Isolation and Cloning of DNA Fragments from a Mycoplasmalike Organism Associated with Walnut Witches'-Broom Disease

J. Chen, C. J. Chang, R. Jarret, and N. Gawel

First and second authors: Department of Plant Pathology, University of Georgia, Griffin 30223. Third author: USDA, ARS, Department of Plant Introduction, 1109 Experiment St., Griffin, GA 30223. Fourth author: Department of Horticulture, University of Georgia, Griffin 30223.

This study was supported in part by state and Hatch funds.
Accepted for publication 10 October 1991 (submitted for electronic processing).

ABSTRACT

Chen, J., Chang, C. J., Jarret, R., and Gawel, N. 1992. Isolation and cloning of DNA fragments from a mycoplasmalike organism associated with walnut witches'-broom disease. Phytopathology 82:306-309.

Total DNA was extracted using CTAB buffer from freeze-dried leaves of mature walnut (*Juglans nigra*) showing severe symptoms of walnut witches'-broom (WWB) disease. DNA of WWB mycoplasmalike organism (MLO) was separated from host DNA by CsCl/bisbenzimide gradient centrifugation. A genomic library was constructed from MLO DNA that contained either chromosomal or extrachromosomal DNA fragments. Extrachromosomal DNA probes hybridized to DNA from WWB and pecan bunch (PB) tissues, but not to DNA from periwinkle (*Catharanthus roseus*) infected with western X (WX), eastern aster yellows (EAY), and western severe aster yellows (WSAY) MLOs, beet leafhopper-transmitted virescence agent (BLTVA), or *Spiroplasma citri* (SC). Chromosomal DNA probes hybridized to DNA from tissues infected by MLOs associated with WX, WSAY, or BLTVA, but not PB, EAY, or SC.

Additional keywords: disease detection.

Walnut witches'-broom (WWB) is a disease affecting *Juglans nigra* L. (walnut) (1,3,16) after infection by a mycoplasmalike organism (MLO). The relationship of WWB MLO to other MLOs is not known. Particularly, there has been concern about whether WWB MLO is related to pecan bunch (PB) MLO (1,17). Both WWB and PB diseases are of economical and ecological importance in the nut-growing regions of the southeastern United States (3,16,17). The ability to accurately detect the presence of WWB MLO and to understand its relationship to PB is essential for disease control.

MLOs are phloem-inhabiting plant pathogenic microorganisms characterized by the absence of cell walls. Efforts to culture MLOs in vitro have not been successful (12), thus limiting the study of MLOs by traditional techniques. However, recombinant DNA techniques are a potentially useful tool for the study of MLOs (2,6,9,15). Cloned fragments of MLO DNA can be used as probes for the rapid and sensitive detection of MLOs in plant tissues and for the study of interrelationships between different types of MLOs (2,6,9,15).

MLO DNA has been isolated from insect vectors and infected herbaceous host plants (2,6,9,15). Information on the isolation of MLO DNA directly from woody plant tissues is limited. This report describes a technique for the isolation of WWB MLO DNA from infected *J. nigra* leaf tissue and the cloning of WWB MLO DNA fragments for MLO studies.

MATERIALS AND METHODS

Plant disease materials and chemicals. Two WWB MLO-infected and two asymptomatic walnut trees (approximately 800 m apart) located on the Georgia Experiment Station, Griffin, GA, were used as sources of tissue in this study. PB MLO-infected and asymptomatic pecan (*Carya illinoensis* (F. A. Wagenheim) K. Koch) tissues were collected in Thomasville, GA. Tissues from asymptomatic trees were used as healthy controls. Eastern aster yellows (EAY) MLO (originally provided by T. A. Chen, Department of Plant Pathology, Rutgers University) and *Spiroplasma citri* (SC) (originally provided by D. A. Golino, Department of Plant Pathology, University of California, Davis) were maintained by graft inoculation in periwinkle (*Catharanthus roseus* (L.) G. Don) under greenhouse conditions. Periwinkles infected with western severe aster yellows (WSAY) MLO, western X (WX) MLO, or beet leafhopper-transmitted virescence agent (BLTVA) were provided by B. Kirkpatrick, Department of Plant Pathology, University of California, Davis. Unless otherwise noted, all chemical reagents were purchased from Sigma Chemical Company, St. Louis, MO.

