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Molecular Cloning and Detection of Chromosomal and Extrachromosomal DNA of Mycoplasmalike Organisms Associated with Witches'-Broom Disease of Pigeon Pea in Florida

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A Florida isolate of the pigeon pea witches'-broom (WB) mycoplasmalike organism (MLO) was transmitted from naturally infected pigeon pea (*Cajanus cajan*) to periwinkle (*Catharanthus roseus*) and induced symptoms typical of a "yellows" group disease. Preparations enriched with MLOs were obtained from periwinkle by differential centrifugation after tissues were ground in an osmotically augmented buffer. Total DNA was extracted from these preparations and MLO DNA was separated from host DNA by cesium chloride-bisbenzimide buoyant density gradient centrifugation. *Eco*RI-*Hin*dIII restriction fragments of MLO-associated DNA were cloned in pUC19 and transformed *Escherichia coli* DH5α. Selected recombinant plasmids or their excised inserts were labeled with [^{32}P]dATP and used as probes in dot and Southern hybridizations with total DNAs from plants affected by various MLO-associated diseases. Nine of 14 probes hybridized with DNA from WB-affected periwinkle and pigeon pea but not with DNA from plants infected with Eastern aster yellows, Western dwarf aster yellows, periwinkle witches'-broom, maize bushy stunt, beet leafhopper-transmitted virescence, Western-X, and lethal yellowing MLOs, nor with DNA from healthy plants. Southern hybridization analyses also demonstrated the presence of extrachromosomal DNA associated with the WB MLO. Probes containing fragments of chromosomal DNA hybridized more broadly to DNA of other MLOs than did extrachromsomal DNA probes.

Pigeon pea (*Cajanus cajan* (L.) Millsp.), an edible grain legume, is an important food crop in the Caribbean and elswhere in the tropics, where a estimated 2.9 million hectares are grown (Morton 1976; Kannaiyan *et al.* 1984). The influx of a large Latin American population into south Florida during recent years has created a regional market for this crop, and local production of pigeon pea has increased significantly. Pigeon pea witches'-broom (WB), a disease associated with nonculturable mycoplasmalike organisms (MLOs), has become a limiting factor in the cultivation of the crop, affecting as many as 75% of plants within certain fields located in the Homestead area (McCoy *et al.* 1983).

WB disease has also been reported to affect pigeon pea in Costa Rica, Dominican Republic, El Salvador, Jamaica, Panama, Puerto Rico, and Trinidad (Licha-Baquero 1979; Maramorosch *et al.* 1974; Vakili and Maramorosch 1974). Pigeon pea rosette, a similar disease, occurs in India (Maramorosch *et al.* 1976). Initial descriptions of the disease reported the presence of both MLOs and a rhabdovirus in diseased plants (Hirumi *et al.* 1973; Maramorosch *et al.* 1974). However, a subsequent study in Puerto Rico (Licha-Baquero 1979) found MLOs to be associated with plants exhibiting a bushy canopy condition, whereas the rhabdovirus occurred in plants showing a pale mosaic. Similarly, in Florida, symptoms associated with infection by MLOs were identical to those initially described for MLO-rhabdovirus infections of pigeon pea elsewhere in the Caribbean. However, rhabdovirus was found to be associated with a separate syndrome that included vein yellowing (McCoy *et al.* 1983). The insect vectors of both disease agents are not known.

A resurgence in recent years in research efforts dealing with MLOs has mainly been attributable to the development of improved serological methods (Chen and Jiang 1988; Clark *et al.* 1989; Jiang *et al.* 1989; Lin and Chen 1985) and the application of recombinant DNA technologies (Davis *et al.* 1988a; Davis *et al.* 1990a; Kollar *et al.* 1990; Kirkpatrick *et al.* 1987; Lee and Davis 1988; Lee *et al.* 1990; Sears *et al.* 1989) to the detection and diagnosis of these fastidious plant pathogens. Use of nucleic acid hybridizations, in particular, is proving to be more versatile, sensitive, and specific than previous detection methods. Dot hybridizations, in which cloned random fragments of MLO DNA are used as probes, have enabled detection of several MLOs in both their plant and insect hosts (Davis *et al.* 1988a; Davis *et al.* 1990a; Kirkpatrick *et al.* 1987). Also, dot hybridizations that use combinations of probes are capable of distinguishing MLOs from one another (Bertaccini *et al.* 1990a; Davis *et al.* 1988b; Davis *et al.* 1990b; Lee and Davis 1988) and are providing information concerning genetic interrelatedness among MLOs from various hosts and geographic locales (Bertaccini *et al.* 1990b; Lee and Davis 1988). In addition, cloned rRNA genes have been used to study the phylogeny of plant pathogenic MLOs (Kirkpatrick and Fraser 1988; Lim and Sears 1989).

