DNA Probes as Molecular Markers to Monitor the Seasonal Occurrence of Walnut Witches'-Broom Mycoplasmalike Organism

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ABSTRACT

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A rapid and sensitive method was developed to monitor the occurrence of walnut witches'-broom (WWB) mycoplasmalike organism (MLO) in infected walnut (*Juglans nigra*) trees. Walnut leaf tissues from both asymptomatic and symptomatic trees were collected monthly during the growing seasons of 1990 and 1991 and freeze-dried. Southern blots of CTAB-extracted DNA without endonuclease digestion were used for hybridization with ^{32}P-labeled chromosomal and extrachromosomal WWB MLO DNA probes. The presence of WWB MLO DNA was detected in DNA extracts from a single tree at least 1 mo prior to the seasonal appearance of WWB symptoms when extrachromosomal DNA probes, but not chromosomal DNA probes, were used. Also, hybridization results and observation of symptom development indicate that WWB MLO may not be uniformly distributed within infected walnut trees. Results from two-dimensional electrophoresis experiments suggested that there were two double-stranded, circular, extrachromosomal DNA molecules, approximately 5 and 1.5 kb.

Black walnut (*Juglans nigra* L.), one of the most valuable American forest trees, is grown for its beautiful foliage, superior quality wood, and edible nuts. Black walnut is distributed from Massachusetts to Florida and west to Minnesota and Texas (21). Walnut witches'-broom (WWB) disease is found throughout the walnut-growing area (16). The disease is endemic to the United States and presents a serious problem when growing walnuts for either pleasure or profit (12). Yield and quality of nuts may be severely affected by WWB; also, the disease causes brittleness in the wood, making limbs highly susceptible to storm damage (4,16).

WWB disease was first reported in 1932 in Delaware (20). Later, the disease was associated with infection by mycoplasmalike organisms (MLOs) (1,15). In

addition to causing WWB in black walnut trees, MLOs are the probable cause of witches'-broom disease in other species of *Juglans* (16). Knowledge of this disease is limited. Diagnoses of WWB have relied on symptomatology, graft transmissibility, and the observation of MLO bodies by transmission electron microscopy (1,9,15), techniques that are relatively insensitive or laborious, or both. The inability to cultivate MLOs in vitro has greatly hindered the study of them. In addition, MLOs associated with tree diseases are usually more difficult to study than those associated with diseases of herbaceous plants, because of the difficulties in isolating MLO DNA from woody tissues and because of the perennial (seasonal) growth habit of the host plants.

We describe a simple and sensitive method, employing WWB MLO DNA probes (2), to monitor the seasonal occurrence of WWB MLO in infected walnut trees. The conformational structure of the WWB MLO extrachromosomal DNAs was also investigated.

MATERIALS AND METHODS

Preparation of DNA samples. Four black walnut trees (*J. nigra*) from two locations on the campus of the Georgia Experiment Station, Griffin, were used for this study. We previously reported that symptomatic tissues consistently gave positive results by using WWB DNA probes (2). In this study, except for the conformational study of extrachromosomal DNA or that of using WWB MLO–positive controls, only asymptomatic twigs were sampled; twigs on some branches, however, expressed WWB symptoms late in the growing season. Trees 1 and 2 (site 1), with their diameters at breast height of approximately 60 cm, have been showing witches'-broom symptoms since 1983. Four lower branches of each tree were selected and marked for this study. Trees 3 and 4 (site 2), with their diameters at breast height of about 24 cm, have never been observed with witches'-broom symptoms. One twig was randomly sampled from each tree. Sites 1 and 2 were about 800 m apart.