Isolation of total DNA. The procedure reported by Gawel and Jarret (4) was used with minor modifications. Plant tissues were stored at −20 C until used. Leaf tissues were freeze-dried and ground into a fine powder with an electric coffee grinder. All procedures were performed at room temperature unless otherwise specified. Ground tissue (0.5 g) was added to 20 ml of preheated (65 C) CTAB buffer (100 mM Tris-HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 4% hexadecyltrimethylammonium bromide [CTAB], and 2.5% [v/v] mercaptoethanol) in a 40-ml centrifuge tube. The mixture was incubated at 65 C for 1 h and mixed occasionally. An equal volume of chloroform-isoamyl alcohol (CIA) (24:1, v/v) was added, and the solution was mixed by inversion for 15 min before centrifugation at 5,110 *g* in a Beckman JA 20 rotor (Beckman Instruments, Palo Alto, CA) for 5 min. The aqueous phase was transferred to a clean tube, and an equal volume of isopropanol was added. The mixture was incubated at −20 C overnight and then centrifuged at 5,110 *g* for 5 min. The resulting DNA pellet was washed once with 70% ethyl alcohol, transferred to a 1.5-ml Eppendorf tube, and dissolved in 400 μl of Tris-EDTA buffer (TE: 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). These crude DNA extracts were used for MLO DNA isolation and hybridizations. DNA was quantified spectrophotometrically after digestion with RNase A and phenol-chloroform extraction (11).

Isolation of WWB MLO DNA. Asymptomatic leaves and leaves with severe WWB symptoms were harvested in September and October 1989. Total crude DNA, isolated as described above, was centrifuged in a CsCl gradient (refractive index 1.925) with 0.1 mg/ml of bisbenzimide at 180,000 *g* (Beckman SW 55.1 Ti) at 18 C for 48 h. UV light was used to visualize DNA bands. MLO DNA (upper band) was collected by piercing the centrifuge

tube wall with a syringe (11), and it was further purified according to Sears et al (15).

Cloning of WWB MLO DNA fragments. WWB MLO DNA was digested overnight with *Hin*dIII and *Eco*R1 and ligated into pUC18. The ligated DNAs were used to transform *Escherichia coli* JM83 or TB1 following the method of Schuler and Zielinski (14). Recombinant clones were screened for MLO-specific probes by colony hybridization or by Southern blot hybridization of all of the recombinant plasmids.

Membranes for colony hybridization were prepared as described by Schuler and Zielinski (14), prehybridized, hybridized at 65 C overnight with ^{32}P-dCTP (Du Pont, Wilmington, DE)-labeled crude DNA extracted from either healthy or diseased walnut trees with a random primer DNA labeling system (Bethesda Research Laboratories, Gaithersburg, MD), and washed as described by Gawel and Jarret (4). Membranes were exposed to X-ray film for 18 h at −132 C with intensifying screens. After autoradiography, colonies that hybridized to the DNA isolated from the diseased but not the healthy tissues were selected as MLO DNA probe candidates.

For the Southern blot screening, recombinant plasmids were isolated by the boiling method (11), except 2.5 M LiCl was substituted for sucrose. One microgram of each plasmid DNA was loaded into 0.8% agarose gels. Bromophenol blue was added in one well as a tracking dye. TAE buffer (11) then was added to the top of the gel. Electrophoresis of the DNA preparation was at 4 V/cm for 5 min. Additional TAE buffer then was added to submerge the gel, and electrophoresis was continued until the bromophenol blue had migrated 3 cm. The gel was stained with ethidium bromide, and the plasmid DNA was visualized with UV light and then Southern blotted (11) onto nylon membranes (ICN Biochemicals, Cleveland, OH). Membranes were hybridized to random primer ^{32}P-labeled DNA from diseased and healthy walnut tissues, washed as described by Sambrook et al (13), and autoradiographed. The selection of MLO-specific probe candidates was similar to that in the colony hybridization method.