The purpose of the present study was to develop a DNA hybridization probe to enable detection of the specific MLO associated with witches'-broom disease of pigeon pea. In this paper we report the transmission of the WB MLO

from pigeon pea to periwinkle, isolation and molecular cloning of MLO DNA from periwinkle, characterization of disease-specific DNA hybridization probes, and use of probes in detecting the witches'-broom MLO in plant hosts.

MATERIALS AND METHODS

Plant material. Cuttings from approximately 9-month-old, field-grown pigeon pea plants of undetermined varieties displaying witches'-broom symptoms (McCoy *et al.* 1983) were collected at three locations in Dade County, Florida, during March 1989. Cuttings were planted in pots containing vermiculite and placed in a greenhouse under overhead intermittent mist irrigation for 6–8 wk to stimulate rooting. Rooted cuttings were maintained as a source of host tissues for extraction of the witches'-broom MLO (WB MLO). Several of these plants were also used to transmit the MLO to periwinkle (*Catharanthus roseus* (L.) G. Don). Pigeon pea plants grown from seed were maintained in a separate greenhouse and served as a source of healthy host tissues for DNA extractions.

Plants affected by several other MLO-associated diseases were maintained. These were sweet corn cultivar Aristogold Guardian Evergreen (*Zea mays* L. 'saccharata') with isolates of maize bushy stunt from Florida (MBSF) and Texas (MBST) (Davis *et al.* 1988a); periwinkle with Florida periwinkle witches'-broom (FWB) (McCoy and Thomas 1980); and periwinkle with Eastern aster yellows (EAY), kindly provided by J. A. Wyman, University of Wisconsin, Madison. Bud tissues of a Chinese fan palm (*Livistona chinensis* (Jacq.) R. Br. ex Mart.) with lethal yellowing (LY) disease was harvested from the palm collection at the Ft. Lauderdale Research and Education Center. Plant material with other MLO-associated diseases was kindly provided by B. C. Kirkpatrick, University of California, Davis, and included: Western-X (WX) in celery (*Apium graveolens* L.); beet leafhopper-transmitted virescence (VR); and Western dwarf aster yellows (DAY) in periwinkle.

Transmission of the WB agent to periwinkle. Dodder seed (*Cuscuta* sp.) was germinated in pots containing rooted, WB-affected pigeon pea plants. Dodder vines were established on the plants for 14 days before coestablishment of the vines on young seed grown periwinkle plants. Vine connections were maintained between plants until foliar and/or floral symptoms indicative of disease transmission became evident on periwinkle, at which time the dodder was removed. For confirmation of transmission of the witches'-broom agent, phloem tissues of leaf midribs from symptomatic periwinkle shoots were examined for the presence of MLOs by transmission electron microscopy. Specimens were prepared for examination by using previously described methods (McCoy *et al.* 1983). MLO-infected periwinkle was perpetuated by periodic graft inoculation of additional healthy plants for use in subsequent molecular cloning experiments.