Samples were collected at monthly intervals during 1990 and 1991, beginning in April, when the young shoots began to resume active growth through September or October, by which time most of the leaves had fallen off. On each sampling date, approximately 50 g of small twigs were harvested from trees with a pole pruner and frozen at −20 C within 1 hr after sampling. Samples were freeze-dried for 2-3 days. Dried leaves were powdered with a coffee grinder. DNA was extracted with CTAB buffer as previously reported (2), with slight modifications. Tissue powder (1 g) was added to 20 ml of preheated (at 65 C) CTAB buffer and incubated at 65 C for 30 min. The mixture was extracted by emulsification with an equal volume of chloroform-isoamyl alcohol (24:1, v/v)

for 5 min. Following centrifugation at 6,500 g for 5 min at room temperature, the aqueous phase was transferred to a clean tube and mixed with an equal volume of isopropanol. DNA was collected by centrifugation at 6,500 g for 5 min. at room temperature and dissolved in 0.5 ml of TE (100 mM Tris-HCl, pH 8.0, and 1 mM EDTA). DNA concentrations were estimated by the minigel method (14). Methods for cloning WWB MLO DNA are described elsewhere (2). Three extrachromosomal DNA clones, namely, pWWBJ4, pWWBJ10, and pWWBJ14, with the insert sizes of 1.63, 1.80, and 1.53 kb, respectively, and one chromosomal DNA clone, pWWBT68, with an insert size of 0.94 kb, were used as probes throughout the study. Either the entire recombinant plasmid or the MLO DNA insert alone was used for DNA hybridization experiments.

WWB MLO detection. Ten μg of each sample DNA was electrophoresed in a 0.8% agarose gel at 6 V/cm, using 1× TAE (40 mM Tris-acetate, 1 mM EDTA, pH 7.0) buffer. DNA in gels was visualized by UV light after staining with ethidium bromide, then blotted by the method of Southern (17) as described by Sambrook et al (14) to nylon membranes (Bio-Trans, ICN Nutritional Biochemicals, Cleveland, OH) for use in hybridization experiments. Probe DNA was labeled with ^{32}P dCTP (Du Pont, Wilmington, DE) by using random oligoprimers (Random Primer DNA labeling system, BRL Life Technologies Inc., Gaithersburg, MD). Membranes were prehybridized at 65 C for 4 hr. Following addition of the probe (approximately 200 ng of DNA), hybridizations were performed at 65 C for 18 hr. Membranes were washed once in 2× SSC (sodium chloride–sodium citrate) + 0.1% SDS (sodium dodecyl sulfate) for 30 min (at 65 C) and twice in 0.1× SSC + 0.1% SDS for 30 min (at 65 C) (14), and were exposed to Kodak X-Omat AR film with intensifying screens at −130 C for 24 hr.

Conformational structure of WWB MLO extrachromosomal DNA. Two-dimensional agarose gel electrophoresis (8) was used to separate open circular (OC) and closed circular (CC) conformational forms of the WWB MLO extrachromosomal DNA. Ten μg of total DNA from infected walnut tissue was electrophoresed in a 0.8% agarose gel at 4 V/cm for 3 hr, stained with ethidium bromide, and viewed under UV light. The excised gel slice containing the electrophoretic DNA profile was exposed to 254 nm UV light (Eprom Eraser, Model DE-4, UVP, Inc. San Gabriel, CA) for 5 min. The treated agarose block was then embedded into a second gel and electrophoresed as before at 90° to original orientation. The position of extrachromosomal DNAs was located by UV illumination after ethidium bromide

staining. To confirm the locations of the extrachromosomal DNAs, the final gel was blotted to a nylon membrane and probed separately with ^{32}P-labeled extrachromosomal DNA clones as described above.

RESULTS AND DISCUSSION

Impurities in crude DNA preparations can greatly interfere with DNA–DNA hybridizations (11). We alleviated this potential problem by using agarose gel electrophoresis to separate the sample DNA from impurities, thereby increasing the sensitivity of pathogen detection (Figs. 1 and 2).

Figure 1A and B illustrates the detection of MLO in tree 2 at site 1, using pWWBT68 chromosomal MLO DNA probe. Hybridization occurs with high-molecular weight DNA, with smearing tails. These smears probably resulted from the shearing of MLO chromosomal DNA during the extraction procedure plus the very rapid separation during electrophoresis.

The extrachromosomal DNA probe pWWBJ10 gave a much stronger hybridization signal with DNAs from infected walnut tissues in the detection of the pathogens than did the chromosomal DNA probe (Figs. 1 and 2). This is possibly due to a higher copy number of the low molecular weight extrachromosomal DNA (2,3).

