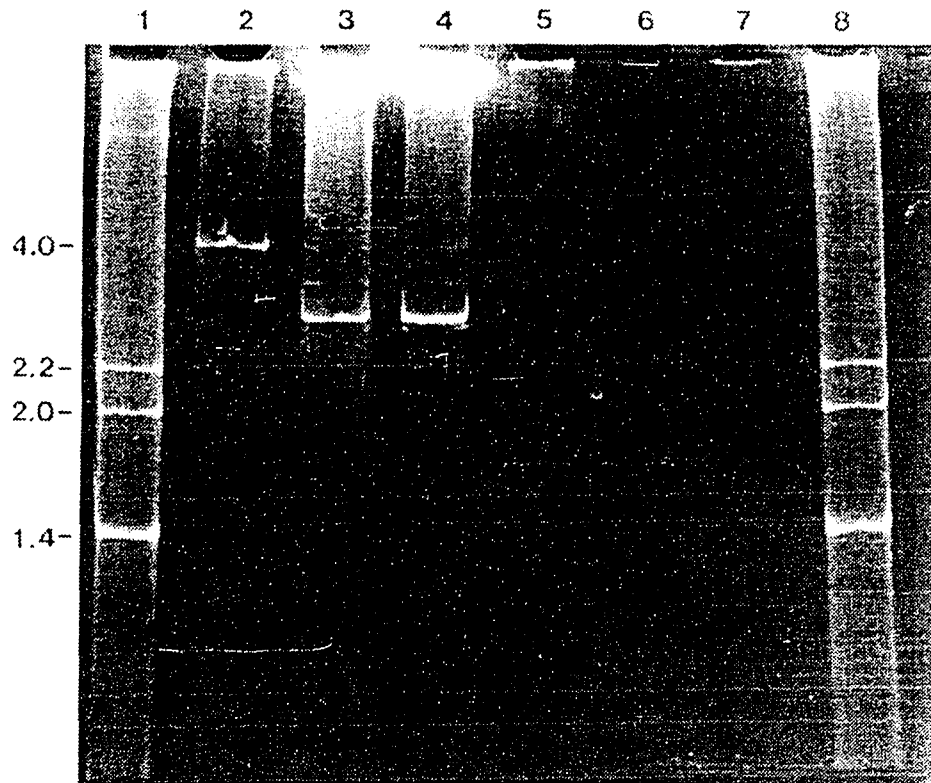
### **Virus Isolation**

The protocol for the isolation of virus particles from diseased plants utilized differential centrifugation. All steps in the protocol were carried out at 4°C.

Symptomatic leaves and young twigs were collected from diseased *D. viscosa* in the field and immediately processed for isolation of virus. Tissues were ground in liquid nitrogen and extracted in buffer (50-100g tissue/l) containing 300 mM Tris, 400 mM NaCl, 50 mM Na<sub>2</sub>SO<sub>4</sub>, 10 mM MgCl<sub>2</sub>, and 3%(w/v) Triton X-100 (pH 8.0) by stirring for 30 min. 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 buffer (100 mM Tris; 400 mM NaCl; 50 mM Na<sub>2</sub>SO<sub>4</sub>; pH 8.0) by stirring overnight. 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 resuspension buffer as above and stored at 4°C. The purification protocol was monitored by observing the yields of each step using TEM.

### **Transmission Electron Microscopy**

Aliquots of virus isolated by the above procedure were applied to carbon-stabilized, formvar-coated copper grids (Ted Pella, Inc.), washed with 200 µl bacitracin solution in water (300 µg/ml) and negative-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 TEM at 50 kV. For width determinations, TMV prepared from *Nicotiana tabacum* cv. Xanthi was used as a standard. To determine the modal lengths of virions isolated from diseased *Dodonaea*, 60 virus particles were measured using 109 nm diameter latex spheres (Ted Pella, Inc.) as standards. Electron micrographs of virions and spheres were measured using a Kontron IBAS image analyzer.



**Plate II. PAGE of double-stranded RNA (dsRNA) isolated from symptomatic *Dodonaea viscosa*.** Lanes 1 and 8, brome mosaic virus dsRNAs; lane 2, tobacco mosaic virus dsRNA; 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 ( $\times 10^{-6}$ ).

## RESULTS

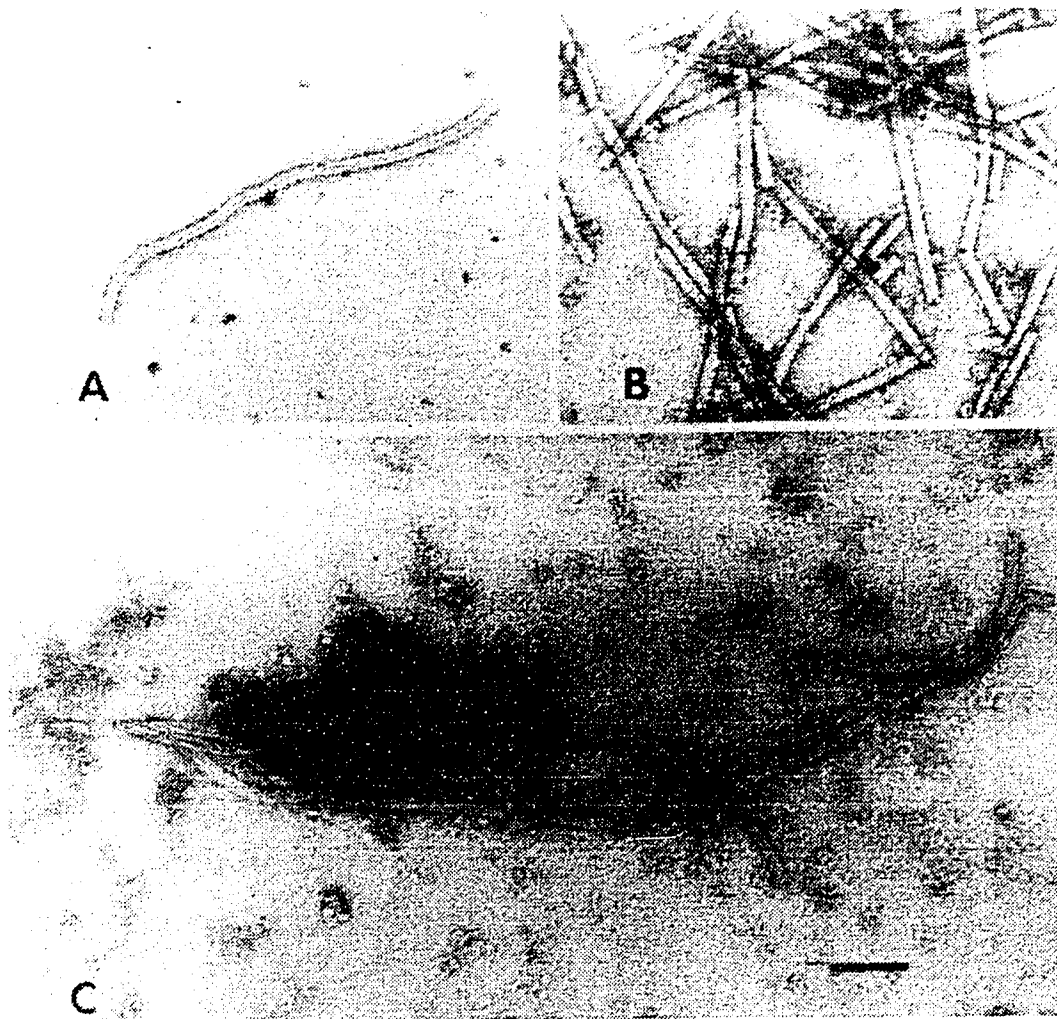
### Double-stranded RNA

PAGE analysis of RNAs isolated from diseased *D. viscosa* revealed the presence of a double-stranded RNA in symptomatic tissues that was not present in healthy tissues (Plate II). 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 showed variability throughout the year with the lowest levels occurring 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  $3 \times 10^6$  daltons.

### Virus Isolation

The isolation of virus particles from diseased *Dodonaea* was difficult due to 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 materials and methods gave the most reproducible yields of virions from symptomatic leaf tissues. Yields were maximized by utilizing only fresh tissues for all extractions. It was not possible to obtain sufficient quantities of virus particles for analysis of either coat protein subunit molecular weight or virion nucleic acids. Uranyl formate negative staining of virus particles associated with diseased *D. viscosa* suggested a symmetric helical arrangement of coat protein subunits around a hollow central core (Plates III, IV). Based upon an 18 nm diameter for TMV virions, the width of the flexuous, rod-shaped particles from diseased





**Plate III. TEM of viruslike particles isolated from diseased *Dodonaea viscosa*.**

(A) Particle about 700 nm long, showing central core and coat protein subunit texture.

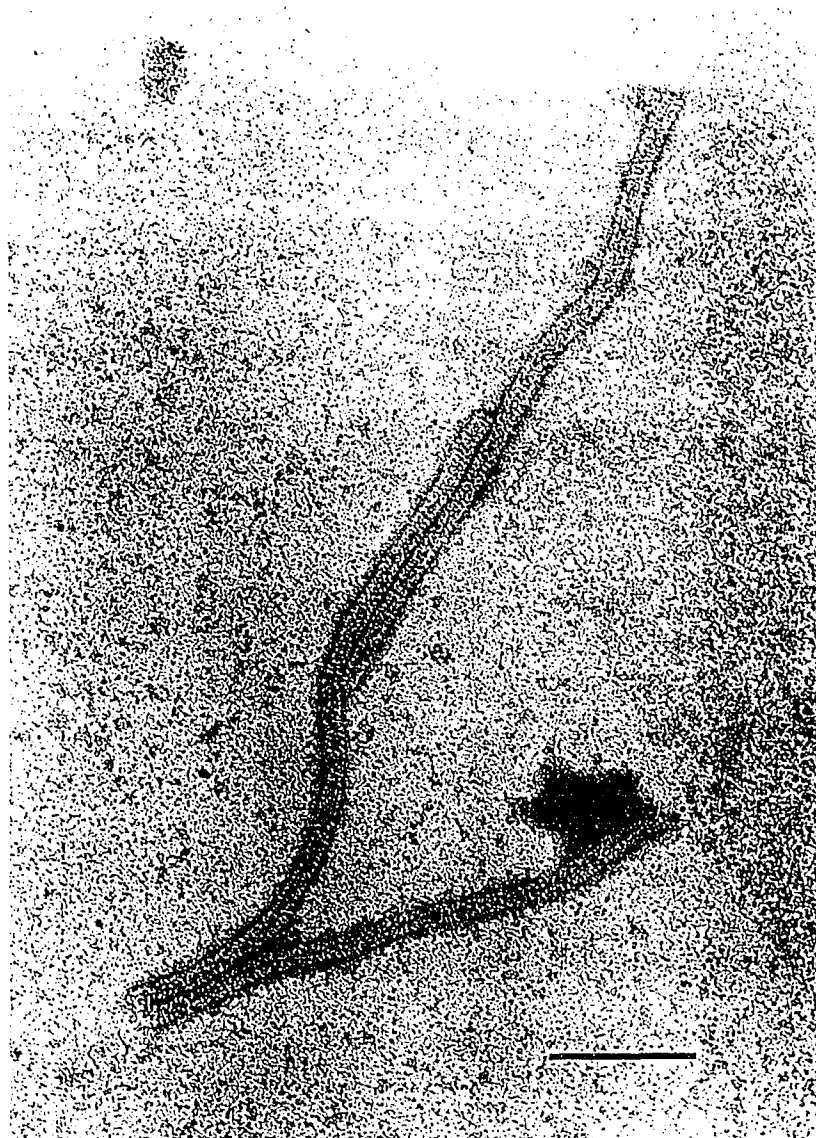
(B) Tobacco mosaic virus isolated from infected *Nicotiana tabacum*.

(C) Group of particles from diseased *D. viscosa*, showing aggregation and breakage commonly

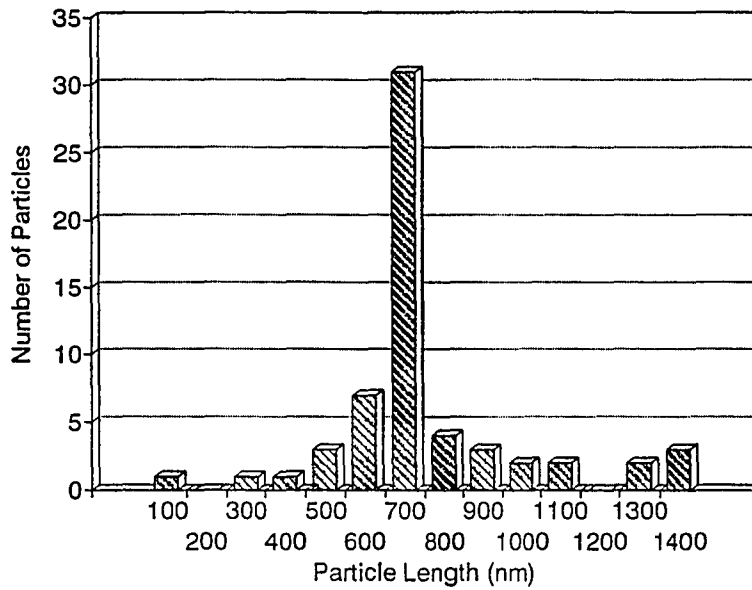
observed in partially purified preparations. Particles were negatively stained with

uranyl formate in methanol. All micrographs are at the same magnification; the scale

bar represents 100 nm.



**Plate IV.** High magnification transmission electron micrograph of viruslike particles from diseased *Dodonaea viscosa*. Regularly arranged protein subunits can be distinguished surrounding the hollow central core. Stained in uranyl formate in methanol. Scale bar represents 100 nm.



**Figure 1. Length distribution of viruslike particles isolated from diseased *Dodonaea viscosa*.** Latex spheres of known diameter were used as size standards to estimate the modal length of VLPs as around 700 nm (n = 60).

*D. viscosa* was estimated to be 16 nm. Using latex spheres of 109 nm diameter as standards, the modal lengths of the particles was estimated at about 700 nm (Fig. 1).

## DISCUSSION

The yellows disease of *D. viscosa* in Hawai'i displays symptoms resembling those caused by mycoplasma-like organisms in other woody hosts (76,77). However, no evidence has been presented which conclusively demonstrates the etiology of this disease. We have demonstrated here the presence of a flexuous, rod-shaped virus in diseased tissues by direct isolation of virions and by inference based upon the presence of double-stranded RNA in diseased plants which is absent in healthy plants. Although the dimensions of these particles do not allow the certain assignment of the isolated virus into any known group, they resemble PVX, PVY, or closteroviruses. The analysis of plants for the presence of dsRNA is a useful tool in the diagnosis of many plant diseases of viral origin (17,18), despite the fact that dsRNA has been found in tissues of certain plant species not infected with RNA plant viruses (64,65,79).

The presence of virus particles in diseased plants does not preclude the possibility that spiroplasmas or MLOs could also be present in these tissues, or that they may be involved in the etiology of *Dodonaea* yellows disease. Examples are widely known of dicots and monocots infected with both viruses and mollicutes (2,3,4,5,26,29,38, 40,44,83). Such dual infections may alter the disease symptoms displayed on susceptible hosts infected with either agent alone (4).

## CHAPTER 3

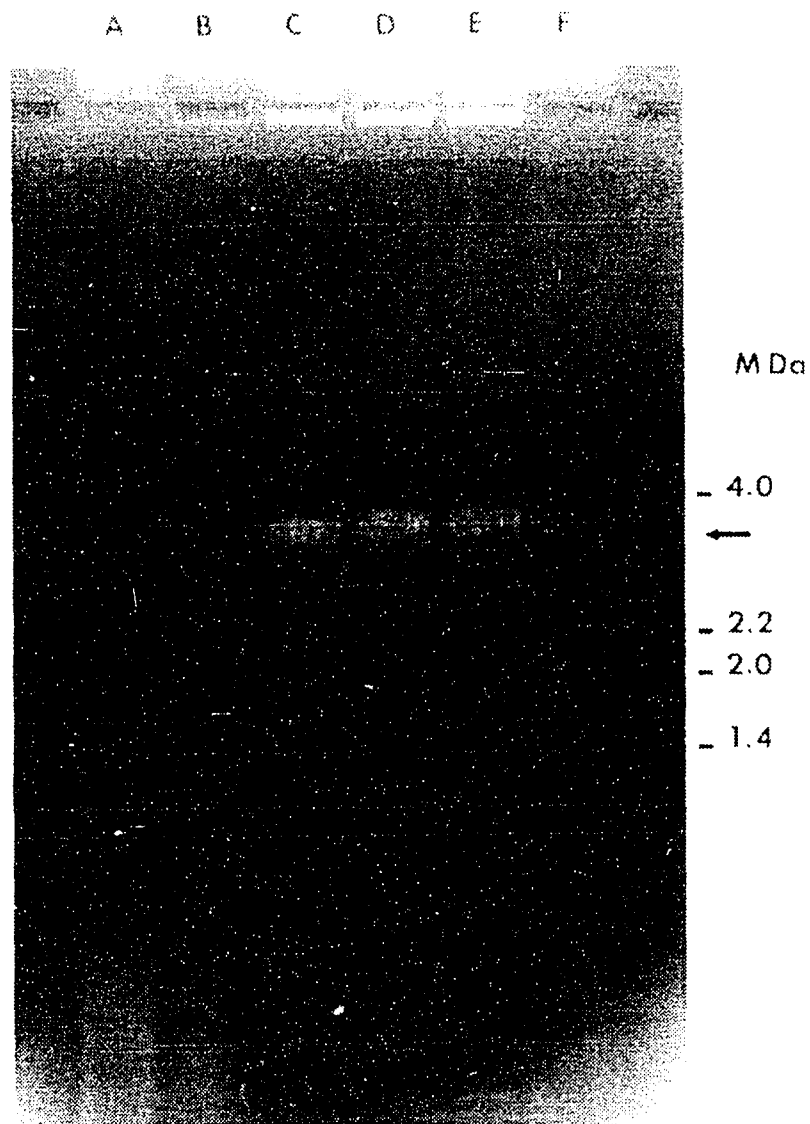
# Cloning of dsRNA Associated with *Dodonaea* Yellows Disease

Isolated dsRNA was cloned into *E. coli* to create a probe which could be used to detect the possible presence of these sequences in *Dodonaea* and to confirm the nature of the origin of this dsRNA in diseased plants.

## MATERIALS AND METHODS

### dsRNA Isolation

Double-stranded RNA isolated essentially as described above was precipitated, collected and dissolved in TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0) before being electrophoresced on a 0.8% (w/v) SeaPlaque agarose gel in 1X TAE buffer (40 mM Tris acetate; 1 mM EDTA; pH 8.0) containing 0.5% (w/v) ethidium bromide at 5 volts/cm for 5 hr at 4°C. Double-stranded RNAs isolated from BMV- infected barley seedlings and TMV-infected tobacco were used as molecular weight standards. Nucleic acids were visualized by ultraviolet light (Plate V) and the dsRNA from *D. viscosa* was carefully excised from the gel avoiding marker dsRNAs and only taking the minimum amount of material. Excised agarose containing the dsRNAs of interest were added to 400 µl sterile TE buffer, heated to 65°C for 5 min to melt the agarose, and quench-cooled on ice. This solution containing dsRNA and agarose was extracted once with an equal volume of redistilled H<sub>2</sub>O-saturated phenol containing 0.1% (w/v)



**Plate V. Agarose gel of dsRNA isolated for use in cloning.** Lane A, dsRNA from BMV infected barley; lane F, dsRNA from TMV infected tobacco; lanes B, C, D, E, dsRNA from symptomatic *D. viscosa*. Molecular weights of BMV and TMV standards given at right in megadaltons. Arrow indicates the position of bands eluted for use in cloning.

8-hydroxyquinone and 0.1% 2-mercaptoethanol, and once with an equal volume of chloroform. Nucleic acids were then precipitated with 1/10 volume 3M sodium acetate (pH 5.2) and 2.5 volumes ethanol at -20°C overnight. Precipitated nucleic acids were collected by centrifugation and reprecipitated as above before being used for synthesis of complementary DNA.

Complementary DNA (cDNA) was synthesised from dsRNA using the Riboclone cDNA synthesis system from Promega Corp. The method is based upon that described by Okayama and Berg (68) as modified by Gubler and Hoffman (33). Complementary DNA synthesis was primed by random hexadeoxynucleotides supplied by the manufacturer.