DNA extractions. Leaves, petioles, and stems (50 g) of a WB MLO-infected periwinkle plant were ground with a Waring blender in 200 ml of ice-cold, modified phosphate-buffered sucrose (PS) (Kirkpatrick *et al.* 1987). The resulting tissue brei was filtered through two layers of cheesecloth, and the filtrate was then centrifuged at 3,000 $\times$ *g* for 10 min to sediment starch, intact chloroplasts, and nuclei. The supernatant liquid was centrifuged again at 20,000 $\times$ *g* for 30 min to pellet MLO and mitochondria. DNA was extracted from MLO-enriched pellets by the method of Dellaporta *et al.* (1983). Each pellet was resuspended in 15 ml of DNA extraction buffer (100 mM Tris-HCl, pH 8, 50 mM disodium EDTA, pH 8.0, 500 mM NaCl, and 10 mM mercaptoethanol) to which 1 ml of 20% sodium dodecyl sulfate (SDS) was added. The mixture was incubated at 65° C for 10 min with periodic gentle agitation. After 5 ml of 5 M potassium acetate was added to the lysate, the mixture was chilled on ice for 30 min and centrifuged at 25,000 $\times$ *g* for 20 min. The aqueous supernatant was filtered through two layers of facial tissue to remove remaining debris, and nucleic acids were precipitated from solution by the addition of a 0.6-volume of cold isopropanol.

Precipitated nucleic acids were sedimented by centrifugation at 20,000 $\times$ *g* for 15 min, resuspended in 2–3 volumes of 50 mM Tris-HCl, pH 8.0, 20 mM EDTA, then extracted twice with equal volumes of Tris-neutralized phenol:chloroform: isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1) alone. Nucleic acids were precipitated again with isopropanol, collected by centrifugation as before, then dried *in vacuo* and resuspended in TE (10 mM Tris, 1 mM EDTA; pH 8.0) buffer containing 50 μg of RNAse per milliliter. After incubation at 37° C for 1 hr, DNA solutions were stored at 4° C.

Separation of WB MLO DNA from host DNA. Cesium chloride-bisbenzimide buoyant density gradient centrifugation, which separates A+T rich MLO DNA from mixtures with plant DNA (Kollar *et al.* 1990; Sears *et al.* 1989), was used to obtain fractions enriched for WB MLO DNA. Approximately 250 μg of DNA sample was added to each 5.1-ml quick-seal polyallomer gradient tube containing both 892 mg of CsCl and 127 μg of bisbenzimide per milliliter of TE, pH 8.0 buffer. The gradients were adjusted to a final density of 1.68 g/cm^3 with additional TE buffer, and centrifuged at 227,640 $\times$ *g* for 24 hr at 20° C in a Beckman VTi 65.2 rotor. Putative WB MLO DNA, appearing as a unique band uppermost in gradients of DNA from infected plants, was first visualized by UV light, then removed and recentrifuged two more times, by using the same conditions just described, except that no additional bisbenzimide was added. The MLO DNA band was retrieved after final recentrifugations extracted 5$\times$ with equal volumes of NaCl-saturated isopropanol, and diluted with 3 volumes of sterile distilled water. The DNA was precipitated overnight at −20° C after the addition of 20 μg of glycogen (Boehringer-Mannheim Biochemicals, Indianapolis, IN) and cold isopropanol. Upon collection by centrifugation at 20,000 $\times$ *g* for 15 min, the DNA pellet was rinsed with 80% ethanol, dried, resuspended in TE, pH 7.5 buffer and quantified according to methods of Maniatis *et al.* (1982).

Molecular cloning of MLO DNA and screening of recombinants. Approximately 2 μg of CsCl-purified WB MLO DNA was partially digested with the restriction endonucleases *Eco*RI-*Hin*dIII for 1 hr at 37° C. A sample (625 ng) of the resulting fragments was ligated with *Eco*RI-*Hin*dIII-digested pUC19, using a 10:1 vector:insert ratio,

for 16 hr at 15° C. A 0.1-volume of the ligation mixture was used to transform DH5α *Escherichia coli* cells (Gibco BRL Life Technologies Inc., Gaithersburg, MD) according to the manufacturer's instructions. Recombinants were identified as white colonies on Luria-Bertani agar containing ampicillin (100 μg/ml) and X-gal (32 μg/ml).