Cloned plasmids or extrachromosomal DNAs have been used as probes for detection of the plant pathogens *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson (6), *Erwinia amylovora* (Burrill) Winslow et al (5), and many other environmentally important bacteria, such as *Bacillus pumilus* Meyer and Gottheil (7), *Shigella* spp., *Escherichia coli* (Migula) Castellani and Chalmers (19), *Lactobacilli* (18), and several isolates that degrade 4-chlorobiphenyl (13). Klein et al (10) reported that when used for detection of sugarcane white leaf MLO, extrachromosomal sugarcane white leaf MLO DNA probes were 100 times more sensitive than cloned chro-
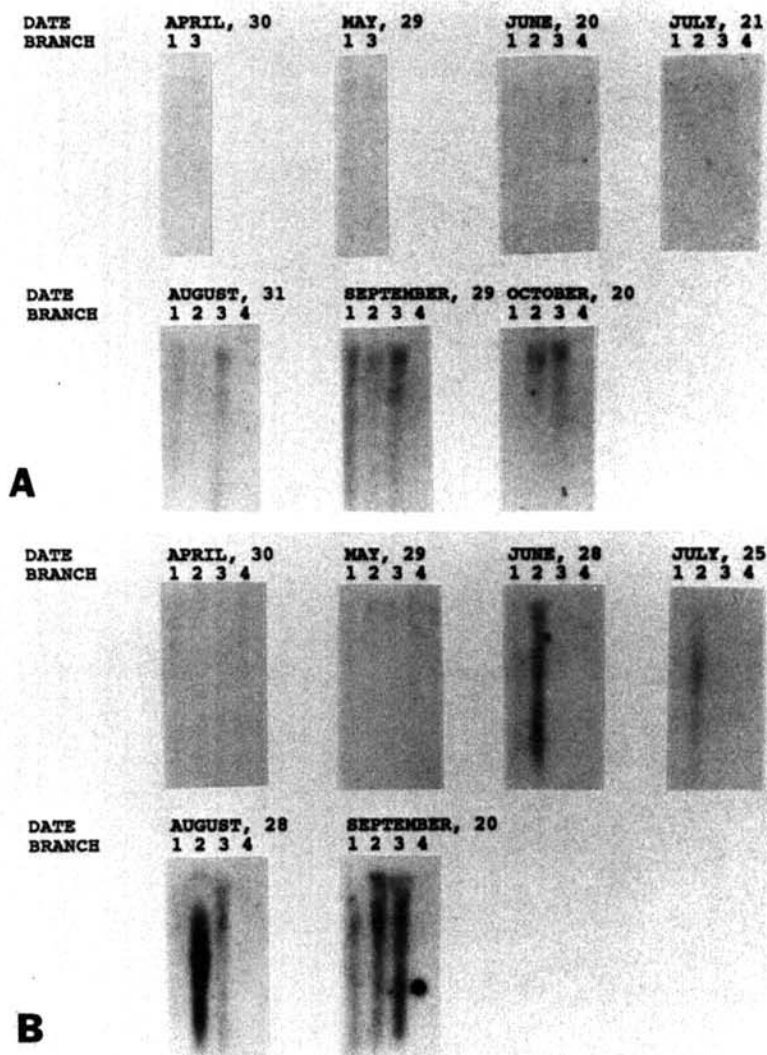

Fig. 1. Southern blot detection of walnut witches'-broom mycoplasmalike organism (WWB MLO) DNA in DNA extracts from an infected black walnut tree (#2) at site 1 with WWB MLO chromosomal DNA probe pWWBT68 during April–October in (A) 1990 and (B) 1991.

mosomal DNA probes.

Davis et al (3) reported the inconsistency of extrachromosomal DNA to maize bushy stunt MLO. We observed the inconsistency in 1990 when positive detection of MLOs occurred only by use of chromosomal DNA probe (Figs. 1A and 2A; branch 3 from 31 August, branch 2 from 29 September, and branch 2 from 20 October). However, this inconsistency was not found in 1991 (Figs. 1B and 2B). One possible explanation is that walnut trees may sustain multiple infections by several WWB MLO isolates, not all of which necessarily contain extrachromosomal DNA. Alternatively, the seasonal titer of extrachromosomal DNA may fluctuate within populations of the WWB MLO.