### **First-strand Synthesis**

Double-stranded RNA dissolved in 8 µl nuclease-free, sterile H<sub>2</sub>O was boiled 5 min and quenched on ice. Random primers (1 µl of 1 µg/µl solution) were added and heated to 70°C for five min then cooled slowly to room temperature. The remaining reaction components were added in the following order:

- 2.5 µl 10X reaction buffer (0.5M Tris, pH 8.3; 750 mM KCl; 100 mM MgCl<sub>2</sub>; 5 mM spermidine)
- 2.5 µl 100 mM Dithiothreitol (DTT)
- 2.5 µl dNTP mix (10 mM each dATP, dGTP, dTTP)
- 1 µl (=40 units) RNasin ribonuclease inhibitor
- 2.5 µl 40 mM sodium pyrophosphate
- 5 µl (=10 units) AMV reverse transcriptase (1:10 dilution in 10 mM KH<sub>2</sub>PO<sub>4</sub> pH 7.4; 2 mM DTT;
- 10 % glycerol; 0.2% Triton X-100)

The reaction was incubated at 42°C for 60 min, after which second strand synthesis was immediately carried out.

## Second-strand Synthesis

To the entire contents of the first-strand reaction mixture (20  $\mu$ l), the following were added:

10  $\mu$ l 10X buffer (400 mM Tris pH 7.2; 850 mM KCL;

30 mM MgCl<sub>2</sub>; 1 mg/ml bovine serum albumen;

100 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>)

3  $\mu$ l 100 mM DTT

10  $\mu$ l 1 mM nicotine adenine dinucleotide

2.3  $\mu$ l (=23 units) *E. coli* DNA polymerase I

1.0  $\mu$ l (=1 unit) *E. coli* ligase

1.0  $\mu$ l (=0.8 units) *E. coli* RNase H

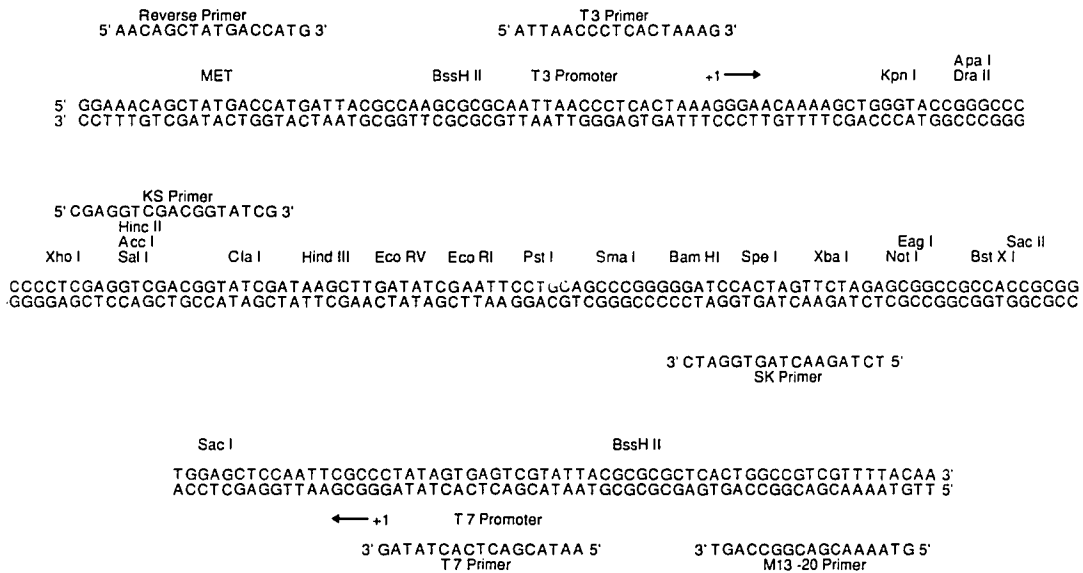
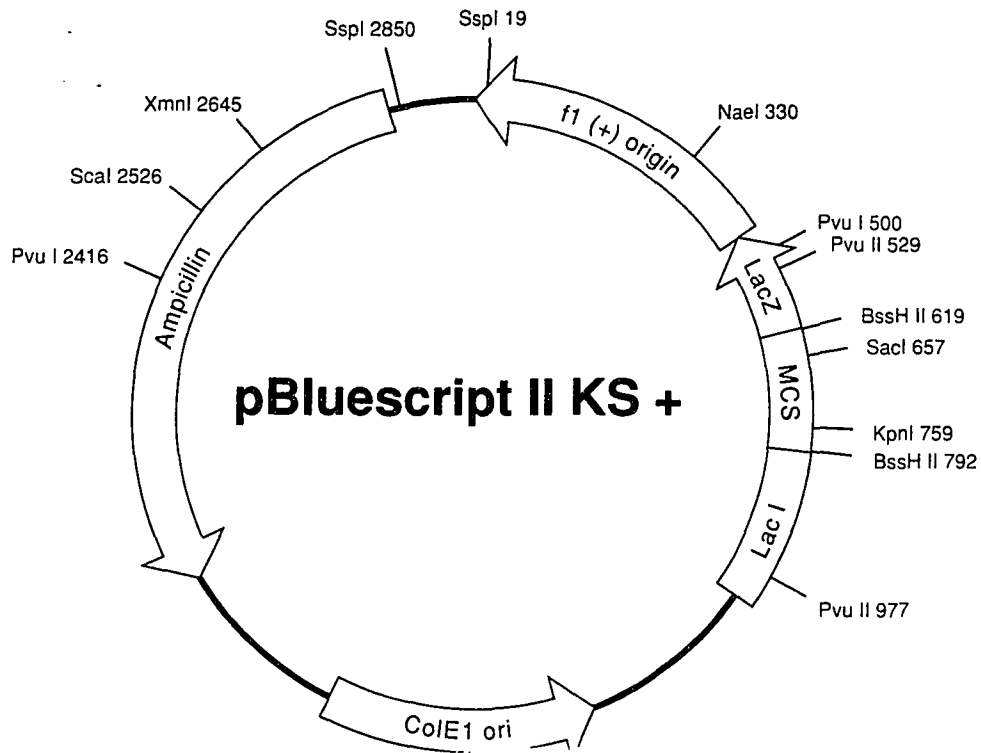
52  $\mu$ l H<sub>2</sub>O (nuclease-free)

The reaction was incubated at 14°C for 2 hr, then at room temperature for 1 hr, and stopped by heating to 70°C for 10 min and quenching on ice. To this reaction mixture was added 0.5  $\mu$ l (=5 units) T4 DNA polymerase. The reaction was incubated at 37°C for 10 min and stopped by the addition of 10  $\mu$ l 0.2 M EDTA. Synthesized nucleic acids were extracted with phenol (as above), then sevag (chloroform:isoamyl alcohol @ 24:1), and precipitated with 1/10 volume 3M sodium acetate and 2.5 volumes ethanol for 15 min at -70°C. Precipitated nucleic acids were collected by centrifugation, dried, and dissolved in 20  $\mu$ l nuclease-free H<sub>2</sub>O.

## Ligation of cDNA into Bluescript II KS (+)

Ligation of newly synthesized cDNA into plasmid Bluescript II KS (+) from Stratagene, Inc.(Fig. 2) (previously cut with restriction endonuclease *Sma* I) was performed under the following reaction conditions essentially from Sambrook et al. (71).





**Figure 2. Plasmid Bluescript II KS (+).** Multiple cloning site (MCS) sequence is given below plasmid map. Recognition sites of restriction endonucleases are indicated.

6  $\mu$ l cDNA from second-strand synthesis

1  $\mu$ l T4 ligase

2  $\mu$ l 5X buffer (250 mM Tris pH 7.6; 50 mM  $MgCl_2$ ; 50mM DTT;  
250  $\mu$ g/ml fraction V BSA)

1  $\mu$ l Bluescript II KS (+) (*Sma* 1 cut)

The reaction was incubated at 14°C for 24 hr and resulting plasmids were used to transform *E. coli* DH5 $\alpha$  competent cells.

### **Transformation**

DH5 $\alpha$  competent *E. coli* cells were purchased from Bethesda Research Laboratories and used according to the manufacturer's protocol. Ligation reaction was diluted 5-fold in TE (pH 7.5), and 10  $\mu$ l diluted ligation reaction was added to 100  $\mu$ l competent cells. They were incubated on ice for 30 min and heat shocked at 42°C for 45 sec without shaking. Cell suspensions were then incubated on ice again for 2 min. Then 0.9 ml S.O.C. medium (2% bactotryptone; 0.5 % yeast extract; 10 mM NaCl; 2.5 mM KCl; 10 mM  $MgCl_2$ ; 10 mM  $MgSO_4$ ) was added and cells were shaken at 225 rpm for 1 hr at 37°C. The cells were plated at 0.2 ml per plate on LB agar containing 100 mg/ml ampicillin and incubated at 37°C.

The multiple cloning site of Bluescript II KS (+) lies between the lac promoter and the lacZ gene (Fig. 2). Insertion of cloned DNA into this site therefore inactivates the lacZ gene. Bacterial transformants which contain cloned segments of DNA at this site are unable to hydrolyze beta-galactosidase, and can be selected for by plating upon a medium which contains chromogenic substrates that are developed by the action of the active beta-galactosidase gene. Selection of colonies which were unable to hydrolyze the substrate and which therefore produced white colonies were picked with a sterile toothpick into 2 ml TB + 100  $\mu$ g/ml ampicillin with shaking at 37°C. They were harvested in late log phase growth and plasmid DNA was prepared according to

the method of Sambrook et al. (43) with slight modification. Cells were harvested by centrifugation, washed once in sterile TE buffer, and resuspended in 0.32 ml ice-cold STET buffer (8% sucrose; 0.5% triton X-100; 50 mM EDTA; 10 mM Tris pH 8.0). To this solution was added 32  $\mu$ l of a freshly prepared lysozyme solution (10 mg/ml in TE buffer), the cell suspension was boiled for 1 minute, quench-cooled on ice, and centrifuged for 15 min at 15,000 g at 4°C. The resulting sticky pellet of cell debris was removed with a sterile toothpick and nucleic acids were precipitated by the addition of 1/2 volume 7.5M ammonium acetate and one volume isopropanol. Precipitated nucleic acids could be recovered immediately or after an overnight incubation at -20°C, by centrifugation as above. Resulting pellets were washed once in 70% ethanol, dried, and redissolved in 50-100  $\mu$ l sterile TE buffer/initial 2 ml volume liquid culture medium.

#### **Restriction Digests of Plasmid Clone DNA**

Plasmid DNA was digested with restriction endonuclease *Not I* to confirm the presence of insertion sequences in transformed clones. The plasmid Bluescript II KS (+) has only a single recognition site for this restriction enzyme, and because its recognition site is composed of six nucleotides, the probability that it would also cleave within the inserted sequences was low. A reaction mixture was prepared as follows:

10  $\mu$ l *Not I* 10X buffer (500 mM Tris; 100 mM MgCl<sub>2</sub>; 1.0 M NaCl;

10 mM dithioerythritol; pH 7.5)

10  $\mu$ l BSA (1 mg/ml)

10  $\mu$ l RNase (DNase-free; 5  $\mu$ g/ml)

3  $\mu$ l *Not I* (=30 units)

17  $\mu$ l H<sub>2</sub>O (nuclease-free)

Nine microliters of this mixture was used to digest 1  $\mu$ l of plasmid DNA from the minipreps. Reaction mixtures prepared without *Not I* enzyme were used as negative controls. Samples were incubated at 37°C for one hr, prepared for electrophoresis by

the addition of 1.5  $\mu$ l loading buffer, and heated to 60°C for 2 min followed by immediate quenching on ice. Agarose gel electrophoresis was performed in 0.8% (w/v) Seakem GTG agarose in 0.5X TBE (45 mM Tris-borate; 1 mM EDTA; pH 8.0) + 0.5  $\mu$ g/ml ethidium bromide at 5 volts/cm and room temperature.

The approximate lengths of inserts in two clones, designated A1 and A4, were further characterized by double digestion with endonucleases *Xba* I and *Xho* I, followed by gel electrophoresis to resolve fragments. Inserted DNA should be released intact from Bluescript II KS (+) plasmid DNA by the actions of these enzymes, since the original blunt-end ligation was at the *Sma* I site of the plasmid. The reaction conditions were as follows:

1  $\mu$ l *Xba* I 10X buffer (500 mM Tris; 100 mM MgCl<sub>2</sub>; 1 M NaCl;

10 mM dithioerythritol; pH 7.5)

0.3  $\mu$ l *Xba* I (=18 units)

0.6  $\mu$ l *Xho* I (=18 units)

1  $\mu$ l BSA (1 mg/ml)

1  $\mu$ l RNase A (DNase-free; 5 $\mu$ g/ $\mu$ l)

6.1  $\mu$ l H<sub>2</sub>O

One  $\mu$ l of plasmid DNA was digested with 9  $\mu$ l of this mixture for one hr at 37°C. Plasmid Bluescript II KS (+) was also digested with this reaction mixture. Parallel digestions without enzymes were also performed as negative controls. Samples were prepared for electrophoresis in 1.2% GTG agarose gels in 0.5X TBE + 0.5% ethidium bromide as above and run at 5 volts/cm and room temperature. Molecular weight standards were included on the same gel. Estimates of molecular weights of inserts were based upon the log of the molecular weight versus the migration distance in the gel.

### **Radiolabelling of Inserts for Use as Probes**

Both the 300 bp and the 1 kbp inserts were purified by gel electrophoresis and radiolabelled by random primer initiation to determine their specificity in molecular hybridization experiments. Miniplasmid preps prepared as in the previous section were digested with *Xho* I and *Xba* I as described above. The fragments were separated by electrophoresis on 1.2% GTG agarose gels in 1xTAE buffer and the 300 bp and 1 kbp fragments were visualized by longwave ultraviolet light. A sandwich of Whatman GF/C filter and dialysis membrane was prepared slightly larger than the band of interest and wider than the gel was thick. The gel was cut just ahead of the band of interest and the sandwich wetted in 1X TAE was inserted into the gel at this position with the GF/C filter towards the plasmid insert band. Electrophoresis was continued at 10 volts/cm until the bands had migrated onto the GF/C filter (about 10 min). This movement was monitored with long-wave ultraviolet light. The sandwich was removed, the dialysis membrane discarded and excess buffer squeezed from the filter, which was then placed in a 0.6 ml eppendorf microfuge tube. A small hole was punched in the bottom of this tube, which was then placed within a 1.5 ml Eppendorf tube, and both were centrifuged for 10 min in a microfuge at 16,000 g to collect the remaining liquid from the GF/C filter. The recovered nucleic acids were precipitated by the addition of 1/10 volume 3M sodium acetate (pH 5.2) and 1 volume isopropanol. These isolated plasmid DNA inserts were collected by centrifugation, washed 2 times in 80% ethanol, dried, and resuspended in nuclease-free H<sub>2</sub>O.

Radiolabelling of these fragments was performed using the Random Primed DNA labelling kit from Boehringer-Mannheim essentially as recommended by the manufacturer. The procedure is based upon that of Feinberg and Vogelstein (27) and utilizes the hybridization of random hexadeoxynucleotides to target DNA and the subsequent synthesis of complementary strands of DNA by the Klenow fragment of

DNA polymerase 1. Radiolabelled  $\alpha^{32}\text{P}$  dCTP added to the reaction mixture is incorporated into the new DNA strand during synthesis. The reaction conditions used to label the insert fragments were as follows:

9  $\mu\text{l}$  DNA fragment (boiled for 10 min and quenched on ice)

3  $\mu\text{l}$  dNTP mixture (containing 0.5mM each dATP, dGTP, dTTP)

2  $\mu\text{l}$  reaction mixture (contains random primers in 10X reaction buffer:

900 mM HEPES pH 6.6; 100 mM  $\text{MgCl}_2$ ; 20 mM DTT)

5  $\mu\text{l}$   $\alpha^{32}\text{P}$  dCTP (800 or 3000 Ci/mM)

1  $\mu\text{l}$  Klenow enzyme

The reaction was incubated at 37°C for one hr and stopped by the addition of 2  $\mu\text{l}$  0.2M EDTA (pH 8.0). Incorporated radioactivity was measured to provide an estimate of the synthesis of complementary strands of DNA. One microliter of the reaction mixture was removed after the addition of EDTA and added to 40  $\mu\text{l}$  calf thymus DNA at a concentration of 1 mg/ml in  $\text{H}_2\text{O}$ . Two 10  $\mu\text{l}$  aliquots were spotted onto GF/C filters which were air dried without washing. The two remaining 10  $\mu\text{l}$  aliquots were each added to 0.5 ml ice-cold 5% trichloroacetic acid (TCA) and kept on ice for 15 min. These were each spotted onto GF/C filters and washed 5 times with 5 ml 5% TCA, one time with 95% ethanol, and air dried. Radioactivity was measured by Cherenkov counting of dried filters in glass scintillation vials. The estimate of percentage incorporation of label into DNA synthesized during the reaction was calculated based upon the formula

$$\frac{\text{CPM washed}}{\text{CPM unwashed}} = \% \text{ incorporation}$$

Typical results were 30-50% incorporation of label. The radiolabelled probes were used directly without removal of unincorporated nucleotides.

## Dot Blots and Hybridizations

Specificity of hybridization of both clones A1 and A4 were checked by preparing random primed radiolabelled probes as described above, and using them to probe dot blots of dsRNA isolated from symptomatic *D. viscosa*. Double-stranded RNA was prepared by CF-11 chromatography as described above and denatured as follows:

10  $\mu$ l dsRNA in H<sub>2</sub>O

20  $\mu$ l deionized formamide

7  $\mu$ l 37% formaldehyde

2  $\mu$ l 20X SSPE (3M NaCl; 0.2M NaH<sub>2</sub>PO<sub>4</sub>; 0.02M EDTA; pH 7.4)

This sample was heated to 68°C for 15 min and quenched on ice. Two volumes of 20X SSPE were then added and the samples were applied to a nylon membrane through a dot-blot apparatus (Millipore, Inc.). Undigested plasmid DNA from Bluescript II KS (+) and clones A1 and clone A4, were prepared from log cultures of *E. coli* containing these plasmids as described above and denatured by adding 1/10 volume 3M NaOH and heating to 68°C followed by quench cooling on ice. These samples were then diluted in 20X SSPE and applied to the membrane. Each well of the apparatus was washed twice with 400  $\mu$ l 20X SSPE after sample application. The membrane was removed and nucleic acids were immobilized by baking the membrane for 30-60 min at 80°C under vacuum.

Dot blots were probed with either radiolabelled A1 or A4 fragments prepared as described above. The hybridization conditions and subsequent washes were as follows:

Prehybridization solutions:

50% deionized formamide

5X SSPE

5X Denhardt's solution (1.0 Ficoll Type 400; 1.0 g polyvinylpyrrolidone;

1.0 g BSA fraction V; 1000 ml H<sub>2</sub>O.

100 µg/ml denatured sheared salmon sperm DNA

0.2% sodium lauryl sulfate

0.5 ml/ cm<sup>2</sup> of this solution was used to prehybridize each membrane at 42°C for 2-6 hr in sealed plastic bags. Hybridization conditions were identical except for the addition of denatured radiolabelled probes to the above solution. The concentration of probes was between  $2 \times 10^5$  and  $2 \times 10^6$  cpm/ml prepared as described above. Before use, probes were denatured by boiling for 10 min and cooling on ice. Hybridizations were carried out at 42°C for 12-18 hr. Filters were washed according to the following schedule:

2X SSPE + 0.5% SDS	5 min @ RT
2X SSPE + 0.1% SDS	15 min @ RT
0.1X SSPE + 0.5% SDS	45 min @ 37°C
0.1X SSPE + 0.2% SDS	45 min @ 60°C
0.1X SSPE	2 min @ RT

Membranes were then wrapped in plastic and exposed to Kodak X-OMAT AR film at -80°C with intensifying screen. Films were developed using standard procedures.

### **Probing of Genomic DNA from *D. viscosa***

To be assured that the dsRNA found in symptomatic *D. viscosa* plants is not a product of the genome of the host, total genomic DNA was isolated from a non-symptomatic *D. viscosa* plant which had been grown from seed collected from greenhouse-grown plants which themselves had been grown from seed collected in the field from non-symptomatic plants. The source of DNA was therefore a third generation plant from two previous generations of non-symptomatic individuals. DNA extractions of fresh leaf material were as described by Doyle and Doyle (21).