Characterization of WWB MLO DNA probes. For Southern blot hybridizations, 10–30 μg of total undigested DNA from all of the plant samples was prepared by electrophoresis and Southern blotted. For dot blot hybridizations, a series of 1.6, 0.6, and 0.08 μg of total DNA was denatured by the alkaline method (11) and spotted onto nylon membrane. MLO DNA probes were labeled with ^{32}P, and hybridizations were performed as described for Southern blot screening. The ^{32}P-labeled pUC18 was used as a control.

RESULTS

Isolation of DNA. DNA solutions were brownish after CTAB extraction and clarification with CIA. Following the isopropanol precipitation, the DNA was difficult to spool with a glass hook, especially DNA extracted from petioles alone. In these instances, DNA was collected by centrifugation. DNA recovery depended on tissue type, e.g., DNA yields from whole leaves were greater than those from petioles alone. The average yield from all tissue was about 300 μg from 0.5 g of freeze-dried plant tissue.

Following CsCl centrifugation, a DNA band was observed directly above the host genomic DNA band (Fig. 1). This narrow band was only present in the DNA extracts from walnut tissues

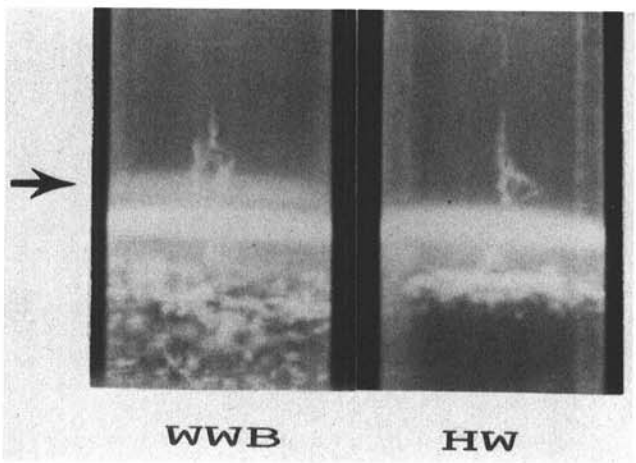

Fig. 1. CsCl/bisbenzimide density gradient preparation of total DNA extracted from petioles of walnut witches'-broom (WWB) mycoplasmalike organism (MLO)-infected trees and from petioles of healthy (asymptomatic) walnut (HW) trees. MLO DNA is marked with an arrow.

TABLE 1. Comparison of recombinant plasmids containing extrachromosomal and chromosomal DNAs of a mycoplasmalike organism associated with walnut witches'-broom (WWB) disease

Cloned plasmids	Insert size (kb)	DNA hybridization	
		WWB tissue	Healthy tissue
Extrachromosomal DNA			
pWWBJ4	1.63	+++[a]	ND[b]
pWWBJ10	1.80	+++	ND
pWWBJ14	1.53	+++	ND
pWWBT13	1.58	+++	ND
Chromosomal DNA			
pWWBJ18	0.94	+	ND
pWWBT68	1.11	+	ND

[a]Strong and weak relative hybridization signals indicated by +++ and +, respectively.
[b]Not detected.