Four low-molecular-weight extrachromosomal DNA bands were observed in electrophoretic profile of undigested DNA from WWB-infected walnut tissues. The four visible DNA bands, designated as bands 1, 2, 3, and 4, are illustrated in Figure 3A. Two-dimensional electrophoresis of these bands indicated that bands 1 and 3 represent OC forms, and bands 2 and 4 are the CC forms, of extrachromosomal elements (Fig. 3B). Staining with ethidium bromide and exposure to UV_{254} light creates nicks on the extrachromosomal DNA, which convert CC form to OC form (8). Only a portion of band 2 CC form was converted to OC form by this treatment. In contrast, all the band 4 CC DNA was converted to the OC form (Fig. 3B). These data suggested that there are two circular extrachromosomal DNA molecules associated with WWB MLO. Bands 1 and 2 represent two conformational forms of an extrachromosomal DNA estimated to be about 5 kb, and bands 3 and 4 are two forms of a about 1.5 kb element. The relationship of the two extrachromosomal DNA molecules has not been determined, although all three extrachromosomal DNA probes were able to hybridize to the two molecules, indicating that these two extrachromosomal DNA molecules share a high degree of sequence similarity.

DNA samples from trees 3 and 4 at site 2 always tested negative for MLOs and both trees were asymptomatic throughout the 1990 and 1991 seasons. The two symptomatic trees at site 1 differed in the severity of their disease infection. Tree 1 at site 1 developed a few symptomatic twigs on the upper branches late in the season (September) of both years. Symptoms were never observed in the lower four branches examined, nor were the MLOs detected in these tissues utilizing any of the DNA probes, suggesting that there was very low or no titer of MLOs in these four branches. In contrast, symptomatic twigs from the higher branches consistently gave positive results to both chromosomal and extrachromosomal DNA probes (data not shown), confirming the association of the disease symptoms with the presence of WWB MLOs (1,2).

Using extrachromosomal probes, WWB MLO was detected from tree 2 at site 1 in June of 1990 (Fig. 2A), but obvious disease symptoms were not present until early August. In 1991, WWB MLO was detected in May (Fig. 2B), and WWB symptoms developed in late June. In contrast, the chromosomal DNA probe did not detect the presence of WWB MLO infection before the occurrence of symptoms elsewhere in the tree.

Early detection of infection is very important for WWB MLO disease control, since prompt eradication has been suggested as the most effective treatment (16,20). The identification of the pathogen, regardless of the presence of symptoms, is also significant for WWB MLO indexing (16). The WWB MLO extrachromosomal DNA probes are potentially useful for early detection of the WWB MLO, but the relationships between the extrachromosomal DNA elements and their MLO hosts need to be clarified. The chromosomal DNA probe used is not as sensitive as extrachromosomal DNA probes but is still able to detect MLO DNA from asymptomatic tissue, suggesting chromosomal DNA probes can also be valuable for disease indexing.