To tentatively identify recombinants containing cloned fragments of MLO DNA, plasmid DNA was extracted from 80 randomly selected white colonies and screened by dot hybridizations using ^{32}P-labeled WB MLO DNA and total DNA from healthy plants as probes. Probe DNA was labeled by nick translation with [^{32}P]dATP according to the manufacturer's instructions (NEN Research Products, Boston, MA). Recombinant plasmid DNA was extracted by the alkaline lysis method of Maniatis *et al.* (1982) from 5 ml of Luria-Bertani broth (containing 100 μg of ampicillin per milliliter) cultures of each individual colony. Approximately 50 ng of plasmid DNA of each preparation was denatured by boiling for 10 min in 50 μl of TE buffer that contained 3 μl of 2 N NaOH, immediately cooled on ice for 5 min, neutralized by the addition of 3 μl of 2 M Tris, pH 7.0, diluted with an equal volume of 12× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and blotted onto duplicate nylon (Nytran) membranes (Schleicher and Schuell, Inc., Keene, NH) by using a Bio-Dot manifold (Bio-Rad Laboratories, Richmond, CA). Membranes were air-dried, then baked at 80° C for 30 min.

Selected recombinant plasmids that hybridized with ^{32}P-labeled MLO DNA, but not with labeled DNA from healthy plants, were examined further for use as probes. The sizes of inserts of each recombinant were determined by restriction endonuclease digestion and agarose gel (0.75% Sea Kem GTG; FMC Bioproducts, Rockland, ME) electrophoresis. Recombinant plasmids (p) used as probes in subsequent Southern hybridizations were labeled by nick translation as described previously. When inserts (I) only from recombinant plasmids were used as probes, these were labeled with [^{32}P]dATP, using random oligoprimers (Random Primed DNA Labeling Kit, Boehringer-Mannheim).

DNA hybridizations. To extract total plant DNA, 1–3 g of shoots (pigeon pea), immature leaf bases (palm), leaf midribs and petioles (periwinkle, celery), or stems (corn) were pulverized in liquid nitrogen and extracted as previously described by Dellaporta *et al.* (1983). DNA samples for dot hybridizations were resuspended in 6× SSC, whereas DNA samples for Southern hybridizations were resus-