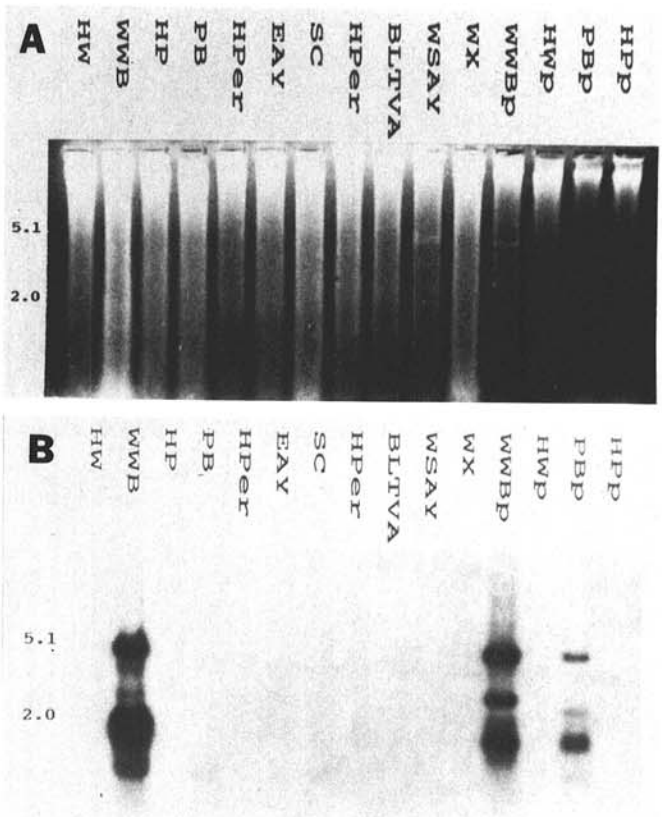

Fig. 2. A, Gel electrophoresis of undigested total DNA. Notice the presence of plasmid DNA in WSAY and extrachromosomal DNA in WWBp. **B,** Hybridization of pWWBJ10 to Southern blot of undigested total DNA preparations from several mycoplasmalike organism- or spiroplasma-infected and healthy plants. HW, healthy walnut; WWB, walnut witches'-broom; HP, healthy pecan; PB, pecan bunch; HPer, healthy periwinkle; EAY, eastern aster yellows; SC, *Spiroplasma citri;* BLTVA, beet leafhopper-transmitted virescence agent; WSAY, western severe aster yellows; WX, western X. Letter p after a sample name represents petiole from which DNA was extracted. Numbers are size markers in kilobases.

showing witches'-broom symptoms. Yields of enriched MLO DNA were about 1.5 μg/0.5 g of freeze-dried tissue.

Cloning of WWB MLO DNA fragments. One hundred and forty-two recombinant colonies were selected and screened by colony hybridization and Southern blot hybridization of recombinant plasmids. Fifty-two clones hybridized strongly only to DNA from diseased tissue, and 87 clones hybridized weakly. Three clones hybridized to DNA from both diseased and healthy tissues.

MLO DNA probe hybridization. Eight recombinant plasmids were selected and labeled with ^{32}P for further experiments. Four probes that hybridized strongly to WWB total DNA hybridized only to the low molecular weight DNAs from WWB and PB tissues (Table 1 and Fig. 2B). These low molecular weight DNAs were assumed to be extrachromosomal DNA. MLO extrachromosomal DNAs also were seen on the agarose gel (Fig. 2A). Two probes hybridized to high molecular weight DNA from WWB tissue (Table 1 and Fig. 3). These two probes were considered to be from MLO chromosomal DNA (2,9,15). Both chromosomal probes had hybridized weakly during the initial screening tests. The two probes that hybridized to DNA from both WWB and healthy tissues during the original screening again hybridized to the DNA from both WWB and healthy walnut tissues. They are assumed to be from host plant DNA.