Electron microscopy is a sensitive method for detecting MLOs. In practice, however, the basis for selection of samples for electron microscopy generally depends upon the expression of the disease symptoms. Large-scale MLO screenings, using electron microscopy, is both time-consuming and expensive. On the other hand, hybridization assays are more versatile and specific, enabling efficient large-scale samplings. In addition, fresh samples can be stored at -20 C for several months before use. This is especially convenient for studies in which
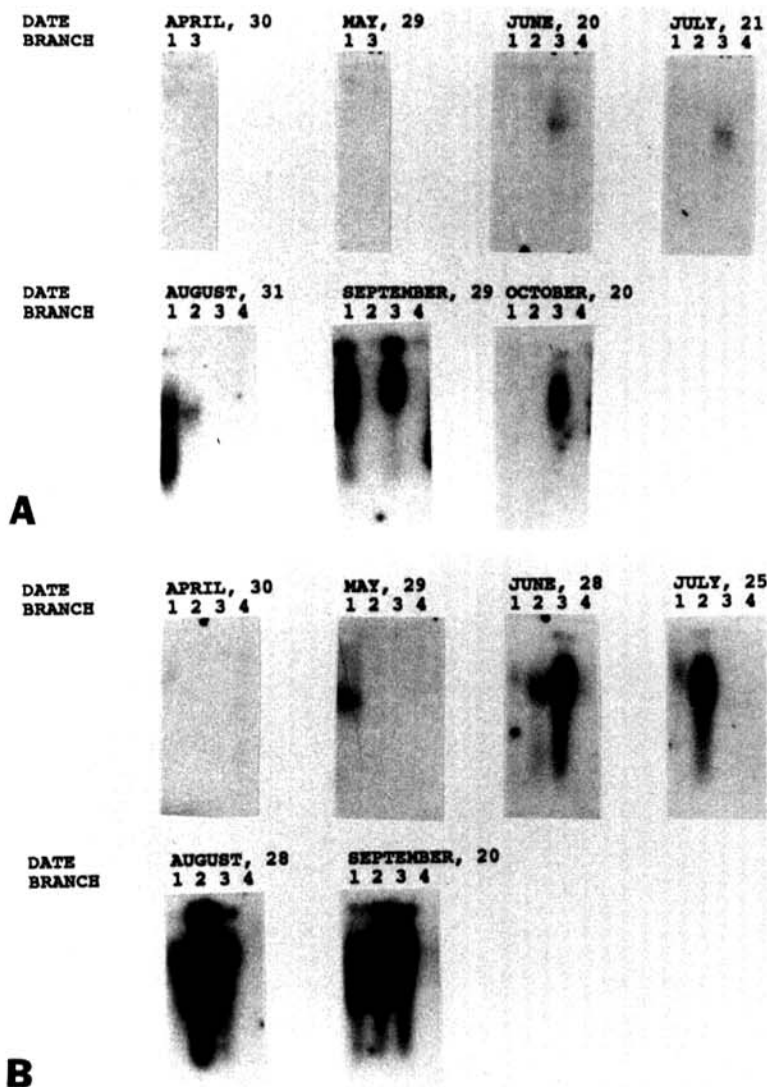

Fig. 2. Southern blot detection of walnut witches'-broom mycoplasmalike organism (WWB MLO) DNA in DNA extracts from an infected black walnut tree (#2) at site 1 with WWB MLO extrachromosomal DNA probe pWWBJ10 during April–October in **(A)** 1990 and **(B)** 1991.
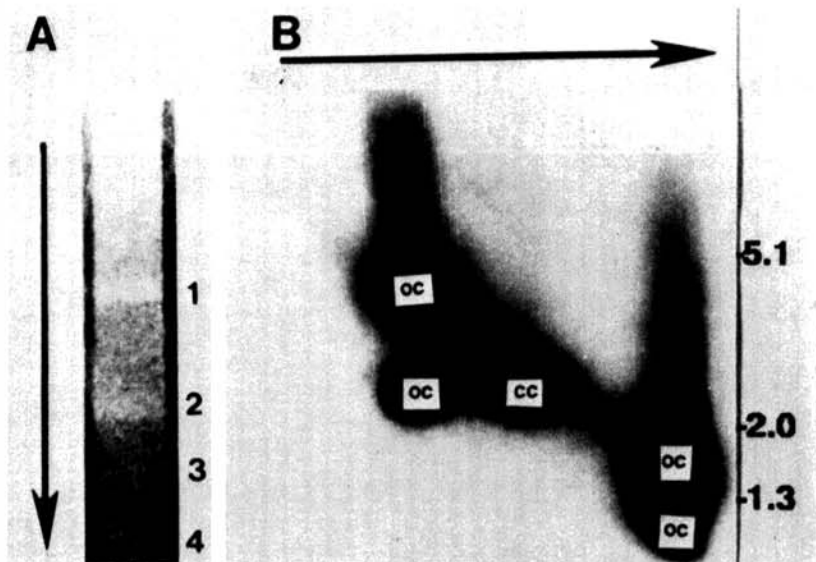
Fig. 3. Two-dimensional agarose gel electrophoresis of a DNA extract from black walnut shoots affected by walnut witches'-broom (WWB) disease. Numbers on the right are DNA size markers from EcoRI–HindIII digested λ DNA. **(A)** Presence of low molecular WWB mycoplasmalike organism extrachromosomal DNA bands (1–4) revealed by UV illumination of the gel after ethidium bromide staining. Because of the photographic process, a considerable loss of resolution of bands 3 and 4 has occurred. **(B)** Southern blot hybridization with pWWBJ10 probe to the two-dimensional agarose gel revealing the open circular and closed circular forms of WWB extrachromosomal DNA elements.

the occurrence of the pathogen must be evaluated at frequent intervals.