One gram fresh weight plant material ground in liquid nitrogen was added to 12 ml extraction buffer at 60°C. Extraction buffer contained:



2.5% (w/v) hexadecyltrimethyl-ammonium bromide (CTAB)

1.4M NaCl

100 mM Tris (pH 8.0)

20 mM EDTA (pH 8.0)

1% (w/v) polyvinylpyrrolidone-40

0.2% (v/v) 2-mercaptoethanol

Samples were incubated at 60°C for 30 min with occasional mixing, then cooled to room temperature before extracting with 2/3 vol sevag (CHCl<sub>3</sub>:isoamyl alcohol @ 24:1) by mixing for 10-15 min at room temperature followed by storage on ice for 15 min. The emulsion was broken by centrifugation at 3000 g for 30 min at room temperature and the nucleic acids were precipitated from the aqueous phase by the addition of 2/3 volume isopropanol and storage at -20°C for 12-16 hr. Precipitated nucleic acids were collected by centrifugation, resulting pellets were washed twice in 80% ethanol, dried, and dissolved in 1 ml TE buffer (pH 8.0) which had been treated with DEPC to remove RNase activity. Spectra were recorded for these nucleic acid preparations and agarose gel electrophoresis was performed to estimate approximate concentrations of nucleic acids. Absorbance at 260 nm and at 280 nm were compared and ratios computed as a criterion of purity. Nucleic acids extracted by this procedure commonly gave A<sub>260</sub>/A<sub>280</sub> ratios of 1.8-2.1. These isolated nucleic acids were then digested with RNase A (DNase-free) and two restriction endonucleases to generate fragments that could be probed with clone A4 following agarose gel electrophoresis and Southern blotting on nylon membranes.

Nucleic acids (150 µl) from healthy *Dodonaea* (approximate concentration 200 µg/ml nucleic acids) was diluted to 600 µl with H<sub>2</sub>O and sheared slightly by repeated passage through a #28 gauge needle. To each of two tubes containing this solution, 10 µl RNase A (DNase-free) at 5 µg/µl in H<sub>2</sub>O was added, and the solution

was incubated at 37°C for one hr. Samples were then extracted with water saturated phenol and sevag (as above) and nucleic acids were precipitated with 1/10 vol 3M sodium acetate and 1 volume isopropanol at -20°C. Resulting pellets were collected by centrifugation and washed once in 80% ethanol before being dried. Pellets were pooled and dissolved in 50 µl H<sub>2</sub>O. DNA was then double digested with restriction endonucleases *Rsa* I and *Cfo* I as follows:

10 µl DNA

2 µl *Rsa* I 10X buffer (100mM Tris; 100mM MgCl<sub>2</sub>; 10mM DTE; pH 7.5)

2 µl *Rsa* I (=40 units)

4 µl *Cfo* I (=40 units)

2 µl BSA (@1mg/ml)

Reactions were incubated at 37°C for one hr. Five µl of loading buffer was added to each reaction, samples were heated to 60°C for 2 min, cooled on ice and applied to 1.0% GTG agarose gel in 0.5X TBE buffer + 0.5 µg/ml ethidium bromide and run at 5 volts/cm. Molecular weight standards and the 1 kbp insert fragment from clone A4 were also run on the gel. Following electrophoresis, the gel was denatured in 0.5M NaOH + 1M NaCl for 30 min with gentle shaking at room temperature, then neutralized by two 15 min washes in 0.5M Tris + 3M NaOH (pH 7.5). Southern transfer onto nitrocellulose membranes (MSI, Inc.) was accomplished in 20X SSC for 16 hr. The blot was then dried for 30 min at 80°C under vacuum and probed with the radiolabelled 1 kbp fragment from clone A4. Prehybridization and hybridization conditions were as follows:

prehybe @ 42°C for 6 hr in:

50% deionized formamide

5X SSC

5X Denhardt's

0.2% SDS

100 µg/ml denatured, sheared salmon sperm DNA

Hybridization were performed at 42°C for 16 hr in the same solution as prehybridization with the addition of  $1.3 \times 10^7$  cpm  $^{32}\text{P}$ -labelled 1 kbp fragment prepared as described above. Probe was boiled for 5 min and quench-cooled on ice before use. Washes were as described above with a final stringency at 60°C in 0.1X SSC + 0.2% SDS. Blots were exposed to Kodak X-OMAT AR film at -80°C with intensifying screen for 4-20 hr and developed as usual.

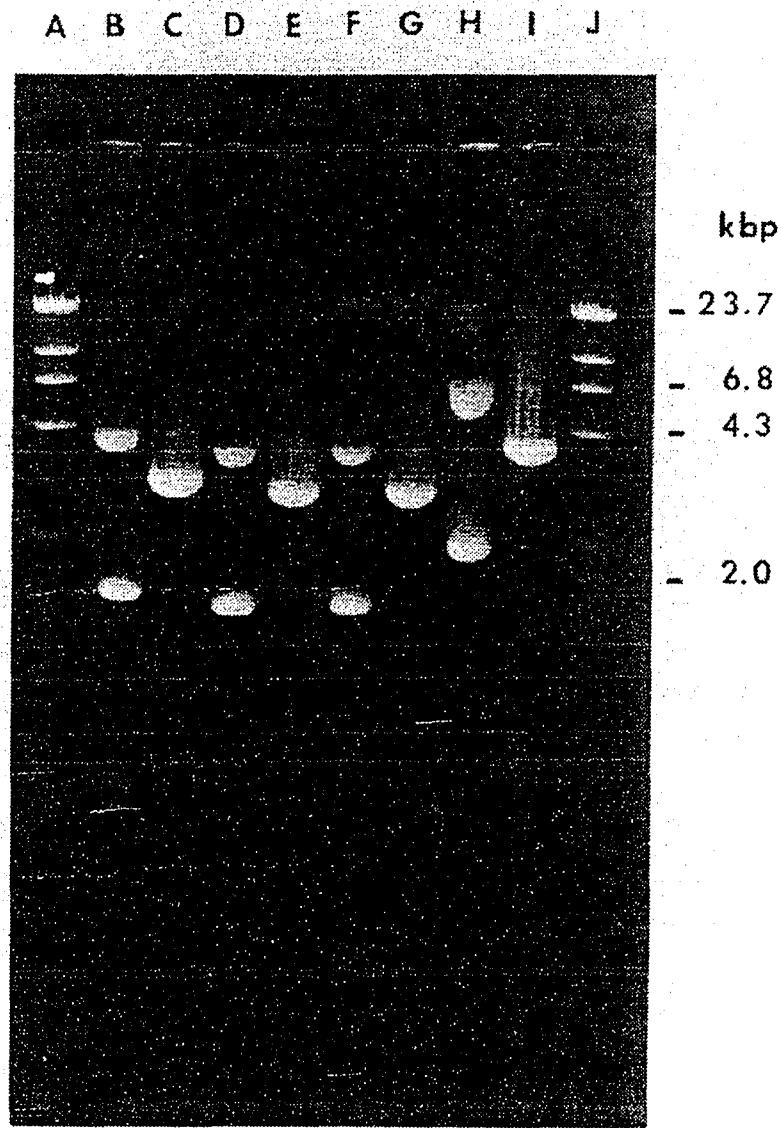
## RESULTS

### Restriction Digests of Plasmid Clones

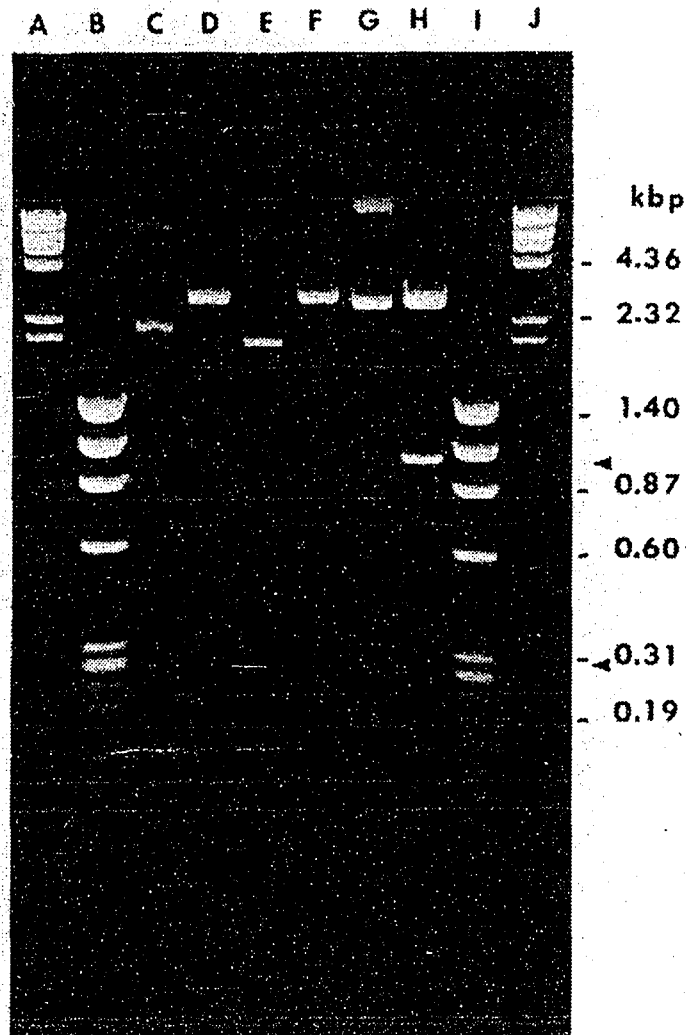
Plate VI shows the results of *Not I* digestion of clones A1 through A4. It can be seen that only 2 of the clones, A1 and A4, contained DNA inserts of reasonable size. Digestion of each of these clones with *Xba I* and *Xho I* restriction endonucleases released these inserts from Bluescript II KS (+) and allowed the estimation of their relative sizes based upon their migration characteristics in agarose gels. From this analysis, it was concluded that clone A1 contained an insert of about 300 base pairs and clone A4 contained an approximately 1 kbp insert (Plate VII and Fig. 3).

### Dot Blots and Hybridizations

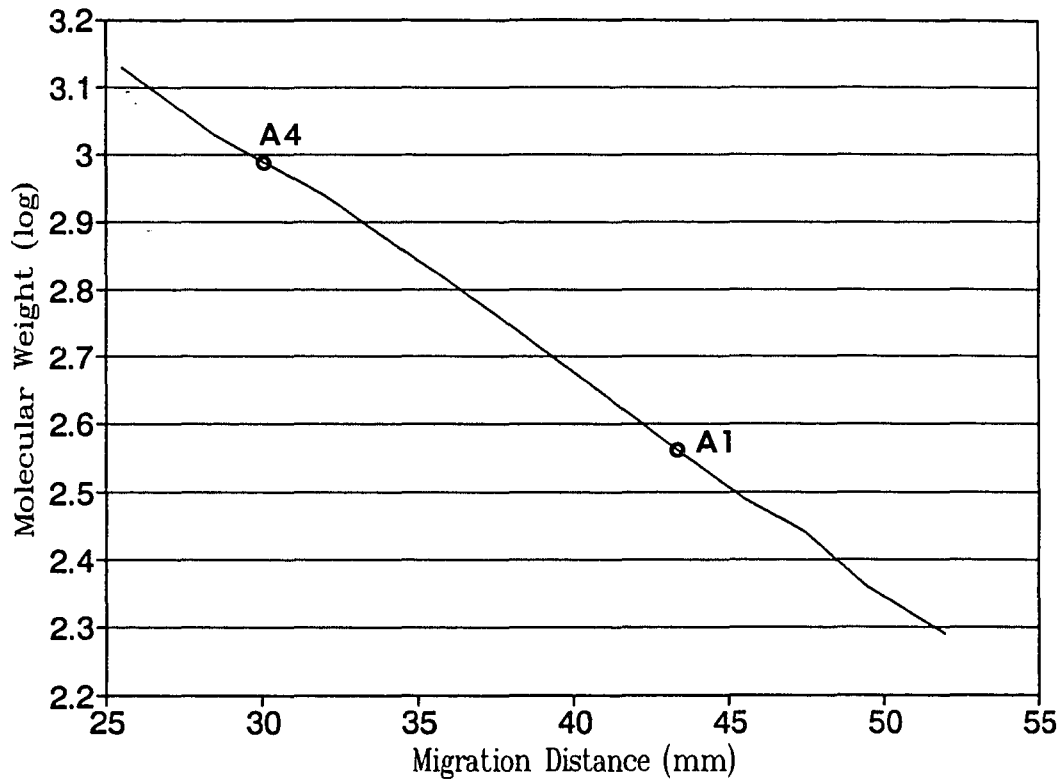
Probes prepared from either the 300 bp fragment from clone A1 or from the 1 kbp fragment from clone A4 did not hybridize to nucleic acids prepared from healthy *D. viscosa* (Plate VIII). In contrast, probe prepared from the 300 bp fragment from clone A1 hybridized to DNA from Bluescript II KS (+), and from uncut plasmids derived from clones A1 and A4, but not to dsRNA prepared from diseased *D. viscosa* (Plate VIIIA). Clone A4 hybridized to DNA from *Eco* RI cut plasmid Bluescript II



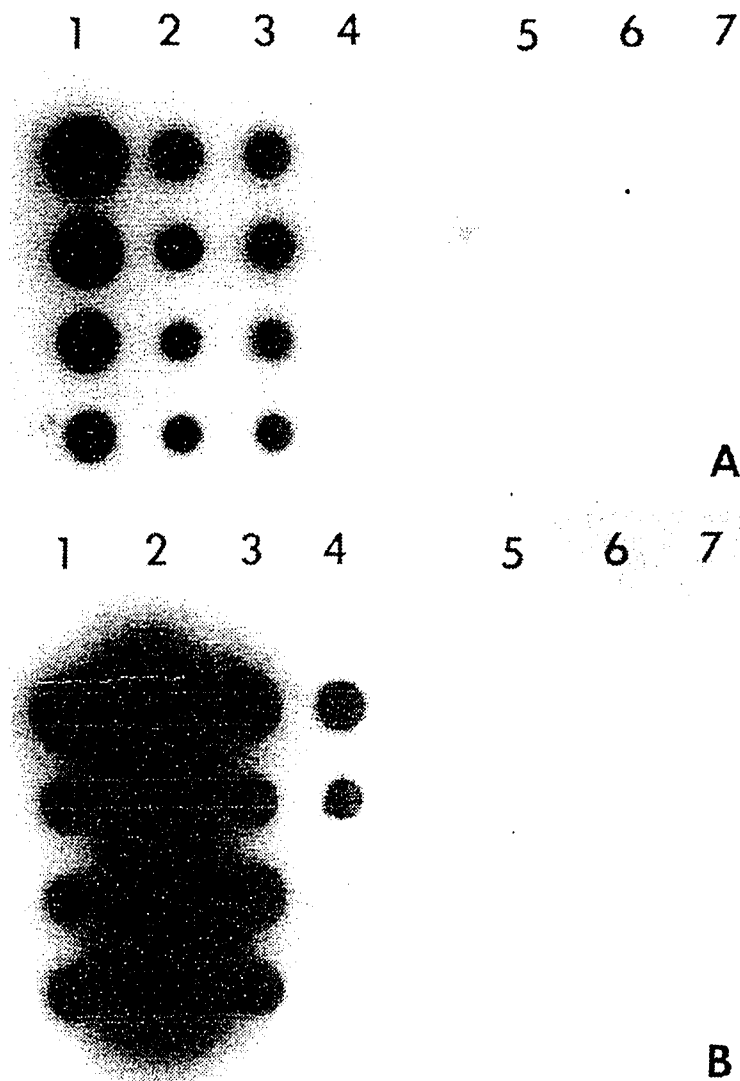
**Plate VI. Agarose gel of clones digested with *Not I* restriction endonuclease.**  
 Lanes A, J,  $\lambda$  *Hind* III digest; lane B, clone A1 undigested; lane C, clone A1 digested;  
 lane D, clone A2 undigested; lane E, clone A2 digested; lane F, clone A3 undigested;  
 lane G, clone A3 digested; lane H, clone A4 undigested; lane I, clone A4 digested.  
 Molecular weights of  $\lambda$  fragments given on the right in kilobase pairs.



**Plate VII. Agarose gel of DNAs isolated from clones A1 and A4.** Lanes A, J, *Hind* III digest of  $\lambda$  DNA; lanes B and I, *Hae* III digest of  $\phi$ X174 replicating form DNA; lane C, undigested plasmid DNA from clone A1; lane D, *Xba* I/*Xho* I double digest of clone A1 plasmid DNA; lane G, undigested plasmid DNA from clone A4; lane H, *Xba* I/*Xho* I double digest of clone A4 plasmid DNA; lane E, undigested DNA from Bluescript II KS (+); lane F, *Xba* I/*Xho* I double digest of Bluescript II KS (+) DNA. Molecular weights (kilobase pairs) of  $\phi$ X174 and  $\lambda$  standards are given on the right. Arrowheads mark the positions of inserts in clones A1 and A4.



**Figure 3. Molecular weights of inserts from clones A1 and A4.** Regression analysis of log molecular weight versus migration distance. Sizes of clones A1 and A4 are indicated.  $R^2=0.99$ .



**Plate VIII. Dot blot hybridizations of inserts from clones A1 and A4 with dsRNA from diseased *Dodonaea viscosa*.** Nucleic acids were immobilized on nylon membranes and probed with radiolabelled, double digested fragments from clone A1 (A), or clone A4 (B). Lane 1, A1 300 base pair fragment; lane 2, A4 1 kilobase pair fragment; lane 3, undigested Bluescript II KS (+) plasmid DNA; lane 4, dsRNA isolated from diseased *D. viscosa*; lanes 5, 6, and 7, total nucleic acids isolated from healthy, greenhouse grown *D. viscosa*.

KS (+), strongly to uncut plasmid DNA isolated from clone A4 and also to dsRNA derived from symptomatic *D. viscosa* (Plate VIII B).

For this reason, further work to characterize the 300 bp insert from clone A1 was not pursued, while clone A4 insert was used to answer further questions about the dsRNA found in symptomatic *D. viscosa*.

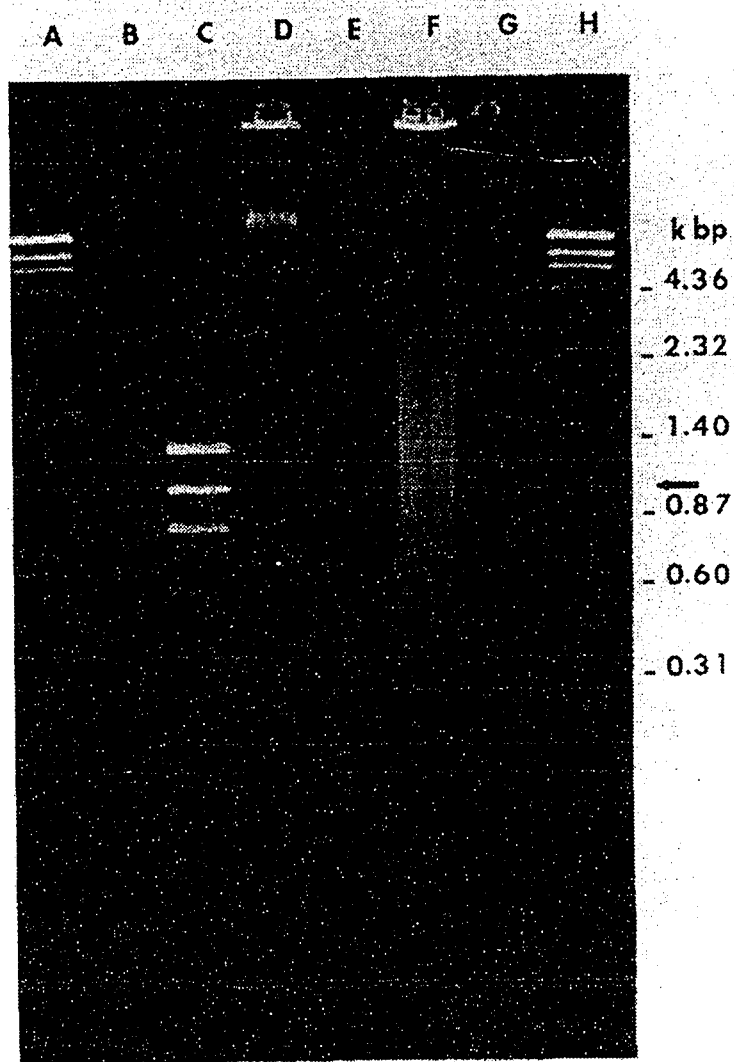
### **Probing of Genomic DNA from *D. viscosa***

The results of the probing of genomic DNA indicated no detectable hybridization of the 1 kbp fragment to genomic DNA from *D. viscosa* following double digestion of the DNA with restriction endonucleases which cut the DNA into many fragments (Plates IX, X). Such lack of hybridization cannot be attributed to poor hybridization conditions since the probe was able to detect less than 5 nanograms of the 1 kbp fragment (Plate IX, lane B; Plate X, lane B) It is therefore confirmed that the source of the dsRNA found in diseased plants is not of host origin and can only be explained by the presence of some non-host element.