Comparison of WWB MLO with other mollicutes. With both dot blot and Southern blot experiments, probes of WWB MLO extrachromosomal DNA hybridized strongly to DNAs from WWB tissue, only weakly to that from PB tissue, and not at all, or very weakly, to DNA from healthy plants and that from periwinkles infected with WX, EAY, and WSAY MLOs, BLTVA, or SC (Figs. 2B and 4). The WWB MLO chromosomal DNA probes hybridized to DNA from plant tissue of only WWB, WX, WSAY, and BLTVA (Fig. 3). The ^{32}P-labeled pUC18 did not hybridize to any DNA extracts from MLO- or spiroplasma-infected or noninfected plant tissues.

DISCUSSION

Currently, symptomatic walnut tissue is the only available source of WWB MLO. Using the described procedure, we were able to isolate approximately 1.5 μg of MLO DNA from 0.5 g of freeze-dried (about 2 g fresh weight) walnut tissue. Our data indicate that WWB MLO DNA is about 0.5% of the total DNA extracted from severe symptomatic leaves. Kollar et al (7) recovered 1–2 μg of MLO DNA from 1 g of lyophilized apple leaf expressing proliferation symptoms, whereas 40 μg of aster yellows MLO nucleic acid was recovered from 6 g of midrib tissues collected from symptomatic periwinkle leaves by Lee et al (9).

Either whole leaves or petioles alone were suitable for DNA extraction. However, the DNA extracted from petioles alone resulted in a more distinct MLO DNA band on CsCl/bisbenzimide gradients than that from whole leaves. This may be because of a higher ratio of phloem tissue/whole tissue, leading to a higher ratio of MLO DNA/host DNA in the petiole extracts.

The use of freeze-dried tissue offers at least two advantages over the use of fresh tissue in MLO DNA isolation: sample oxidation that interferes with DNA isolation is minimized, and freeze-dried tissues are more readily ground into powder. This is especially true when using a coffee grinder to pulverize the woody petioles.

The unique upper band observed in CsCl/bisbenzimide gradients was consistently associated with DNA extracted from WWB-infected tissues but not that of DNA from healthy tissues. The low G-C content or its high degree of methylation contributes to the difference in buoyant density between MLO DNA and host plant DNA (2,6,7,9,15). In our recent experiment (data not shown), no MLO was detected in some asymptomatic leaves collected from the two WWB-infected trees when MLO DNA probes were used. These findings suggest that the unique upper DNA band in the CsCl/bisbenzimide gradients is associated with the WWB pathogen.

The hybridization of WWB extrachromosomal DNA probes with DNA from the PB MLO-infected petiole (Figs. 2B and 4) suggests that these two MLOs possess extrachromosomal DNAs that share sequence homology. The weak hybridization reaction to PB DNA was probably due to a low concentration of MLO in the PB-infected tree, as observed by electron microscopy (data not shown). The reason for the very low concentration of extrachromosomal DNA from PB whole leaf sample is not known. EAY, WSAY, and WX MLOs, BLTVA, and *S. citri* apparently do not have the same extrachromosomal DNAs found in WWB MLO (Fig. 2).

Extrachromosomal DNAs have been identified in several MLOs isolated from herbaceous plants (2,8,9). This is, however, the first report of extrachromosomal DNA found to be associated with an MLO of a woody plant. We used the term extrachromosomal DNA because the information about WWB MLO is limited at the present time.

Extrachromosomal DNA probes from WWB MLO showed stronger hybridization signals than chromosomal DNA probes to DNA from WWB tissues, suggesting higher copy number of the extrachromosomal DNA than chromosomal DNA in WWB MLO. As shown in Figure 4, MLO DNA was detected from 0.08 μg of total DNA extracted from typical symptomatic walnut leaves. If the ratio of 0.5% (MLO DNA/total DNA) is used, MLO DNA can be detected at picogram level in our study.