Three (branches 1, 2, and 3) of four branches on symptomatic tree 2 at site 1 developed WWB symptoms as the season progressed and tested positive for the MLO (Figs. 1 and 2). Branch 4 never showed WWB symptoms during 1990 and 1991 and consistently tested negative for WWB MLO. The presence of WWB MLO within tissues also varied from twig to twig of the same branch (Figs. 1 and 2). Symptom observation also appeared to reflect the uneven distribution of WWB MLO in the infected tree.

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. 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.

3. 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.

4. Duke, J. A. 1982. Pages 190-193 in: CRC Handbook of Nuts. CRC Press, Boca Raton, FL.

5. Falkenstein, H., Bellemann, P., Walter, S., Zeller, W., and Geider, K. 1988. Identification of Erwinia amylovora, the fireblight pathogen, by colony hybridization with DNA from plasmid pEA29. Appl. Environ. Microbiol. 54:2798-2802.

6. Gilbertson, R. L., Maxwell, D. P., Hagedorn, D. J., and Leong, S. A. 1989. Development and application of a plasmid DNA probe for detection of bacteria causing common bacterial blight of bean. Phytopathology 79:518-525.

7. Hendrick, C. A., Smiley, B. K., Shelley, T. H., and Tomes, N. J. 1991. Use of a plasmid DNA probe to monitor populations of Bacillus pumilus inoculant strains in hay. Appl. Environ. Microbiol. 57:686-693.

8. Hintermann, G., Fischer, H.-M., Crameri, R., and Hutter, R. 1981. Simple procedure for distinguishing CCC, OC, and L forms of plasmid DNA by agarose gel electrophoresis. Plasmid 5:371-373.

9. Hutchins, L. M., and Wester, H. V. 1947. Graft-transmissible blooming disease of walnut. (Abstr.) Phytopathology 37:11.

10. Klein, G., Klinkong, S., Maurer, R., Schneider, B., Saillard, C., and Seemuller, E. 1990. Occurrence and homology of extrachromosomal DNA in plant pathogenic mycoplasmas. Int. Org. Mycoplasmol. 1:570-571.

11. 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.

12. MacDaniels, L. H., and Welch, D. S. 1964. The walnut bunch disease problem. North. Nut Grow. Assoc. Annu. Rep. 55:41-48.

13. Pittigrew, C. A., and Sayler, G. S. 1986. The use of DNA:DNA colony hybridization in the rapid isolation of 4-chlorobiphenyl degradative bacterial phenotypes. J. Microbiol. Methods 5:205-213.

14. Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 1,546 pp.

15. Seliskar, C. E. 1976. Mycoplasmalike organism found in the phloem of bunch-diseased walnuts. For. Sci. 22:144-148.

16. Seliskar, C. E., and Wilson, C. L. 1981. Yellows diseases of trees. Pages 35-96 in: Mycoplasma Diseases of Trees and Shrubs. K. Maramorosch and S. P. Raychaudhuri, eds. Academic Press, New York. 362 pp.

17. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.

18. Tannock, G. W. 1989. Biotin-labeled plasmid DNA probes for detection of epithelium-associated strains of lactobacilli. Appl. Environ. Microbiol. 55:461-464.

19. Venkatesan, M., Buysse, J. M., Vandendries, E., and Kopecko, D. J. 1988. Development and testing of invasion-associated DNA probes for detection of Shigella spp. and enteroinvasive Escherichia coli. J. Clin. Microbiol. 26:261-266.

20. Waite, M. B. 1933. Notes on some nut diseases with special reference to the black walnut. North. Nut Grow. Assoc. Annu. Rep. 23:60-67.

21. Woodroof, J. G. 1979. Tree Nuts: Production Processing Products. 2nd ed. Avi Publishing, Westport, CT. 731 pp.