## **DISCUSSION**

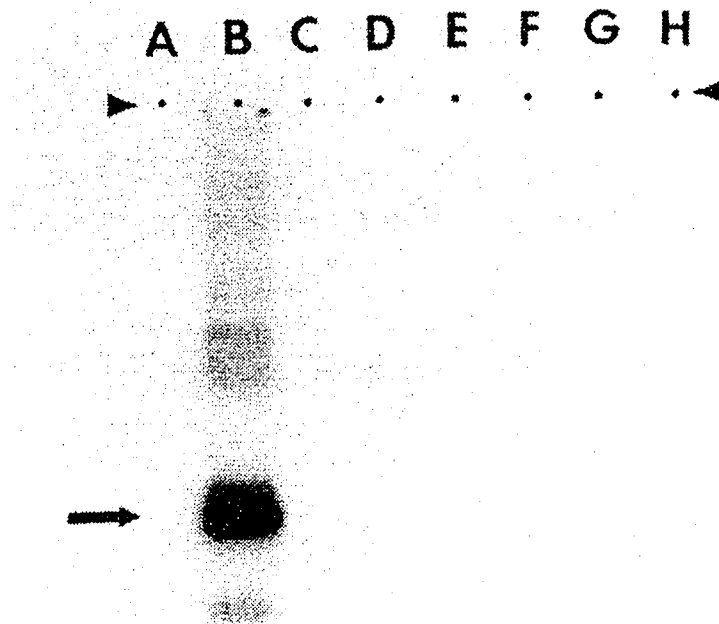
While it is expected that each probe should hybridize weakly to plasmid DNA from Bluescript II KS (+), (since there are some plasmid sequences flanking the *Sma* I insertion site that are excised by *Xho* I/*Xba* I double digestion, and of course to themselves and to a lesser degree to each other, only probe prepared from plasmid A4 was able to detect dsRNA from symptomatic *D. viscosa*. Because the source material for cDNA synthesis and the subsequent cloning procedures was derived from dsRNA that had been purified from diseased material (by CF-11 chromatography followed by agarose gel electrophoresis) that had not been treated with RNAses to remove comigrating or copurifying nucleic acids, it is possible that the 300 bp insert from





**Plate IX. Agarose gel of total genomic DNA isolated from *Dodonaea viscosa*.**

DNA from *D. viscosa* was digested with *Rsa* I and *Cfo* I. Lanes A and H, *Hind* III digest of  $\lambda$  DNA; lane B, clone A4 1 kbp insert; lane C, *Hae* III digest of  $\phi$ X 174 replicating form DNA; lane D, undigested DNA isolated from *D. viscosa*; lanes E, F, *Rsa* I/*Cfo* I double digest of genomic DNA isolated from *D. viscosa*.



**Plate X. Southern blot of total genomic DNA from *Dodonaea viscosa*.**

Radiolabelled 1 kbp fragment isolated from clone A4 was used as probe. The origin is indicated by arrowheads and dots at the top of the gel. Samples in lanes A-H as in Plate IX. Larger arrow indicates the location of the 1 kbp fragment.

clone A1 may have been cloned from fragments of messenger RNAs or from the random primers themselves.

The fragment isolated from clone A4 did not hybridize to any element from genomic DNA of *D. viscosa*, indicating that this element is of non-host origin. It is probable that this element is a plant virus. Plant viruses with (+) or (-) sense RNA genomes replicate via a dsRNA intermediate which can be diagnostic of infection (17,18,62). Many of these (+) sense RNA viruses have filamentous morphologies. It is possible, therefore, that the viruslike particles observed in partially purified extracts of diseased *Dodonaea*, are the origin of the dsRNA detected in these same plants. It is also possible, however, that the dsRNA is not a replicating form of the genome of a plant virus, but is the genome itself. Plant viruses containing dsRNA genomes are known (Reoviridae) but they all have icosahedral morphologies. There are no known filamentous plant viruses which contain a dsRNA genome (57).

## CHAPTER 4

# Association of Mycoplasma-like Organisms with *Dodonaea* Yellow's Disease

Organisms which lack an organized nucleus and do not contain cytoplasmic organelles compose the kingdom Prokaryotae which includes all the bacteria. They are mostly single-celled, so-called primitive life forms which generally are classified into four broad categories (36,56). Those which are primarily found in anaerobic, methanogenic, halophilic and other rather extreme environments are generally distinct from other prokaryotes in a number of characteristics and are grouped into the subkingdom Archaeobacteria. The respiring bacteria, which vastly outnumber the archaeobacteria, are placed into the subkingdom Eubacteria. Within this subkingdom are the Firmicutes (all those gram positive bacteria with well-developed cell walls rich in peptidoglycans but with no lipoprotein outer layer), the Gracilicutes (all gram negative bacteria which generally have less-developed cell walls), and the Tenericutes (which lack a cell wall and are therefore pleiomorphic). There are examples of plant pathogens from all three of these families (1).

The family Tenericutes has usually been divided into genera based upon host range and metabolic requirements. Those tenericutes implicated as plant pathogens have been assigned to two genera, the *Spiroplasma* and *Mycoplasma* (7,58,66). Plant pathogens from the genus *Spiroplasma* have been cultured in pure form from vascular phloem elements of diseased plants (10,12,28,70,82). However, because plant pathogenic mycoplasmas have not yet been brought into pure culture independent

from either host plant or insect vector, these organisms cannot properly be assigned to the genus *Mycoplasma*, and are therefore known as mycoplasma-like organisms (MLOs).

Mycoplasma-like organisms have been implicated in plant disease since 1967 (19). Although the reports of isolation of MLOs from plants and their culture in vitro are fragmentary and not widely accepted (11,22,48,58), the occurrence of MLOs in phloem elements of plants from many families with yellows-type or virescence declines and diseases, coupled with the remission of symptoms in some cases by tetracycline application is evidence of the association of MLOs with particular disease syndromes.

As yet, no thorough treatment of the phylogenetic relatedness of MLOs has been completed. Because they cannot yet be isolated from diseased plants in a form which allows their pure culture in artificial media, relationships between MLOs from different diseased plant species is limited to their symptom expression in naturally occurring hosts or when inoculated into alternate hosts by grafting or transmission with dodder (*Cuscuta* spp.) or insects (34,41,61). Recently, nucleic acid techniques have been developed which may prove to be the basis for a broader understanding of the phylogenic relationships of MLOs. Portions of the genome and plasmid DNA which occur in some MLOs have been cloned from various sources. These clones are able to detect a broad range of MLO DNA in host plants or to distinguish between MLOs from different hosts and areas of the world (6,14,41,42,43,45,46,49,50, 67,69,73).

Diseases believed to be caused by MLOs (those mycoplasmas detected in vascular elements of higher plants) have been documented in nearly 100 plant families (61). Symptoms of MLO infection in plants can vary widely (2,35,54,55,58,61). Declines (either slow or rapid), and chlorosis of infected tissues, sometimes combined with phyllody in which flowering portions of afflicted plants assume a leaflike appearance

occurs. Other growth irregularities such as stunting or rosetting may also be evident. Vein-clearing can also often be associated with MLO infection. A striking growth abnormality often associated with MLO-induced diseases is the formation of so-called “witches’ brooms” on affected plants, which may be the result of imbalances in the within-plant levels of growth regulators.

Definitive evidence for the association of MLOs with diseased plants is usually the detection of MLOs in the phloem elements or sieve tubes of these plants. This is most often obtained through detailed TEM studies, or fluorescence microscopy of phloem elements stained with 4',6-diamidino-2-phenylindole (DAPI) (72,74,75). Because of their often very limited distribution within diseased plants, detection of MLOs by these techniques is problematical. Using TEM, characteristic pleiomorphic bodies have been found in phloem and associated cells of MLO-infected plants (19,37,61,77). Fluorescence microscopic evidence is based upon the presence of DAPI-staining nucleic acids of non-host (not nuclear, mitochondrial, or chloroplast) origin occurring in sieve tubes or other phloem elements of diseased plants.

Further evidence of the involvement of MLOs in disease etiology is the remission of characteristic symptoms in plants following treatment with tetracycline antibiotics (13,32,39,59,60). Such remission does not occur in tissues that are already symptomatic but on the new growth produced after treatment. Direct injection, foliar sprays, and root drenches have all been reported as effective means of antibiotic application in control of diseases associated with MLOs (59). Mycoplasma-like organisms and other prokaryotes are sensitive to this antibiotic, which interferes with the assembly of ribosomes. However, unlike other prokaryotes which have well-defined cell walls and are sensitive to antibiotics which target this structure (e.g., penicillin and streptomycin), mycoplasmas are wall-less and therefore are

insensitive to this class of antibiotics. Symptoms associated with MLO infections are not alleviated by the application of such antibiotics (60).

To address the possibility of MLO association with *Dodonea* yellows disease, four avenues of research were pursued. First, molecular probes specific for particular genomic sequences from MLOs were obtained from the laboratory of Dr. Bruce Kirkpatrick at the University of California at Davis and were used to screen healthy and diseased plants both in the field and in greenhouse situations. Second, transmission electron microscopy (TEM) and fluorescence microscopy were used to observe MLOs in phloem elements of diseased plants. Third, tetracycline antibiotics were applied to diseased plants in pot culture and symptom expression in these plants monitored. Finally, two species of *Cuscuta* were established on non-infected indicator plants and allowed to form haustorial connections with symptomatic *Dodonea* plants established in pots from field-collected specimens. Indicator plants were then observed for symptom development and pathogen presence.

## MATERIALS AND METHODS

### Oligomer Probes and Dot Blots of Nucleic Acids

The sequence of an 18 base oligomer DNA identical to a highly conserved region of the 16S ribosomal gene from the MLO associated with Western X disease of stone fruits was obtained from Dr. Bruce Kirkpatrick of the University of California at Davis. This sequence has been used to detect complementary RNAs of MLOs infecting a wide range of woody and herbaceous hosts (personal communication). A synthetic oligomer of the following sequence: 5'-HO-AAACCGCCTACGCACCCT-OH-3' was constructed at the University of Hawai'i Biotechnology Center. The oligomer was synthesized with terminal OH groups at both the 5' and 3' ends in order to facilitate

radiolabelling with  $\alpha^{32}\text{P}$ -ATP. The oligomer was diluted in sterile TE buffer (10 mM Tris; 1 mM EDTA, pH 8.0) to a concentration of 40 ng/ $\mu\text{l}$  and stored in aliquots at  $-80^\circ\text{C}$  until used. Radiolabelled probe was prepared when needed from this material by enzymatically end-labelling with  $\alpha^{32}\text{P}$ -ATP and T4 polynucleotide kinase. The reaction conditions were as follows:

- 8  $\mu\text{l}$  oligomer DNA (about 320 ng)
- 30  $\mu\text{l}$  DEPC-treated  $\text{H}_2\text{O}$
- 5  $\mu\text{l}$  10X kinase buffer (0.4M Tris; 0.1M  $\text{MgCl}_2$ ;  
50mM dithiothreitol; pH 7.6)
- 2  $\mu\text{l}$  T4 polynucleotide kinase (2 units)
- 5  $\mu\text{l}$   $\gamma^{32}\text{P}$ -ATP (800 or 3000 Ci/ml)

The reaction was incubated for 1 hr at  $37^\circ\text{C}$ , stopped by heating to  $65^\circ\text{C}$  for 10 min to deactivate the kinase and then quenched on ice. The incorporation of radiolabel into the oligomer was assayed by diluting 1  $\mu\text{l}$  of the reaction mixture into 40  $\mu\text{l}$  TE buffer and spotting 10  $\mu\text{l}$  aliquots of this dilution onto DE 81 filters. Two filters were air dried and represented a portion of the total radioactivity in the reaction. Two other filters were washed 5 times for 5 min each in 30 ml 0.5M  $\text{Na}_2\text{HPO}_4$  buffer (pH 7.0), then air dried. These filters represent the amount of radioactivity incorporated into the oligomer. Radioactivity on each filter was quantified by Cherenkov counting in a Beckman Model LS 1801 scintillation counter. Typically 60-80% incorporation of radiolabel into the oligomer occurred under these reaction conditions.

Total nucleic acids used in hybridization analysis were isolated from *D. viscosa* using the hot CTAB procedure of Doyle and Doyle (21). Briefly, plant samples were ground in liquid nitrogen and added to a 10-fold excess of extraction buffer at  $60^\circ\text{C}$ . Extraction buffer contained:



2.5% (w/v) hexadecyltrimethyl-ammonium bromide (CTAB)

1.4M NaCl

100 mM Tris (pH 8.0)

20 mM EDTA (pH 8.0)

1% (w/v) polyvinylpyrrolidone 40

0.2% (v/v) 2-mercaptoethanol

Samples were incubated at 60°C for 30 min with occasional stirring, removed from the water bath and allowed to cool to room temperature for a few min. Two-thirds volume sevag (chloroform:isoamyl alcohol, 24:1) was added to each sample, the tubes were thoroughly mixed by shaking and allowed to sit at room temperature for 15-30 min. They were then centrifuged for 30 min at 3000 g at room temperature. The aqueous phase was collected and the nucleic acids were precipitated by the addition of 2/3 volume of ice-cold isopropanol, gentle mixing and overnight storage at -20°C. Precipitated nucleic acids were recovered by centrifugation at 10,000 g for 30 min at 4°C. The resulting pellets were washed twice in 75% ethanol; 10 mM NH<sub>4</sub>-acetate (pH 7.6) dried, and resuspended in nuclease-free TE buffer (pH 8.0). The concentrations of nucleic acids were determined spectrophotometrically with a Shimadzu model UV-160 recording spectrophotometer, and in 1% (w/v) SeaKem GTG agarose gels in TBE or TAE buffers with *Hind* III cut lambda DNA molecular weight standards. Nucleic acids isolated using this procedure typically had 260/280 ratios of 1.8 to 2.1, depending upon the tissue type and plant species isolated from, and consisted of both DNA and RNA as determined by gel electrophoresis. The RNA was relatively undegraded by this extraction procedure, as evidenced by their migration characteristics in agarose gels (data not shown).

### **Dot Blot Detection of MLO Sequences**

Nucleic acids isolated with the above procedure were immobilized on nytran membranes (MSI, Inc.) as follows: 2 µg total nucleic acids in 50 µl TE buffer were denatured by the addition of 35 µl formalin and 85 µl 20x SSPE and heated at 65°C for 10 min followed by rapid cooling on ice. These samples were then diluted serially 5-fold in 10X SSPE and applied to a nytran membrane held in a dot blot manifold (Millipore, Inc.). The membrane was equilibrated in 20X SSPE before use. Each well containing samples was washed twice with 10X SSPE after which the membrane was removed and the nucleic acids were fixed to the membrane by baking in a vacuum oven at 80°C. for 1 hr. Membranes containing immobilized nucleic acids were stored at 4°C in sealed plastic bags until probed with radiolabelled oligomer.

### **Hybridizations**

Nytran membranes were probed with radiolabelled oligomer prepared as above. Blots were prehybridized at 40°C for 3 to 16 hr in plastic bags containing 0.5 ml/cm<sup>2</sup> prehybridization solution (6X SSPE; 5X Denhardt's solution; 0.2% SDS; 0.05% sodium pyrophosphate; 100 µg/ml denatured, sheared salmon sperm DNA) prefiltered through 0.22 µm polysulfone filters (Gelman, Inc.) before use. Hybridizations were done in the same plastic bags and at 40°C. Hybridization solution contained 6X SSPE; 5X Denhardt's; 0.05% sodium pyrophosphate; 0.1% SDS; 100 µg/µl yeast t-RNA; and 1-5 x 10<sup>7</sup> cpm radiolabelled oligomer. Hybridization solutions were also prefiltered before use, as above. Following an overnight incubation, the membranes were removed from the bags and washed 3 times for 15 min each at room temperature in 150 ml 6X SSPE; 0.05% sodium pyrophosphate, then 1 time in 6X SSPE; 0.05% sodium pyrophosphate for 15 min at 45°C, and finally in 2X SSPE; 0.05% sodium pyrophosphate 1 time for 20 min at 48°C. Filters were then exposed to film (Kodak X-OMAT AR) at -80° with intensifying screens.

### **DAPI Staining**

Stems from symptomatic material were examined with fluorescence microscopy using DAPI staining. Longitudinal sections of 12-20  $\mu\text{m}$  thickness were cut from woody stems about 1-2 mm diameter on a Reichert Cryostat at  $-18^{\circ}\text{C}$ . Sections were stained with DAPI at a concentration of 1  $\mu\text{g}/\text{ml}$  in 0.05M phosphate buffer (pH 7.0) for 5 min at room temperature and examined on a Zeiss epifluorescence microscope using excitation at 365 nm, bandpass filter at 395 and viewing at 420 nm. To aid in tissue distinction and localization, some sections were stained in both DAPI and resorcinol blue simultaneously. Resorcinol blue stains callose (24,52) which is a major carbohydrate component of the sieve plates of many plant species. This dual staining allowed positive identification of mature phloem elements by both their prominently staining sieve plates under incandescent light and their lack of nuclei, which stain brightly fluorescent with DAPI under ultraviolet illumination. Both diseased and healthy stems from *Dodonaea* were examined and compared using this technique, and observations were recorded on Ektachrome ISO 400 film.

### **Transmission Electron Microscopy**

Plants from pot cultures which had tested strongly positive for MLOs in root tissues were sampled for examination using transmission electron microscopy. Different fixation schedules were tried in order to achieve good fixation of root tissues. The best fixation was obtained with fixative concentrations of more than 6% glutaraldehyde. Roots of about 1 mm diameter were excised from plants and immediately placed into 6% glutaraldehyde in 0.05M sodium phosphate buffer (pH 7.8). From these roots smaller feeder roots were selected based upon their apparently healthy condition (lack of obvious necrotic areas), and cut into about 2 mm lengths under fixation, as above. Samples were then exposed to gentle vacuum for 10 to 30 min under fixative. Pressure was released and fixation was continued

overnight at room temperature. Samples were then rinsed four times in 0.1M phosphate buffer (pH 6.8) and post fixed in 2.0% osmium tetroxide in 0.1M phosphate buffer (pH 6.8) overnight at room temperature. Dehydration was in a graded ethanol series, followed by substitution with propylene oxide and infiltration with Spurr's resin. Infiltration was performed in pressure vessels under continuous rotation. Samples were embedded in fresh Spurr's at 70°C. Silver sections were cut with a DDK diamond knife on an M7 RMC ultramicrotome. Sections were stained in 2% uranyl acetate in 50% ethanol and counterstained in lead citrate. Sections were examined in a Phillips model 200 transmission electron microscope at 80 kV.

### **Tetracycline Treatments**

**Application.** Plants which tested positive with the MLO probe were selected for treatment with the antibiotic oxytetracycline (OTC). The use of this antibiotic for the suppression of symptoms associated with diseases caused by MLOs has been documented for a number of diseases (13,32,39,59,60). Most often, the compound has been injected into plants, although root drenches and foliar applications have also been reported. Direct injection of OTC solutions into stems of woody plants has reduced the symptoms associated with MLO diseases in a number of hosts, and is the preferred method of treatment for these diseases. It is based upon the premise that the compound, usually in an acidic medium, can be taken up by the active xylem elements through the driving force of transpiration at the leaf surface, and from there translocated to the photosynthesizing leaves where it is loaded into the active phloem elements along with photosynthates produced in the leaves. Once in the phloem, the antibiotic is able to exert its influence on the MLOs which reside there. Ideally, the levels of antibiotic in the phloem are below those which result in damage to the plant cells.