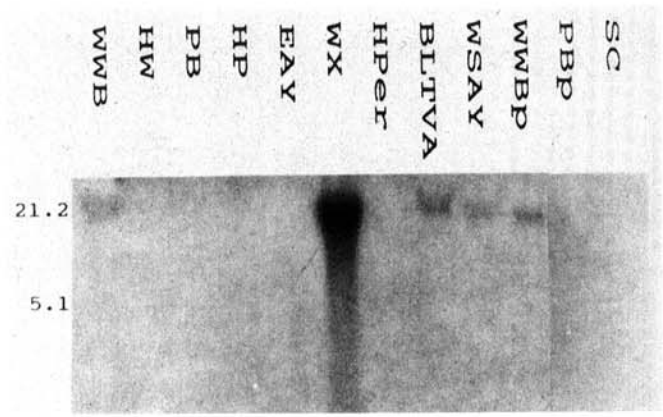

Fig. 3. Hybridization of pWWBT68 to Southern blot of undigested total DNA extracts from several mycoplasmalike organism- or spiroplasma-infected and healthy plants. WWB, walnut witches'-broom; HW, healthy walnut; PB, pecan bunch; HP, healthy pecan; EAY, eastern aster yellows; WX, western X; HPer, healthy periwinkle; BLTVA, beet leafhopper-transmitted virescence agent; WSAY, western severe aster yellows; SC, *Spiroplasma citri.* Letter p after a sample name represents petiole from which DNA was extracted. Numbers are size markers in kilobases.

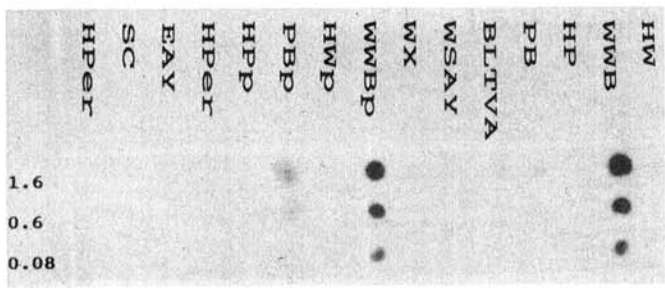

Fig. 4. Dot hybridization of pWWBJ14 to total DNA extracts from several mycoplasmalike organism- or spiroplasma-infected and healthy plants. HPer, healthy periwinkle; SC, *Spiroplasma citri;* EAY, eastern aster yellows; HP, healthy pecan; PB, pecan bunch; HW, healthy walnut; WWB, walnut witches'-broom; WX, western X; WSAY, western severe aster yellows; BLTVA, beet leafhopper-transmitted virescence agent. Letter p after a sample name represents petiole from which DNA was extracted. Numbers are concentration of total DNA in micrograms.

Additionally, extrachromosomal DNA is readily separated from host DNA in agarose gels. This minimizes the background interference from crude DNA extracts during MLO detection. However, there are disadvantages of using extrachromosomal DNA, particularly, extrachromosomal DNA may not be consistently associated with the host MLO, as was observed in maize bushy stunt MLO (2). In this case, the use of chromosomal DNA clones would be more reliable for detection purposes.

The weak hybridization signal made it more difficult to identify WWB MLO chromosomal DNA probes when using the colony hybridization screening method. The screening method using Southern blot recombinant plasmids provides purer DNA on the membrane and reduces the background hybridization signal. Lee et al (10) used a dot blot method instead of colony hybridization to screen an MLO library. Based on our experience, the dot blot method is easy to perform when a library is small (< 100 clones). The Southern blot screening method is a relatively fast procedure when dealing with a large number of samples. Similarly, crude DNA preparations from a large number of infected plant tissue can be Southern blotted and detected with MLO DNA probes.

Probes from WWB MLO chromosomal DNA hybridized to total DNA extracts from WX MLO-infected tissues (Fig. 3). WX MLO DNA probes also can hybridize to DNA extracts from WWB tissue (5). This suggests that WWB and WX may be closely related.

It should be pointed out that the samples we used were total DNA (including the host plant DNA and MLO DNA). Given the same amount of total DNA, the concentration of MLO DNA could be very different because the titer of MLOs from different tissues could vary. For this reason, we have not interpreted our results in terms of definitive MLO relationships, except that between WWB and WX.