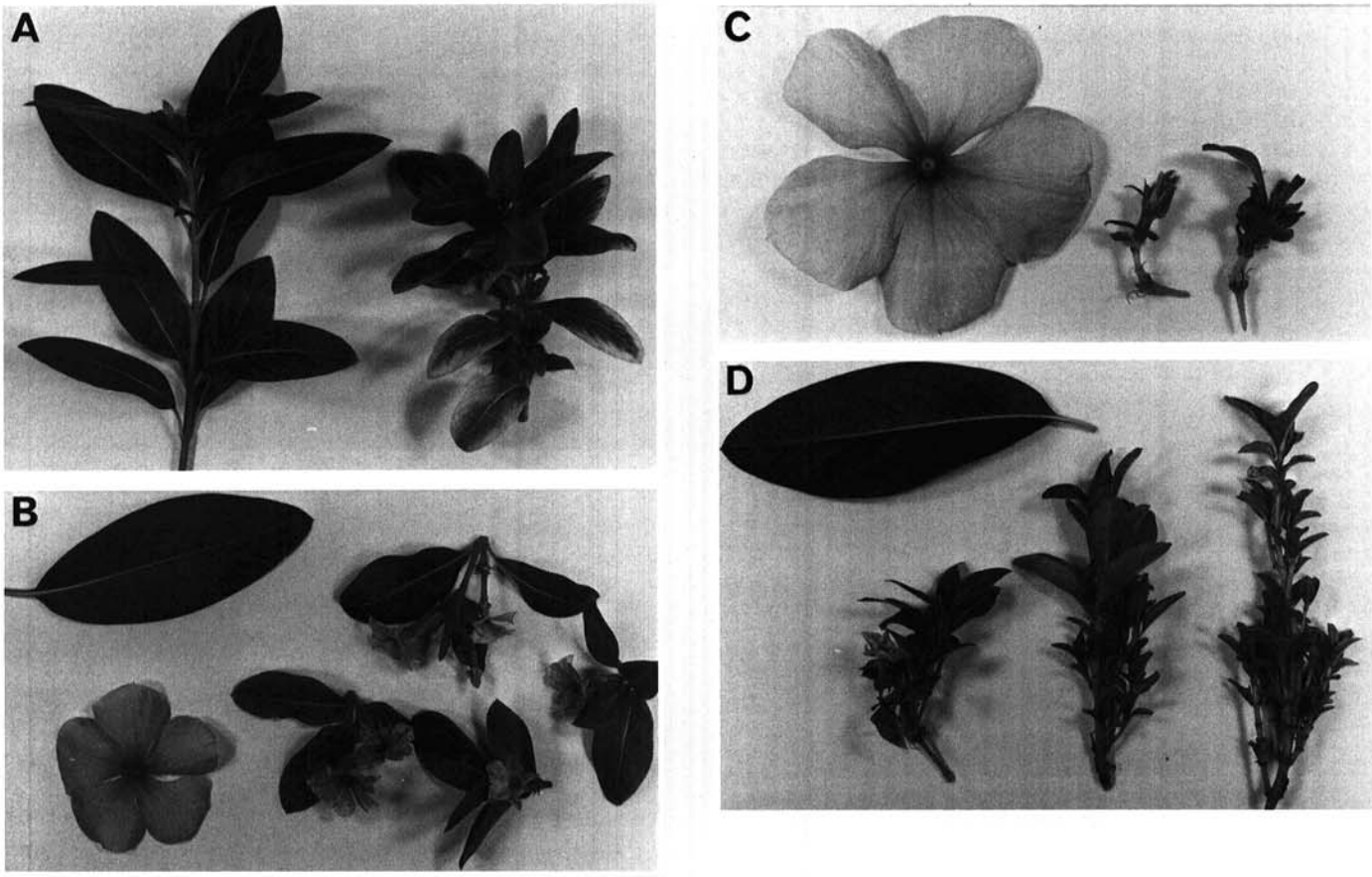

Fig. 1. Appearance of symptoms in *Catharanthus roseus* (periwinkle) after dodder graft inoculation with mycoplasmalike organisms from witches'-broom diseased *Cajanus cajan* (pigeon pea). **A,** Foliar yellowing and production of lateral shoots from axillary buds, normal shoot on left. **B,** Floral virescence showing greening and dwarfing of petals, normal flower and leaf on left. **C,** Small phylloid flowers bearing leaflike petals, normal flower on left. **D,** Proliferation of virescent flowers and stems with both short internodes and dwarf leaves from floral organs producing witches'-brooms, normal leaf on upper left.

pended in TE, pH 7.5 buffer.

For Southern hybridizations, DNA samples from healthy host plants and plants infected with WB, WBP, EAY, FWB, MBSF, or LY were electrophoresed in 0.75% Sea Kem GTG agarose gels and blotted (Southern 1975) to Nytran membranes by using 6× SSC as the transfer buffer. For dot hybridizations, approximately 2 μg of each test DNA was denatured and neutralized as previously described for plasmid DNA, and then blotted as a series of twofold dilutions (initial DNA dilution = 1 μg) to Nytran membranes. All membranes were air-dried, baked at 80° C for 30 min, washed in 0.1× SSC containing 0.5% SDS at 65° C for 1 hr, then prehybridized and hybridized as described below.

Prehybridizations of membranes were performed at 68° C for 4 hr or overnight, according to the manufacturer's instructions. For hybridizations, this solution was replaced with a similar solution that also contained the denatured, labeled probe DNA. Hybridizations with labeled probes were performed for 16 hr at 68° C, after which membranes were subjected to moderate stringency wash conditions consisting of two washes in 2× SSC, 0.1% SDS at 25° C (30 min each wash), one wash in 0.2× SSC, 0.1% SDS at 55° C (30–60 min) and once again in 0.2× SSC, 0.1% SDS at 25° C (30 min). Membranes were then sealed in plastic wrap and exposed to X-Omat AR diagnostic film (Eastman Kodak, Rochester, NY) with an intensifier screen (Lightning Plus, DuPont, Newark, DE).