Oxytetracycline hydrochloride was dissolved in sterile H<sub>2</sub>O at a concentration of 10 mg/ml, and diluted to a concentration of 100, 250, and 500 µg/ml in 0.5% (w/v) unbuffered citric acid. It has been shown that an acidic medium promotes the translocation of the antibiotic in intact xylem elements (16). The diluted OTC solution was filter sterilized (0.22 µm) and used immediately for injections into plants.

Four healthy, symptomless plants of *D. viscosa* growing in pot culture were injected with OTC at each of the above concentrations by pressure infiltration. Plants selected for treatment had stem diameters of about 0.5-0.75 cm at 5 cm above the soil line. A single hole was drilled about 2-5 cm above the soil line into the stems at about 45° from the horizontal to a depth of about 3 mm with an electric drill fitted with a 1.2 mm diameter bit. A sterile #18-gauge needle was inserted into this hole with the bevel of the needle oriented vertically, thus allowing the widest possible vertical cross-sectional intersection with the vascular elements of the plant combined with a minimum obstruction of these elements. The needle was sealed to the stem by application of quick-curing epoxy cement to its exterior and the area locally around the injection site. After the epoxy had cured, a narrow cannula was inserted down the hollow shaft of the needle and 3 ml of sterile H<sub>2</sub>O was used to flush any trapped air out of the needle. A 60 cc disposable plastic syringe was then attached to the needle. This syringe had been modified by the attachment of six #8 rubber bands as a pressurizing force. The insertion of a stiff metal rod locked the position of the plunger at 10 cc. While the syringe plunger was locked at the 10 cc volume, it was filled with 10 ml of sterile OTC solution and tightly connected to the needle inserted into the plant. The weight of the assembly was supported by string attached to the barrel of the syringe and tied to the plant above the point of injection. The metal rod was then removed and the pressure exerted by the rubber bands forced the OTC solution into the stem of the plant. Typically, 8-10 ml of solution could be injected in 12 hr by this method.

Based upon the results of the above preliminary tests, a concentration of 100 µg/ml of OTC in 10 ml of 0.5% (w/v) citric acid was chosen to inject four symptomatic *D. viscosa* plants growing in pots. Injections of vehicle alone were done on four other symptomatic *D. viscosa* growing in pots. These symptomatic plants were selected for treatment based upon symptom expression and positive hybridization with the MLO-specific oligomer probe.

All plants were maintained in a growth chamber with 14 hr photoperiod and temperature of 24°C. Treated plants were sampled before injection and daily thereafter. Visual symptoms were monitored over a period of six months.

**Bioassay.** The uptake of OTC by plants was monitored by bioassay. The bacterium *Bacillus cereus* subsp. *mycoides* was obtained from American Type Culture Collection and has been selected for use specifically for the bioassay of tetracycline antibiotics. The technique used here is basically that of McCoy and Sinha (59). Briefly, 1 ml of *B. cereus* at 10<sup>7</sup> cfu/ml in sterile water was added to 3 ml sterile Penassay Base Agar at 45°C. Penassay Base Agar formula is as follows:

Bacto beef extract	1.5 g/l
Bacto yeast extract	3.0 g/l
Bacto peptone	6.0 g/l
Bacto agar	15.0 g/l

This entire 3 ml suspension was then poured directly onto petri plates which each contained 20 ml Penassay Base Agar.

Extracts of treated plants were prepared by randomly selecting leaves from plants injected with OTC solutions, freezing them in liquid nitrogen and grinding to a fine powder with mortar and pestle. This powder was added to 2.5 volumes (w/v) of 0.05M phosphate buffer at pH 7.0 and vortexed vigorously for one minute. Extracts were centrifuged at 4000 g for 10 min and the resulting aqueous phase removed. The

pH of clarified extracts was checked and adjusted to 7.0 if necessary with dilute sodium hydroxide solution. One-tenth ml of this extract was then spotted onto 1.2 cm diameter filter discs (S&S # 28446) and allowed to air dry for about 1 hr in the dark. These discs were then placed on the seeded lawn of *B. cereus* on PBA. Standards of OTC in 0.05 M phosphate buffer (pH 7.0) were prepared and included for each bioassay performed.

Plates with discs were incubated at 25°C for 8-12 hr and zones of inhibition around each disc were measured. Estimates of OTC concentration in unknowns were based upon comparisons to standard regressions and were calculated based upon  $\mu\text{g}$  OTC/g fresh weight tissue.

### ***Cuscuta* Transmission**

Two species of dodder were obtained and used in experiments designed to test the transmissibility of the agents associated with *Dodonaea* yellows disease. *Cuscuta campestris* Yuncker and *Cuscuta sandwichiana* Choisy were identified based upon the characteristics described in Wagner et al. (78). Periwinkle (*Catharanthus roseus* (L.) G. Don.) grown from seed was chosen as the alternate host plant because it has been used often as an indicator species for MLO infections. *Dodonaea* used in the transmission tests as primary host were selected from those plants in pots which previously had shown strong hybridizations with the MLO probe.

*Cuscuta campestris* was obtained from field-collected material parasitizing *Wedelia trilobata* (L.) Hitch. near the U.H. Manoa campus. It was established on *Catharanthus roseus* grown from seed and allowed to flower and set seed on that host. Seeds of *C. campestris* were then obtained from these plants and were germinated and allowed to form attachments to new *C. roseus* plants.

*Cuscuta sandwichiana* was collected from Makapu'u point on Oahu where it was found parasitizing beach naupaka (*Scaveola sericea* Vahl.) and I'ilima (*Sida falax*

Walp.). It was established on periwinkle, as above, and allowed to mature. Seeds were collected and germinated. However, attachment to and parasitization of periwinkle by *C. sandwichiana* seedlings was never successfully accomplished, and subsequent transmission experiments were conducted using vegetatively propagated material.

Both species of dodder were maintained on *C. roseus* in a growth chamber with 14 hr photoperiod and 23-25°C temperatures. Tendrils from either species were allowed to attach to symptomatic *D. viscosa* in pots from field collected plants. The connections between dodder and periwinkle were broken and the dodder was allowed to establish haustorial connections with symptomatic *Dodonaea*. These plants were maintained in the growth chamber where eventually new periwinkle plants were allowed to form haustorial connections. The three plant association was kept in the growth chamber for about 4 weeks after which the periwinkle, along with attached dodder, was removed from the *Dodonaea*. The dodder was left attached to the periwinkle for an additional 3 weeks and then carefully and completely removed. Periwinkles were then allowed to mature under the same environmental conditions as for the transmission experiments, and were monitored for symptom expression.

## RESULTS

### Detection of Oligomer Sequences

The probe was able to detect MLO sequences in less than 70 ng of total nucleic acids isolated from aster plants infected with aster yellows MLOs. MLO probes were able to detect MLO sequences from material collected in the field from different locations in Hawai'i. Of the 15 samples from the HVNP area of the Big Island, 12 samples tested positive for MLOs. Interestingly, of the 13 samples from non-symptomatic plants, 5 were positive for MLO infection. From material collected on



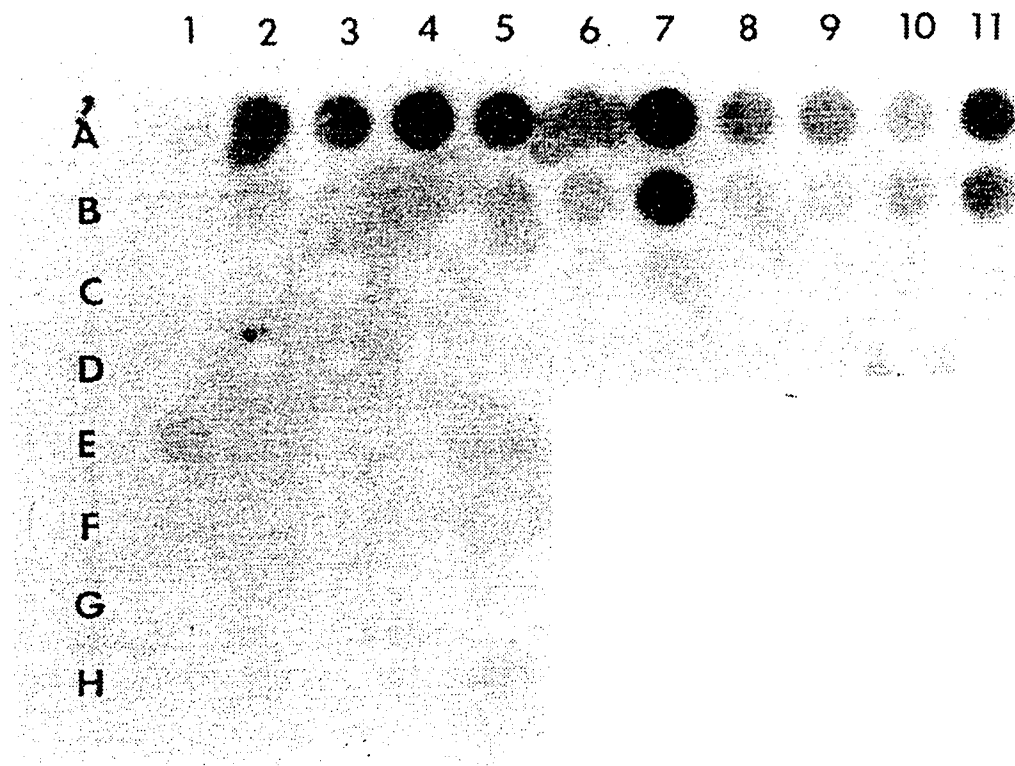
Maui, of the 5 samples from diseased plants, 4 tested positive. From the 3 symptomless plants, 1 was positive. From the island of Kauai, 6 of 7 samples tested from diseased material were positive, while 1 of 4 from non-symptomatic plants were positive (Plate XI). Overall from field-sampled material, about 80% of the symptomatic plants tested positive with the MLO probe, while only about 30% of the non-symptomatic plants tested positive. Importantly, screening of roots from greenhouse-grown, second generation *D. viscosa* which were non-symptomatic were all negative with the probe (Plate XII).

For samples from symptomatic plants in pots, the probe was unable to detect MLO ribosomal sequences in above-ground parts of plants. However, when root material from these plants was probed, 12 of 18 plants tested reacted positively to the probe (Plate XII). Negative controls again failed to bind the MLO-specific probe.

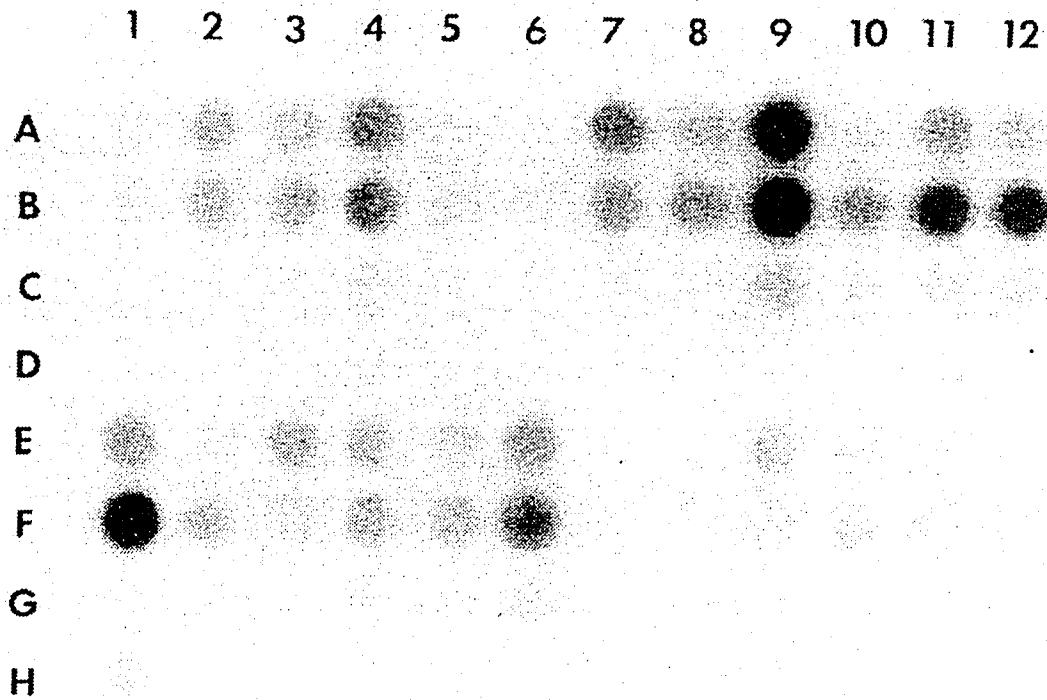
It appears then that this probe is capable of detecting MLO infections in field-collected and greenhouse-grown, symptomatic *D. viscosa*. Also of importance is the apparent detection of MLOs in some apparently non-symptomatic *D. viscosa* growing in field situations in proximity to symptomatic *D. viscosa*, indicating either that certain individuals may be tolerant to infection in field situations or that the appearance of symptoms may be delayed following infection. Positive results were never obtained on material collected from seed-grown *D. viscosa*.

### **DAPI Staining**

Phloem tissues in roots and stems of *D. viscosa* with yellows symptoms could be distinguished from other tissue types by their general shape, presence of characteristic structures including sieve plates, and by their general location within the overall structure of the roots and stems. When stained with resorcinol blue and viewed under visible light, sieve plate areas of mature phloem elements could be readily discerned in both root and stem tissues (Plate XIII A). In stem tissues, mature phloem elements are



**Plate XI. Dot blot of total nucleic acids isolated from field-grown *Dodonaea viscosa*.** Five-fold dilution series were applied vertically in columns 1-11 of rows ABCD and EFGH. Radiolabelled MLO-specific 18 bp oligomer was used as probe. Lanes 1-7 (ABCD), symptomatic *D. viscosa* from Kauai; lanes 8-11 (ABCD), non-symptomatic *D. viscosa* from Kauai; lanes 1-5 (EFGH), non-symptomatic, greenhouse-grown *D. viscosa*.

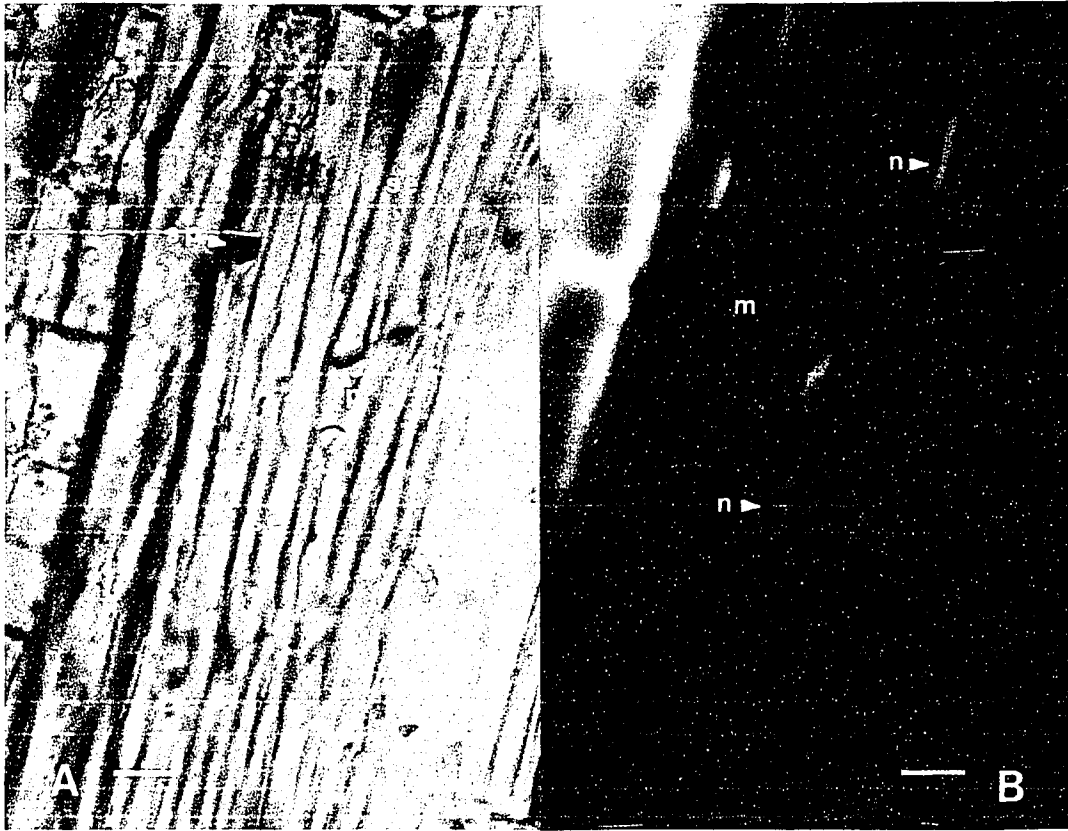


**Plate XII. Dot blots of total nucleic acids isolated from roots of pot-cultured *D. viscosa*.** Five-fold dilution series were applied vertically in columns 1-12 of rows ABCD and EFGH. Radiolabelled, MLO-specific 18 bp oligomer was used as probe. Lanes 1-12 (ABCD) and 1-6 (EFGH), symptomatic plants. Lanes 7-12 (EFGH), greenhouse-grown, non-symptomatic plants.

found surrounding the xylem tissue and within a layer of sclerophyllous tissue at the outer edge of the phloem. This organizational structure was similar to that found in woody roots of *D. viscosa*.

When viewed under ultraviolet light, mature phloem elements from healthy *D. viscosa* exhibited very little autofluorescence, in contrast to the xylem and sclerenchyma tissues which autofluoresced brightly. This characteristic of the phloem tissues was of benefit when viewing mature phloem stained with DAPI, since the lack of autofluorescence allowed easy visualization of DAPI-positive structures within these tissues. Under the conditions used here, DAPI stained nuclei in all cell types intensely and uniformly. Nuclei in phloem tissues were characteristically elongated in longitudinal sections both in stems and roots of *D. viscosa*. Nuclei were not present in all cells of the phloem, since mature sieve elements are devoid of cell contents. These cells appeared uniformly dark when stained with DAPI and examined by fluorescence microscopy. Other internal structures that fluoresce with DAPI staining are mitochondria which appear as very small points within some cells of the phloem. These structures could be easily distinguished from nuclei by their much smaller size and rather uniform distribution within cells (Plate XIII B).

Distinct from either mitochondria or nuclei in phloem tissues, other structures which stained DAPI-positive were observed in root and stem samples collected from symptomatic *D. viscosa*. These structures were variable in shape and appeared to be aggregations of many smaller, brightly fluorescing spots in otherwise empty phloem elements. These aggregations are distributed very irregularly in diseased tissue and were not detected in tissues from non-symptomatic *D. viscosa* (Plate XIVA, B). Careful work by others has shown that similar aggregations detected in other hosts are MLOs in phloem tissues (20,72), leading to the possibility that the aggregations seen in stems of symptomatic *D. viscosa* are MLOs.