The MLO DNA probes reported here provide a useful tool to identify the presence of the insect vector(s) transmitting WWB disease and to monitor replication during in vitro cultivation of the MLO.

LITERATURE CITED

1. Chang, C. J., Impson, L. K., and Cunfer, B. M. 1986. Walnut witches'-broom disease in Georgia. (Abstr.) Phytopathology 76:1139.
2. Davis, M. J., Tsai, J. H., Cox, R. L., McDaniel, L. L., and Harrison, N. A. 1988. Cloning of chromosomal and extrachromosomal DNA of the mycoplasmalike organism that causes maize bushy stunt disease. Mol. Plant-Microbe Interact. 1:295-302.
3. Duke, J. A. 1989. CRC Handbook of Nuts. CRC Press, Boca Raton, FL. pp. 190-193.
4. Gawel, N. J., and Jarret, R. L. 1991. Chloroplast DNA restriction fragment length polymorphisms (RFLPs) in *Musa* species. Theor. Appl. Genet. 81:783-786.
5. Kirkpatrick, B. C., Fisher, G. A., Fraser, J. D., and Purcell, A. H. 1990. Epidemiological and phylogenic studies on western X-disease mycoplasma-like organisms. Zentralbl. Bakteriol. Suppl. 20:288-297.
6. Kirkpatrick, B. C., Stenger, D. C., Morris, T. J., and Purcell, A. H. 1987. Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. Science 238:197-200.
7. Kollar, A., Seemüller, E., Bonnet, F., Saillard, C., and Bové, J. M. 1990. Isolation of the DNA of various plant pathogenic mycoplasma-like organisms from infected plants. Phytopathology 80:233-237.
8. Kuske, C. R., and Kirkpatrick, B. C. 1990. Identification and characterization of plasmids from the western aster yellows mycoplasma-like organism. J. Bacteriol. 172:1628-1633.
9. Lee, I.-M., and Davis, R. E. 1988. Detection and investigation of genetic relatedness among aster yellows and other mycoplasma-like organisms by using cloned DNA and RNA probes. Mol. Plant-Microbe Interact. 1:303-310.
10. Lee, I.-M., Davis, R. E., and DeWitt, N. D. 1990. Nonradioactive screening method for isolation of disease-specific probes to diagnose plant diseases caused by mycoplasma-like organisms. Appl. Environ. Microbiol. 56:1471-1475.
11. Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
12. McCoy, R. E., Caudwell, A., Chang, C. J., Chen, T. A., Chiykowski, L. N., Cousin, M. T., Dale, J. L., deLeeuw, G. T. N., Golino, D. A., Hackett, K. J., Kirkpatrick, B. C., Marwitz, R., Petzold, H., Sinha, R. C., Sugiura, M., Whitcomb, R. F., Yang, I. L., Zhu, B. M., and Seemüller, E. 1989. Plant diseases associated with mycoplasma-like organisms. Pages 546-640 in: The Mycoplasmas. Vol. 5. R. F. Whitcomb and J. G. Tully, eds. Academic Press, New York.
13. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
14. Schuler, M. A., and Zielinski, R. E. 1989. Methods in Plant Molecular Biology. Academic Press, San Diego, CA.
15. Sears, B. B., Lim, P-O., Holland, N., Kirkpatrick, B. C., and Klomparens, K. L. 1989. Isolation and characterization of DNA from a mycoplasmalike organism. Mol. Plant-Microbe Interact. 2:175-180.
16. Seliskar, C. E. 1976. Mycoplasmalike organism found in the phloem of bunch-diseased walnuts. For. Sci. 22:144-148.
17. Seliskar, C. E., KenKnight, G. E., and Bourne, C. E. 1974. Mycoplasmalike organism associated with pecan bunch disease. Phytopathology 64:1269-1272.