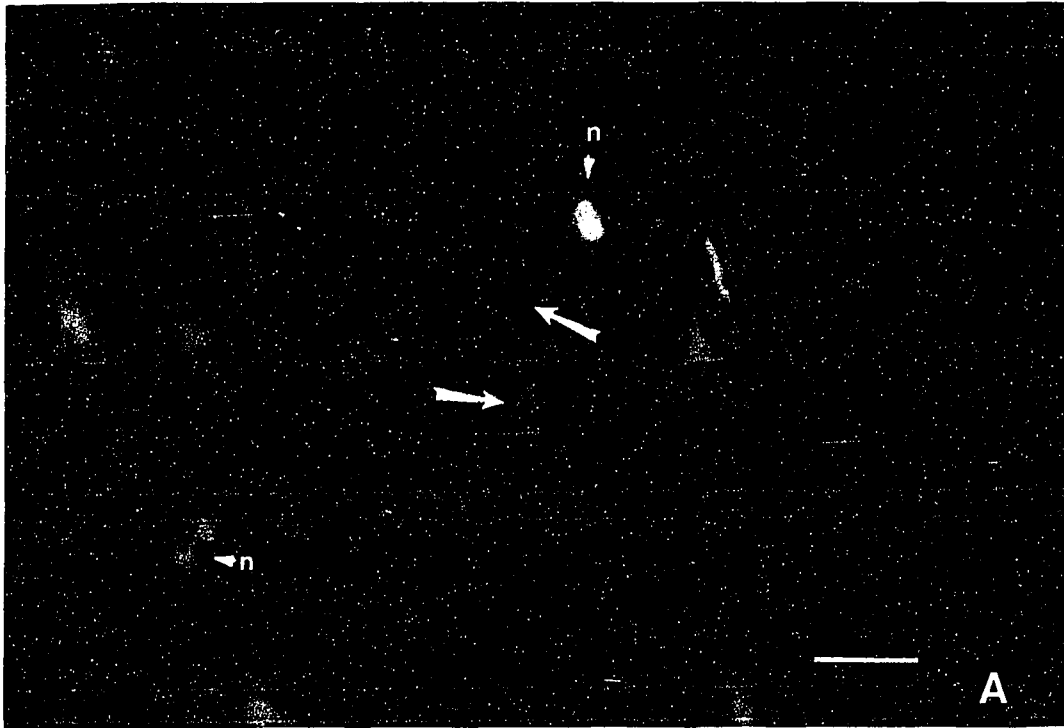


**Plate XIII. Longitudinal sections of stem from healthy *Dodonaea viscosa*.**

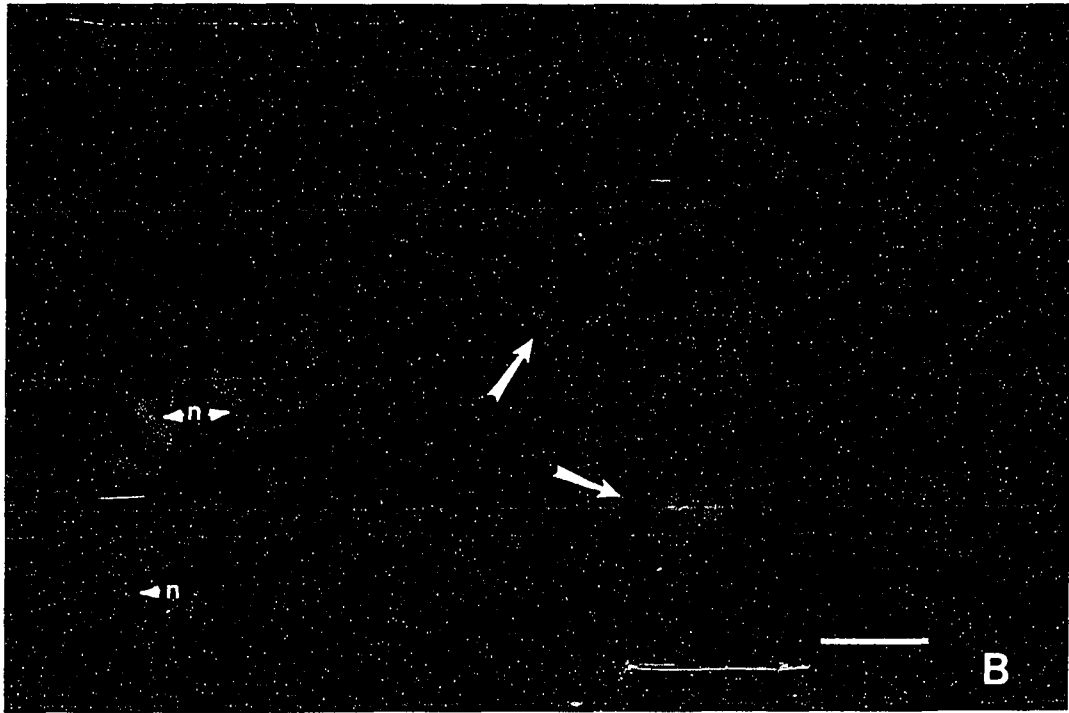
(A) Light micrograph of phloem tissues stained with resorcinol blue and viewed under visible light. Mature sieve elements (se) and blue-staining sieve plates (sp) are marked.

(B) Fluorescence micrograph of phloem tissue stained with DAPI and viewed under ultraviolet light. Nuclei (n) and what may be mitochondria (m) are indicated.

Scale bars = 5  $\mu\text{m}$ .



**Plate XIVA. DAPI-stained phloem tissues from symptomatic *Dodonaea viscosa*.** Nuclei (n) appear as brightly staining masses. The area in the center of the micrograph contains brightly stained structures (indicated by arrows) distinct in shape from nuclei. Scale bar = 5  $\mu$ m.



**Plate XIVB.** See Plate XIVA for explanation of symbols.

## **Transmission Electron Microscopy**

Roots of *D. viscosa* proved very difficult to prepare for TEM. Most frequently, the ultrastructure of the vascular elements in the fine roots were poorly fixed as evidenced by the lack of tonoplast integrity and the disruption of cytoplasmic contents. A range of fixative concentrations from 3 to 8% and various buffer compositions including cacodylate, phosphate and PIPES were tried. Osmoticum was not monitored.

The anatomy of the fine roots of this species may contribute to the problems with proper fixation. The vascular cylinder is located within a densely staining layer of cells which compose the well-developed endodermis. This structure may preclude efficient penetration of fixative into the phloem tissues.

Samples from a single specimen did, however, display relatively good fixation, as evidenced by the integrity of the tonoplast membrane and the well-defined contents of the ground cytoplasm in vascular parenchyma and phloem companion cells.

The vascular tissues of fine roots in *D. viscosa* are organized within a well-developed stele which is very electron dense. Portions of the casparian strip can be distinguished between cells of the endodermis in cross-section. Directly inside this layer of cells, a layer of highly vacuolated cells containing a well-developed cytoplasm and organelles is found. These two structures, the endodermis and the pericycle, surround the vascular elements of the fine roots which are arranged centripetally from the pericycle to the central axis of the root (Plate XV) Adjacent to the pericycle is the phloem tissues, composed of phloem parenchyma, sieve elements, and companion cells. Mature sieve elements can be distinguished by their generally oval cross-sectional profiles, well-developed cell walls (and occasional sieve plates), and their lack of cytoplasmic contents. In close association with mature sieve elements, companion cells which contain only small vacuoles and well-developed cytoplasm complete with various organelles, can also be seen (Plate XV). Development of the phloem in most



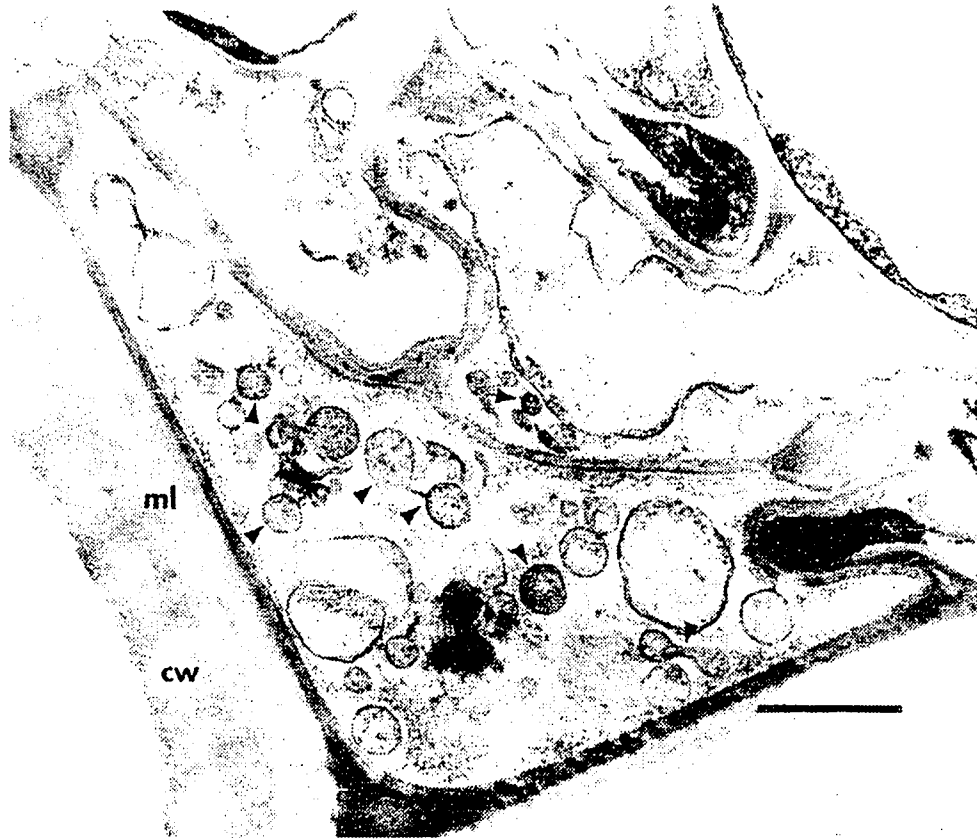


**Plate XV. Transmission electron micrograph of fine roots from symptomatic *D. viscosa*.** Sieve elements (se) and xylem elements (x) are labelled. Endodermis and Casparian strip appear at the top of the photo as darkly staining cell layer. Arrows indicate the location of necrotic tissues in which mycoplasma-like bodies are found. Scale bar = 5  $\mu$ m.

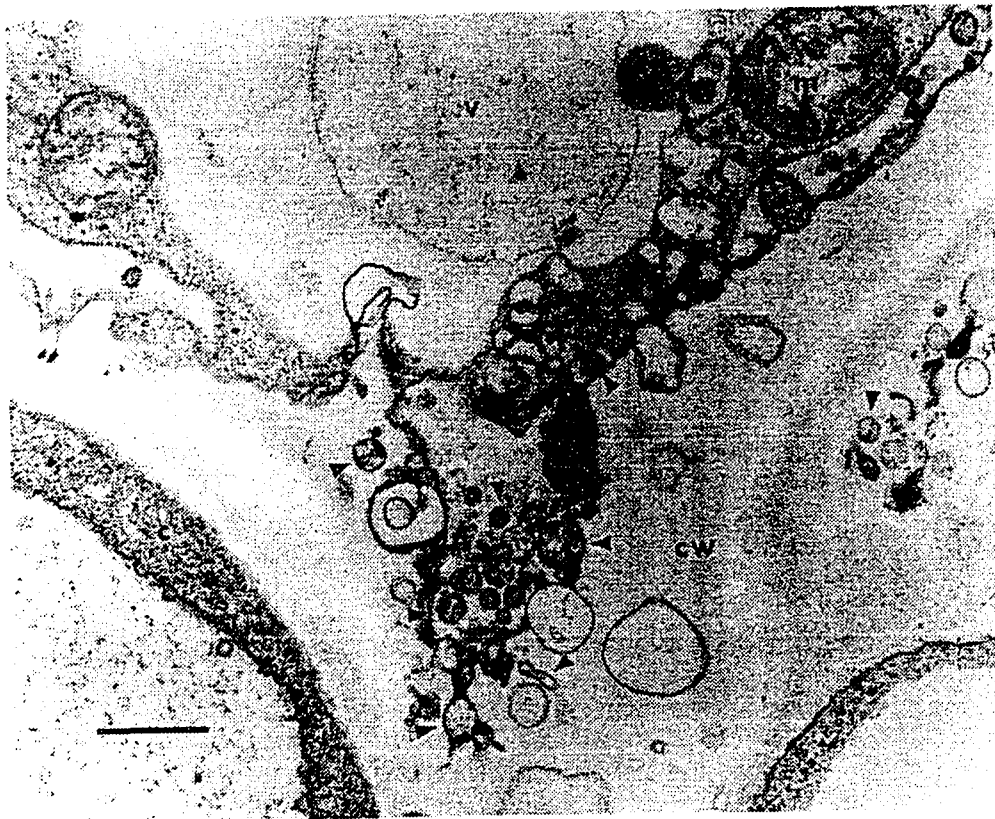
dicotyledons proceeds from the inner to the outer layers (23,25). As a result, the oldest phloem elements are to be found closer to the root axis. The central portion of young roots in *D. viscosa* is occupied by xylem tissues, which are composed of xylem elements with greatly thickened cell walls and which are devoid of cytoplasmic contents, the xylem parenchyma, and what appear to be fiber cells with very thickened cell wall and almost no interior space (Plate XV). In healthy root tissues from *D. viscosa* there is a region between the xylem and phloem tissues that contains cells with rather thin walls and cytoplasm with many well-developed organelles. This layer of tissue is probably root cambium. No necrosis of any tissue type was seen in fine roots from healthy, non-symptomatic *D. viscosa*.

In diseased roots, a layer of what appears to be necrotic tissue can often be found between the xylem and phloem tissues. The position of this necrotic tissue suggests that it may be composed of older, mature phloem. This tissue is characterized by rather thick-walled cells with disorganized cytoplasmic contents containing organelles, small vacuoles and electron-dense deposits abutting the cell walls (Plates XV, XVI). The disorganization of this tissue can not be attributed to poor fixation since adjacent cells are well-fixed (as judged by the condition of the tonoplast membrane) as are the contained sub-cellular organelles (Plates XV, XVII). Within this necrotic tissue are found pleimorphic structures bounded by a unit trilaminar membrane and containing what appear to be ribosomes and fibrillar material suggestive of nucleoprotein. Such structures can occasionally be found with appendages and constrictions which further suggest their pleiomorphism (Plates XVI, XVII, XVIII).

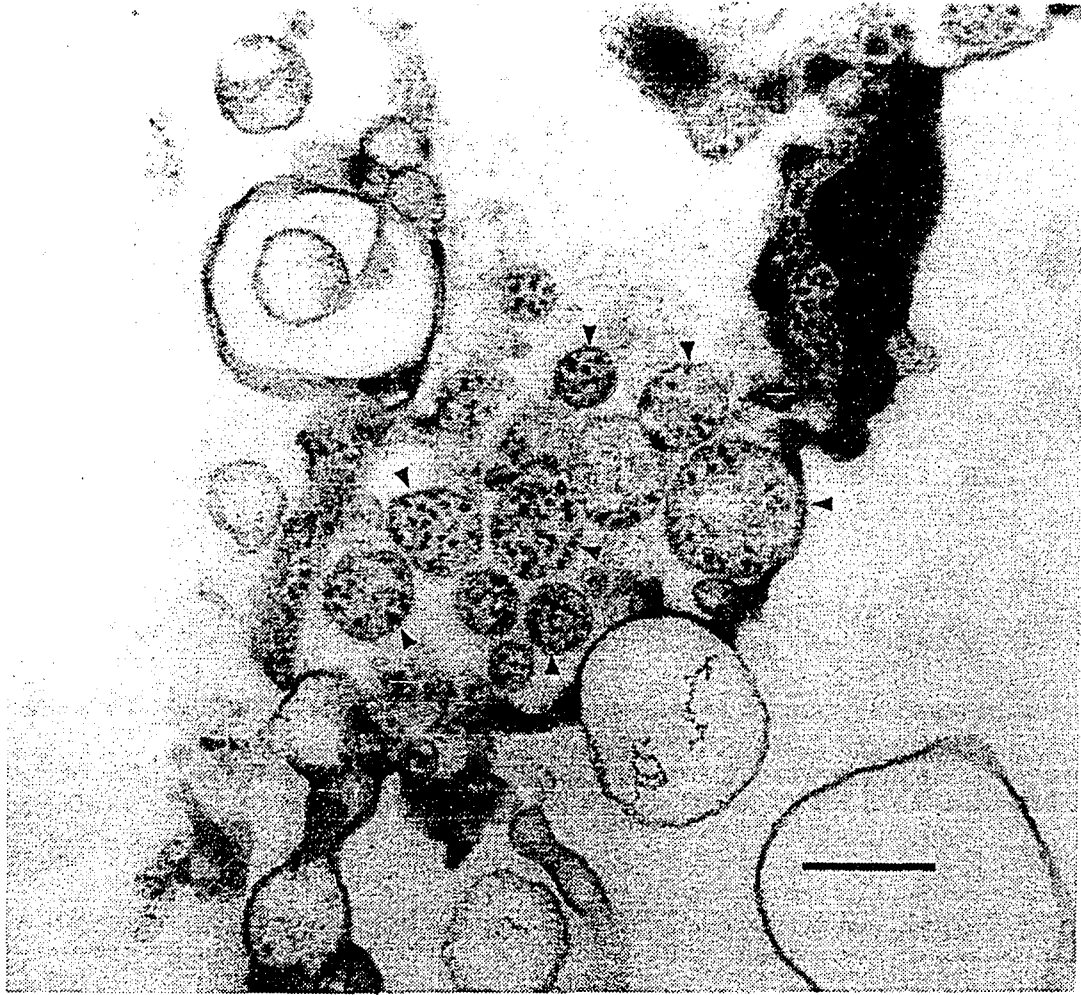
The location of these bodies within what is probably phloem tissue in diseased plants which have previously tested positive for MLOs using the oligo-probe, together with their general morphology and internal contents which are consistent with previously reported characteristics of MLOs in a variety of plants, and their absence in



**Plate XVI. Transmission electron micrograph of phloem tissue from symptomatic *D. viscosa*.** Cell wall (cw) and middle lamella (ml) are labelled. Arrows indicate mycoplasma-like bodies. Scale bar = 1  $\mu$ m.



**Plate XVII. Transmission electron micrograph of phloem tissue of symptomatic *D. viscosa*.** Cell wall (cw), well-preserved cytoplasm (c) with intact tonoplast, vesicles (v) and mitochondria (m) are indicated. MLOs are indicated with arrowheads. Scale bar = 500 nm.



**Plate XVIII. High magnification of portion of Plate XV. MLOs containing ribosomes and fine fibrillar substructure are indicated with arrowheads.**

Scale bar = 200 nm.

roots from non-symptomatic *D. viscosa*, strongly suggest that these structures are MLOs associated with disease symptoms in *D. viscosa*.

### **Tetracycline Treatments**

Bioassay using *B. cereus* subsp. *mycoides* under these conditions was linear from less than 0.1 µg to at least 30 µg OTC (Fig. 4). By incorporating standards with every assay, the uptake of OTC by *Dodonaea* could be rapidly and easily quantified.

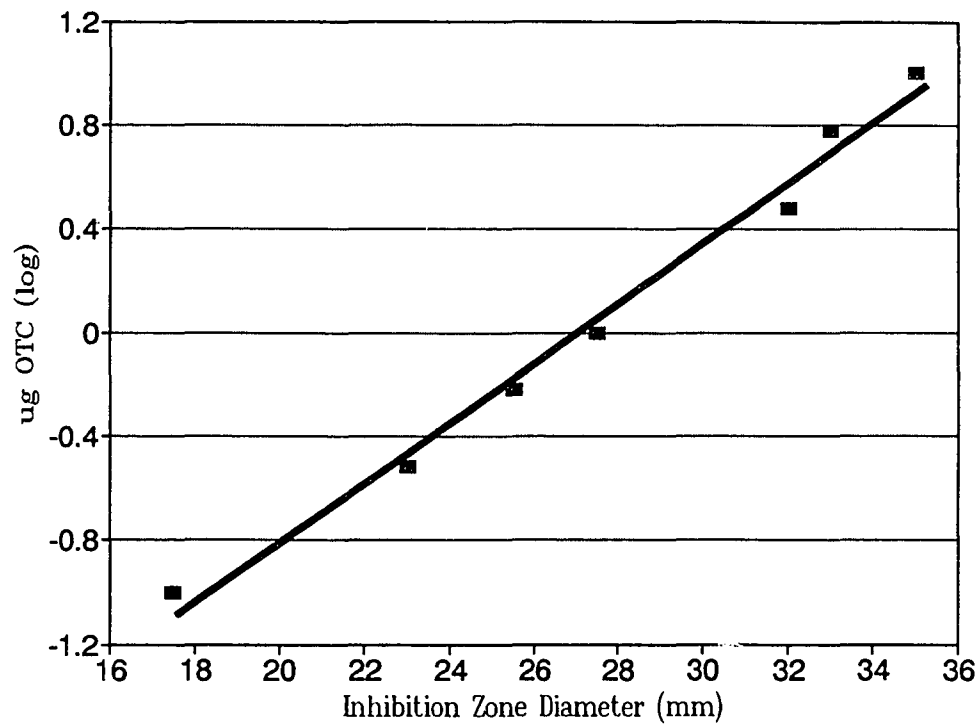
The 250 and 500 µg/ml concentrations of OTC were phytotoxic to healthy plants as evidenced by the browning of leaf and stem tissue about a week after injections, followed by the death of entire plants or portions thereof. This phytotoxic effect was not attributable to the vehicle since plants injected with it alone were unaffected. This level of OTC phytotoxicity was, of course, dependent upon plant size, with smaller plants showing generally more sensitivity.

The diseased *Dodonaea* injected with 10 ml of 100 µg/ml OTC solutions took up the compound and rapidly translocated it to their leaves (Fig. 5) However, two of the smaller plants (6E, 8F) succumbed to the applied dosages. The remaining two plants showed measurable levels of OTC in their tissues two weeks after application.

Visual monitoring of injected plants did not reveal any clear-cut remission of symptoms following uptake of OTC solutions. However, after 6 months, new growth near the areas of symptom expression appeared to be of more normal morphology than plants which received only vehicle.

### ***Cuscuta* Transmission**

None of the *C. roseus* plants displayed any alterations of morphology following transmission experiments with *C. campestris*. The growth of *C. campestris* was vigorous when the dodder parasitized periwinkle alone or simultaneously with *D. viscosa*, but this parasite was not a successful colonizer of *D. viscosa* under the



**Figure 4. Bioassay regression.** Log oxytetracycline ( $\mu\text{g}$ ) regressed against inhibition zone diameter in the *B. cereus* bioassay.  $R^2=0.98$ .

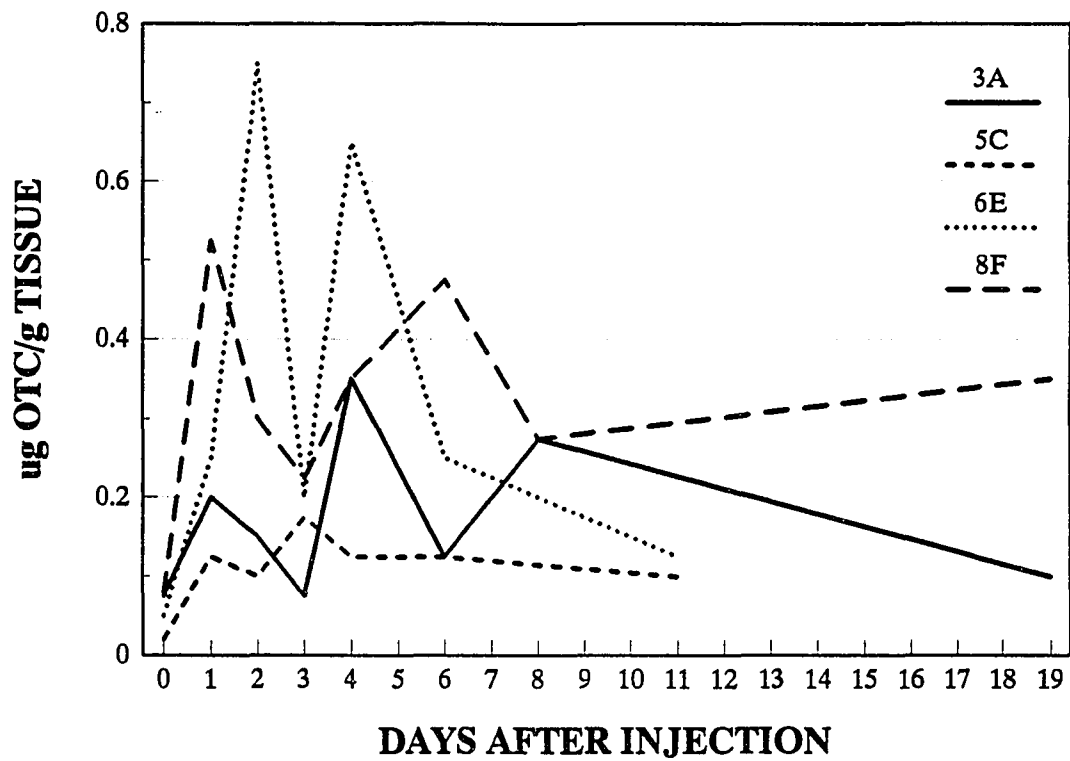


Figure 5. Bioassay for oxytetracycline in treated *D. viscosa*. Individuals are indicated as 3A, 5C, 6E, 8F.



conditions used in this study. This was evidenced by its rather poor survival on isolated *D. viscosa*. *Cuscuta sandwichensis*, on the other hand, was a very successful colonizer of *D. viscosa* alone or in conjunction with periwinkle.

Some of the periwinkle plants developed abnormal morphologies after separation from *C. sandwichensis*/*D. viscosa* association. These growth abnormalities included delayed or cessation of flower production, reduced flower size, reduced leaf area, epinastic drooping of branches, slight yellowing of leaves, and premature abscission of proximal leaves.

### DISCUSSION

Conclusions of MLO etiology for many diseases of plants is based upon a variety of observations including symptoms, the occurrence of characteristic structures in sieve elements of symptomatic plants, the detection of MLO nucleic acid sequences in symptomatic plants, and the remission of symptoms following tetracycline treatments of diseased plants (35,41,54,60,66). Nucleic acid extracts of plants showing symptoms of yellows disease both in the field and in pot culture have been shown to contain sequences of mycoplasma origin based upon the results of probing with the 18-base oligomer derived from the highly conserved 16S ribosomal RNA from Western X disease of stone fruits. This probe has been used to detect mycoplasma nucleic acid sequences from many different plants afflicted with a number of diseases of presumed MLO etiology. However, not all symptomatic plants of *D. viscosa* reacted positively to this probe. While the great majority (about 80%) of symptomatic plants from field samples tested positively, plants in pot culture displayed a less uniform distribution of MLO sequences in their tissues. Only roots of pot cultured plants tested positively with the probe, indicating that environmental conditions may play an important part in the within-plant spread of MLOs. This scenario is consistent with published work by others on the occurrence of MLOs in woody species (20,35,54,75), who found that

MLOs could readily be detected in roots of infected plants but not necessarily in above-ground parts of these plants.

Further proof of the presence of MLOs in diseased *D. viscosa* is the occurrence of characteristic structures in phloem elements of these plants. Although the DAPI-staining of shoots from diseased plants did document the presence of non-nuclear material in mature sieve elements, the distribution and occurrence of these structures was far from uniform. Many samples had to be examined before the presence of such structures could be demonstrated. Nonetheless, these structures closely resemble those detected by others using this technique (20,72,74,75).

Of greater significance is the finding of pleiomorphic structures in the phloem tissue of roots from diseased *D. viscosa* using transmission electron microscopy. Such structures can be seen to contain ribosomes and fine fibrillar material (which may be DNA) and are bound by a simple unit membrane without a cell wall. The appearance of some of these bodies give the impression of pleiomorphism by their apparently elongated shapes. Again, the characteristics of these structures in what is probably older, degenerating phloem elements strongly suggests that they are in fact MLOs present in diseased *D. viscosa*.

Taken together, the MLO probe data, the DAPI staining and the observation of pleiomorphic structures in the phloem of diseased plants confirms the presence of MLOs in symptomatic *D. viscosa* and their absence in cultivated healthy plants.

An interesting result of the field survey with the MLO probe is the apparent presence of MLOs in non-symptomatic *D. viscosa* growing in the vicinity of severely diseased individuals. A number of explanations for this result are possible. Symptoms may take a relatively long time to develop fully in plants infected with the MLO, or some plants may be immune to infection, or the disease is of complex etiology and may not simply be caused by infection with MLOs.

## CHAPTER 5

### Epidemiology of *Dodonaea* Yellows Disease

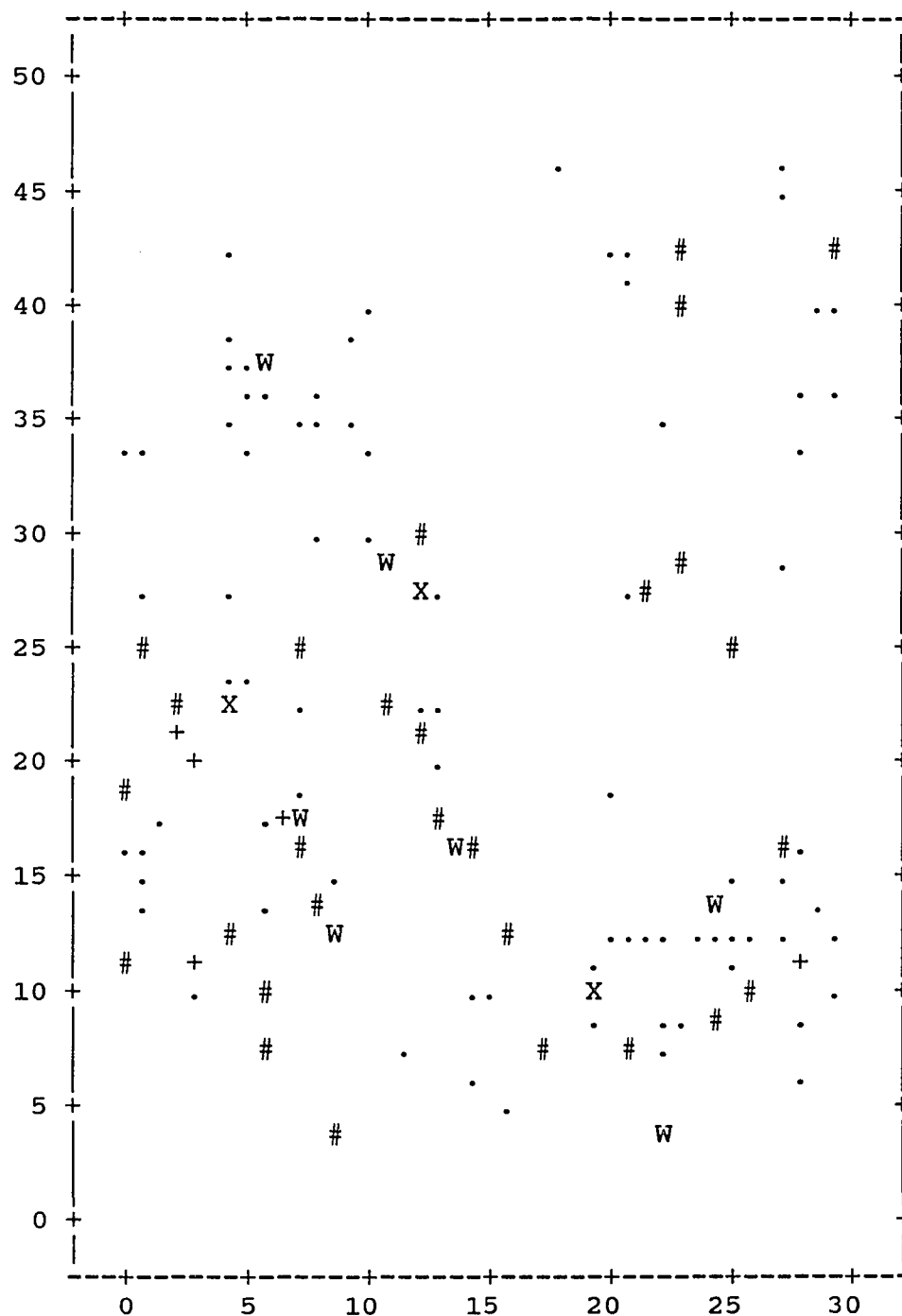
A study was undertaken to assess the incidence of disease in a native plant community dominated by *Dodonaea viscosa*. Following initial observation of the percentage of disease as determined visually, follow-up surveys were conducted after two and three year intervals to assess the dynamics of spread of *Dodonaea* yellows disease in an established population of *D. viscosa*. It was hoped that epidemiological trends could be revealed in this time frame, and that conclusions could be drawn about possible consequences of such spread.

#### MATERIALS AND METHODS

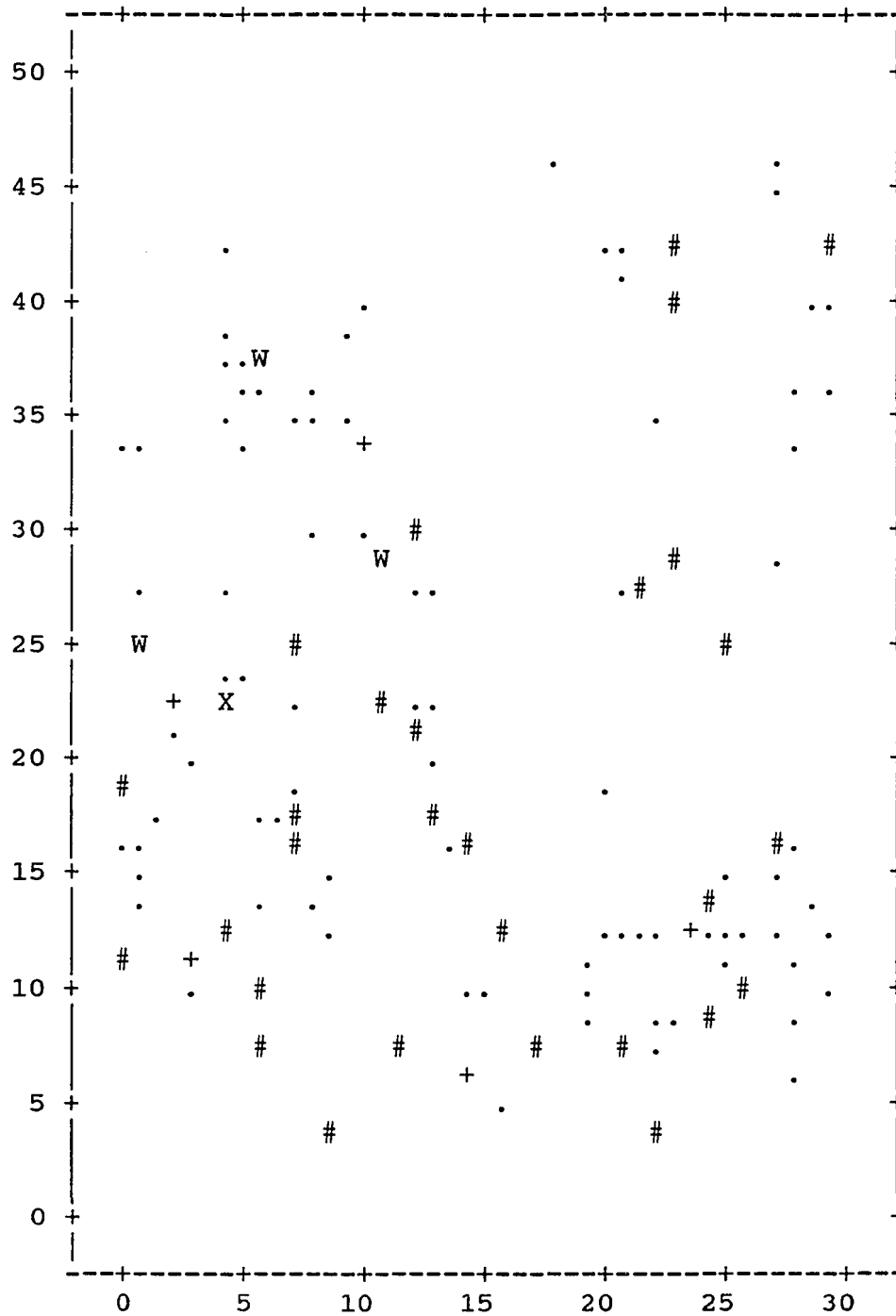
The study site was chosen near Mauna Loa strip road primarily because the site was composed of a thick stand of *D. viscosa* with many symptomatic individuals. The area was an open, southeast-facing, relatively flat shrubland at an elevation of about 1600 m. The soil was a decomposing lava that was loamy in places. Individual *D. viscosa* within this area were both shrub and tree forms 1-4 m in height. Young seedlings were also present, although they were not included in the survey. The plant community was composed primarily of *D. viscosa*, with *Styphelia tameiameia* [(Cham.) F. Muell.] and *Coprosma* spp. intermixed. Within this community, a location was chosen in which the population of *D. viscosa* included

both symptomatic and non-symptomatic individuals. *Dodonaea viscosa* was the largest species in terms of canopy cover.

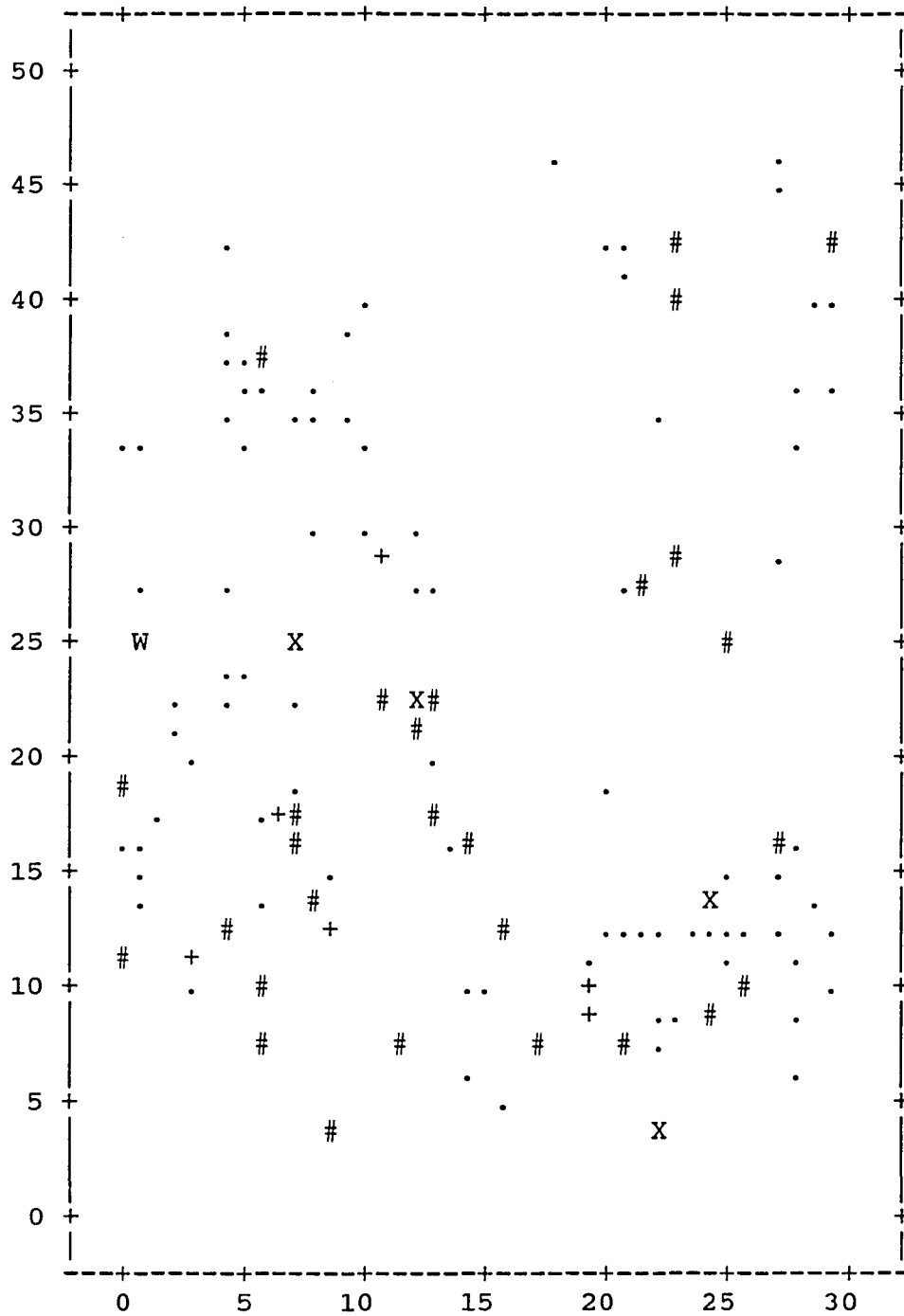
Transect lines 50 m long were laid out at 10 m intervals to describe a rectangle 50 x 30 m oriented north to south along the 50 m axis. The location of each *D. viscosa* individual was recorded as the distance along a transect line and the perpendicular distance of the plant from the nearest transect line. Each individual was numbered and labelled for future identification. Gross morphology was recorded for each plant in terms of height, width and the number of branches 1 cm or more in diameter. Disease incidence was estimated visually as the percentage of the total canopy area of each individual showing any characteristics of yellows disease, including epinastic brooms, distally reddened stems, and small, chlorotic, deformed or crinkled leaves. Although estimation of disease incidence as the proportion of the plant displaying visual symptoms is notoriously difficult (51), such an approach should at least reveal any rapid spread of disease within the study site and may reveal environmental factors which may be important in the spread of this disease at this site. Each plant in the study site was evaluated in March 1988, March 1990, and March 1991. Data were analyzed using a SAS data management system. Changes in disease incidence were calculated and compared to the percentage disease and plant distribution. Correlation between measured variables were calculated for all the plants monitored and for each year's observation (Figs. 6-11).



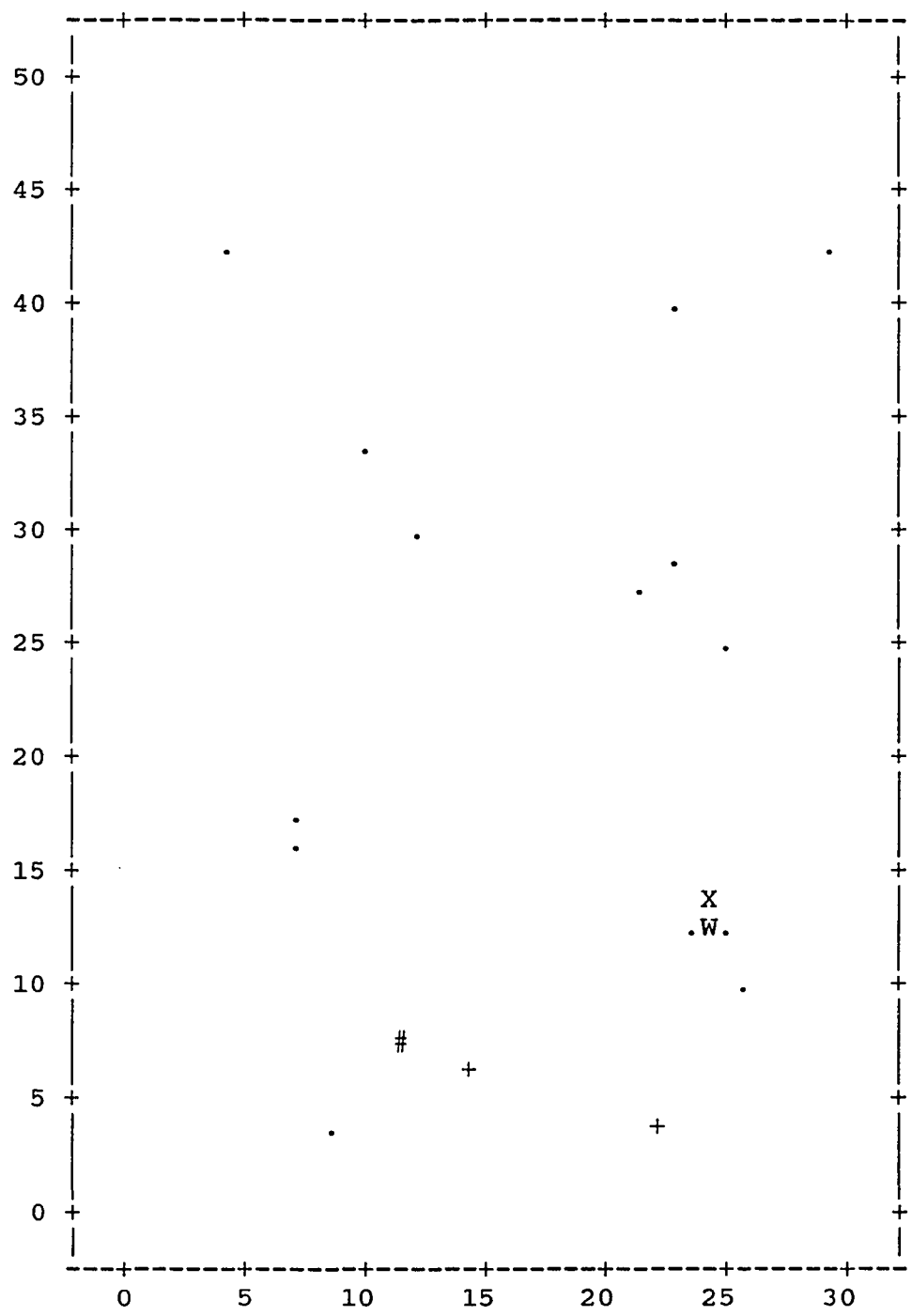
**Figure 6. Field plot, SAS analysis: 1988.** Percentage disease per plant estimated visually: . ., 0-20%; ++, 20-40%; XX, 40-60%; WW, 60-80%; ##, 80-100%. Twenty-one observations were obscured by analysis.



**Figure 7. Field plot, SAS analysis: 1990.** Percentage disease per plant estimated visually: . ., 0-20%; ++, 20-40%; XX, 40-60%; WW, 60-80%; ##, 80-100%. Twenty-one observations were obscured by analysis.



**Figure 8. Field plot, SAS analysis: 1991.** Percentage disease per plant estimated visually: · ·, 0-20%; ++, 20-40%; XX, 40-60%; WW, 60-80%; ##, 80-100%. Twenty-one observations were obscured by analysis.

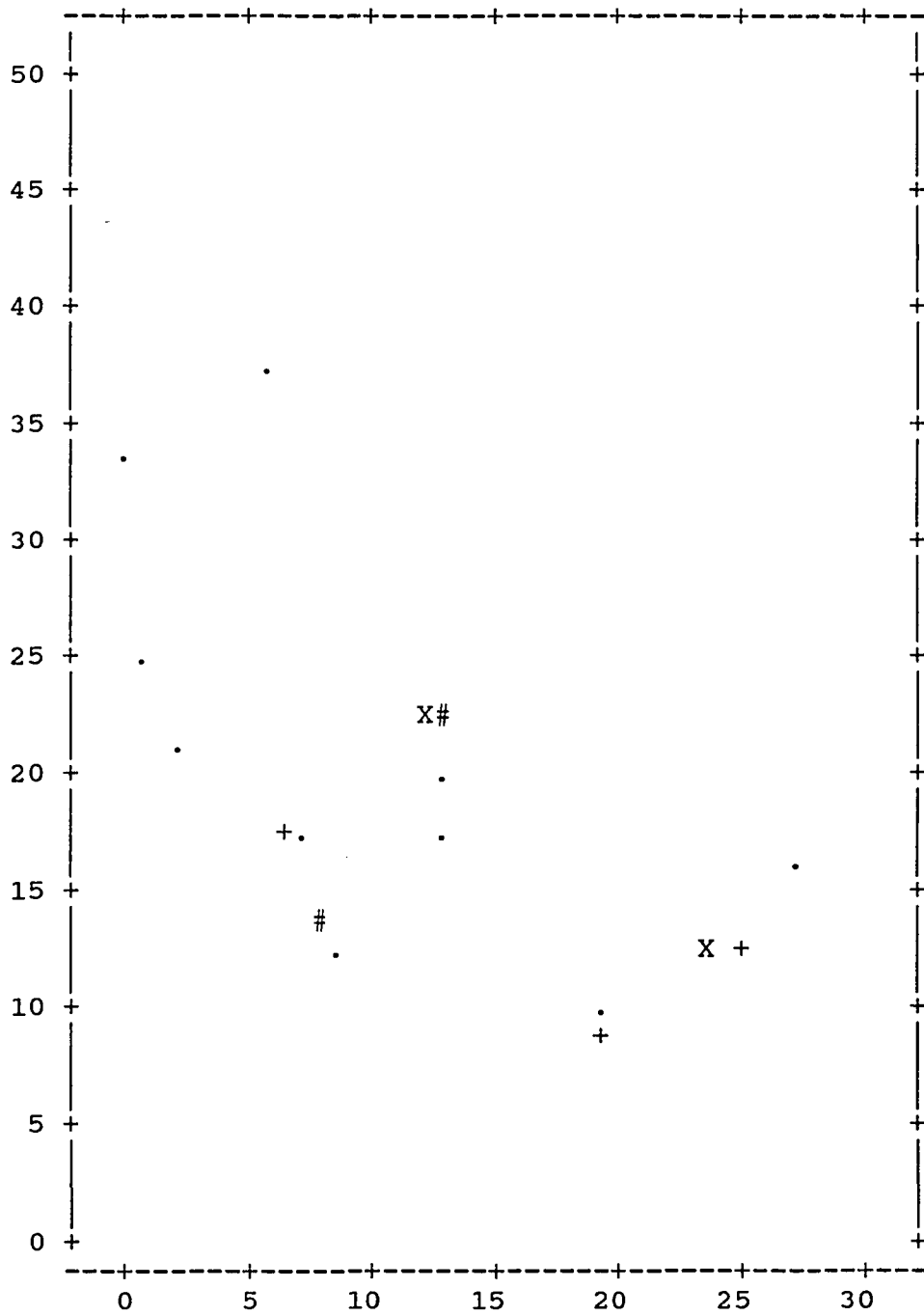


**Figure 9. Field plot, percentage disease change: 1988-1990.** Percentage change:

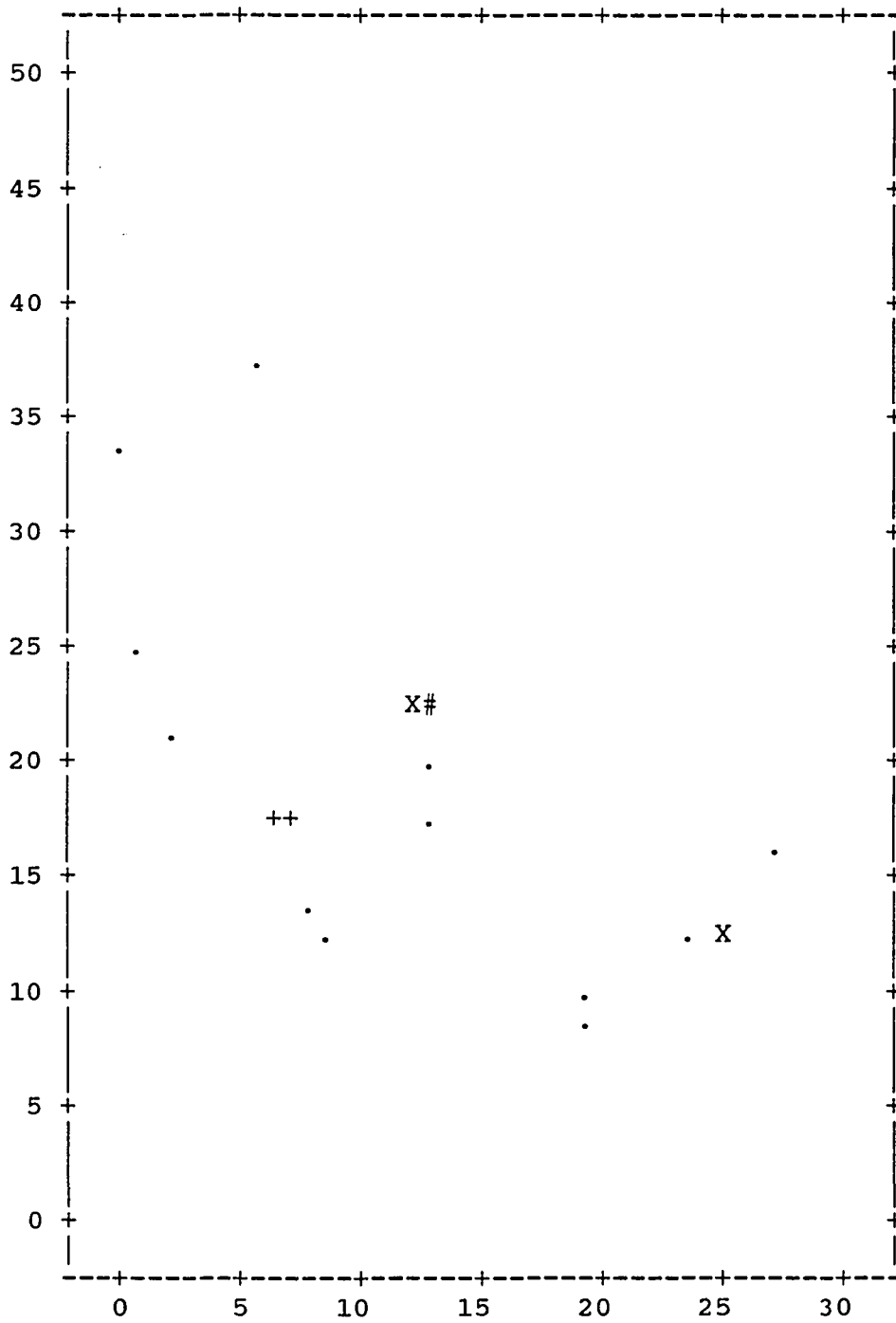
••, 0.0-17.5%; ++, 17.5-35.0%; XX, 35.0-52.5%; WW, 52.5-70.0%;

##, 70.0-87.5%.





**Figure 10. Field plot, percentage disease change: 1990-1991.** Percentage change: ••, 0.0-17.5%; ++, 17.5-35.0%; XX, 35.0-52.5%; WW, 52.5-70.0%; ##, 70.0-87.5%.



**Figure 11. Field plot, percentage disease change: 1988-1991.** Percentage change: ··, 0.0-17.5%; ++, 17.5-35.0%; XX, 35.0-52.5%; WW, 52.5-70.0%; ##, 70.0-87.5%.

## RESULTS

The results of the monitoring of this selected stand of *D. viscosa* for yellows disease indicates that at this site the spread of the disease is rather slow and cannot be correlated with any of the parameters monitored over the course of the study. Most of the spread appears to have occurred in areas where the density of the plants was the greatest.

In addition, the progress of the disease on an individual basis, that is, the spread of symptoms within afflicted individuals is also rather slow. There were only four plants which had increases in disease percentage of greater than 50% in three years, while there were 24 plants which showed slight increases in percentage disease.

## DISCUSSION

The spread of *Dodonaea* yellows disease in the field situation was not rapid at this site during the time frame of the study. It is difficult to ascribe this lack of spread to any clearly definable characteristic of either the pathogens, vectors or host. Certainly, insect families with species that have been shown to be vectors of MLO-induced diseases in other parts of the world are also present in the Hawaiian islands. Species of leafhopper, planthopper, and psyllid have been collected from symptomatic *D. viscosa* in the field. However, studies to determine their suitability as vectors of this disease have not been conducted and conclusions as to their suitability as vectors of disease cannot be drawn.

There is also the possibility that the spread of this disease in the field may be limited by the genetic background of the host. *D. viscosa* is a genetically diverse species with many different growth forms endemic to Hawai'i. Its breeding system is complex, and while the species is generally considered dioecious, individuals may

produce flowers of one sex at one time of year and flowers of the other sex at other times. Occasionally, perfect flowers will be produced on a plant that was previously male or female. Given such sexual polymorphism, the relative amounts of outcrossing and inbreeding may be difficult to assess. Consequently, the genotypic background of individuals established in the field may be complex and the relatedness of individuals may be uncertain. It is probable that the populations of *D. viscosa* in Hawai'i are extremely diverse with respect to genotype and that this could be reflected in the relative susceptibilities of individuals to *Dodonaea* yellows disease.

## CHAPTER 6

# Conclusions

The etiology of the yellows disease of *Dodonaea viscosa*, an ecologically important component of the native vegetation in the Hawaiian Islands, remains unproven. Results presented here indicate that the disease is presently spreading slowly in field populations on the island of Hawai'i. A limitation of the analysis of this spread is the estimation of the frequency and severity of this disease by strictly visual methods, which is further complicated by the great phenotypic variability of this species. This variability may reflect inherent genetic variability that could impact the spread of disease within mixed populations. The relationship of host genetic background to the development of yellows disease symptoms in *D. viscosa* merits further investigation. A significant advance in understanding the spread of this disease could be made if it could be shown that genotypes are differentially susceptible to disease in field situations. The selection of suitable enzyme polymorphisms may be feasible and may shed light on this aspect of the disease. Another approach would take advantage of the polymerase chain reaction (PCR) to determine the genetic diversity of individuals in field populations. PCR has recently been shown to be capable of distinguishing genotypic groupings in species for which enzyme polymorphisms showed no distinctions (80,81). By sampling symptomless and affected *D. viscosa* in mixed populations in field situations with either PCR or enzyme polymorphism analysis, disease susceptibility may be found to be correlated with genotype.

This dissertation demonstrates the presence of two potential pathogens which occur in diseased *D. viscosa*. First, viruslike particles which resemble type 3 closteroviruses have been detected in symptomatic *D. viscosa*. In addition, dsRNA has been isolated from these plants and shown to be absent in non-symptomatic plants in the field and in healthy plants raised from seeds collected from symptomless plants. There was variability in the amounts of dsRNA detected from diseased individuals. In samples collected from diseased plants in the field, there was a decrease in the amount of dsRNA detected in the summer compared with that detected in the cooler months before and after summer. This may be correlated with decreased replication of virus particles in diseased *D. viscosa* during the months when temperatures are generally high during daylight hours. In contrast, levels of dsRNA detected in symptomatic plants grown under controlled conditions were relatively constant throughout the year. Although the molecular weight of this dsRNA is less than that present as replication intermediates of closteroviruses, the possibility remains that it is in fact the replicating form of the genome of the viruslike particles shown to be associated with symptomatic *D. viscosa*. The possibility that this dsRNA is generated as a result of environmental stress is discounted by the lack of complementary sequences in the genome of *D. viscosa*, as demonstrated by the failure of the dsRNA probe to hybridize with genomic DNA isolated from *D. viscosa*.

Second, mycoplasma-like organisms have also been detected in diseased *D. viscosa* by a variety of techniques. Initially, a nucleic acid probe, which has been shown to be capable of detecting specific sequences from the 16S ribosomal gene of the MLO which causes Western X disease was obtained and used to test symptomatic *D. viscosa*. In screenings of field populations of *D. viscosa* from different islands it was found that approximately 80% of symptomatic individuals and nearly 30% of the symptomless individuals growing in conjunction with diseased *D. viscosa* reacted

positively with this probe. This result can be interpreted in at least three ways. Disease symptoms may develop over a long period of time, as suggested by the rather slow spread documented in field surveys. Individual susceptibility may be variable, with only certain morphs of *D. viscosa* able to develop the characteristic symptoms of Dodonaea yellows disease following infection. A third possibility is that full-blown symptoms of Dodonaea yellows disease occur only when both MLO and virus are present in the infected plants. The results presented here do not allow a distinction to be made between these possibilities.

Further documentation of the presence of MLOs in diseased *D. viscosa* was obtained through DAPI staining in conjunction with fluorescence microscopy and through transmission electron microscopy. DAPI staining revealed the presence of brightly staining amorphous structures, distinct from either nuclei or mitochondria, in mature phloem elements from stems of symptomatic *D. viscosa*. These were not present in phloem tissues of healthy plants raised under controlled conditions from seed collected from symptomless plants. These structures resemble those which have been found in plants afflicted by diseases known to be associated with MLOs. In addition, using TEM, pleiomorphic bodies bounded by a single unit membrane and lacking a cell wall were observed in collapsed metaphloem elements within the vascular cylinder of roots from diseased *D. viscosa*. These bodies are very like the MLOs known to occur in sieve elements of plants afflicted with diseases associated with MLO etiology. Their size, shape, contents, and ultrastructure, together with their location in what appear to be necrotic, metaphloem elements are similar to the characteristics of MLOs which parasitize many higher plants.

Although evidence obtained from the oxytetracycline injection experiments and the transmission attempts using *Cuscuta* species were not conclusive, this study demonstrates the feasibility of such tests. The treatment of a greater number of

symptomatic individuals with tetracycline may reveal differences between the symptoms produced on infection by viral agents alone or in conjunction with MLOs. In addition, the fact that *C. sandwichiana* has been demonstrated to easily parasitize *D. viscosa* should allow its use in more thorough experiments possibly involving additional alternate hosts. Such experiments may allow the separation of viral and mycoplasmal agents in different host plants, which would greatly facilitate a more detailed analysis of their characteristics and their relative importance in producing symptoms in *D. viscosa*.

Various nucleic acid probes have recently been developed which open the possibilities of establishing the relatedness of MLOs which cause disease in woody species. It would be of great values to make use of these probes to establish the relationships of the MLO of *D. viscosa* in Hawai'i to those which cause disease in other species. Such probes may allow a comparison to be made between the MLO of *D. viscosa* in Hawai'i and that reported from *D. viscosa* in India, where a similar disease has been noted in this species parasitized by sandalwood trees affected with sandal spike disease.

Lastly, with the availability of probes for the detection of MLOs and dsRNA from *D. viscosa*, the opportunity to search for possible insect vectors of either or both of these agents becomes possible. Such a study may identify the most likely vector species from the arthropod population known to occur on *D. viscosa* in the field and may indicate other possibly economically valuable species which may be at risk from the known diseased populations of *D. viscosa* on the major Hawaiian islands.



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