RESULTS

Transmission of the WB MLO. Development of foliar yellowing in periwinkle 6–8 wk after transmission tests were initiated provided the first evidence that MLO infection of these plants had occurred. The appearance of this symptom was closely followed by the production of virescent flowers and a proliferation of small shoots from axillary buds on new growth (Fig. 1A,B). When symptoms further intensified, the development of small phylloid flowers (Fig. 1C) was also evident, as was the development of shoots with shortened internodes and stunted leaves from floral organs (Fig. 1D), which gave a pronounced witches'-broom appearance to affected branches. Grafting of witches'-broomed scions to additional healthy periwinkle plants resulted in the appearance of identical symptoms within 21–28 days, as infections progressed systemically in these plants. Transmission electron microscope examination of petiole vascular tissues from symptomatic plants revealed the presence of typical polymorphic MLOs in phloem sieve tube elements; these bodies were absent from similar tissues of noninoculated plants (data not shown).

Isolation and cloning of MLO DNA. Density gradient centrifugation of DNA from extracts of healthy periwinkle tissues resulted in the separation of one major band with a buoyant density (ρ) of approximately 1.6 g/cm^3 located between 21.5 and 29 mm from the top of the gradient tube and two minor, less buoyant bands, at 5 and 9 mm below the main DNA band, respectively. Centrifugation of DNA from WB-affected plants produced an identical banding pattern to that observed for healthy extracts, except that an additional faint band (ρ = 1.59 g/cm^3), located at 2.5 mm above the main host band, was also observed. This unique band was removed from each of three gradients, pooled, and recentrifuged as before, revealing two sharply defined, narrowly separated bands. The uppermost of these bands corresponded in approximate location and buoyant density to the unique band that was visualized following the earlier separation. When only the uppermost band was removed and recentrifuged, a single discrete band was resolved. This putative MLO DNA was used for molecular cloning. An estimated 5 μg of MLO DNA was recovered from the final gradient. This quantity represented only 0.67% of the total DNA loaded in the initial gradients.

A total of 440 recombinant *E. coli* colonies were obtained from cloning MLO DNA isolated from WB-diseased periwinkle plants. Labeled DNA from healthy plants hybridized with recombinant plasmids from 10 of 80 arbitrarily selected, individual colonies, whereas labeled MLO DNA hybridized with recombinant plasmids from all 80 colonies.

Table 1. Results from dot hybridizations of ^{32}P-labeled cloned pigeon pea witches'-broom mycoplasmalike organism (MLO) DNA to DNA extracts from healthy plants or plants affected by various MLO-associated diseases

Probe DNA[a] (insert size, kbp)	Hybridization with DNA extracts from[b]											
	HP	HPP	WB	WBP	EAY	DAY	FWB	MBSF	MBST	LY	VR	WX
WBA3I(1.1), WBA9I(2.5), WBA16I(1.5), pWBA27(0.6), WBA28I(1.3), WBA36I(3.2), pWBA40(0.5), WBB3I(2.0), WBB5I(2.1)	−	−	+	+	−	−	−	−	−	−	−	−
WBA13I(1.6), pWBA48 (1.7), WBB7I(2.9)	−	−	+	+	W	−	W	−	−	−	−	−
WBB2I (3.1)	−	−	+	+	+	−	+	−	−	−	−	W
WBA12I(1.4)	−	−	+	+	+	+	+	W	W	−	W	W

[a] Probes consisted of either cloned MLO DNA inserts (I) only, or the recombinant plasmids (p).

[b] +, Positive hybridization; W, weak hybridization; −, no hybridization signal. Each data point represents results of hybridizations of labeled probes with DNA from: HP, healthy periwinkle; HPP, healthy pigeon pea; WB, witches'-broom infected periwinkle; WBP, witches'-broom infected pigeon pea; periwinkle with EAY, Eastern aster yellows; DAY, Western dwarf aster yellows; FWB, Florida periwinkle witches'-broom; MBSF, sweet corn with maize bushy stunt, Florida isolate, or MBST, Texas isolate; LY, Chinese fan palm (*Livistona chinensis*) with lethal yellowing; VR, beet leafhopper-transmitted virescence agent infected periwinkle; WX, Western-X infected celery. None of the probes hybridized to DNA from healthy corn, palm, or celery.

Also, MLO DNA, but not healthy plant DNA, produced discernible hybridization signals with pUC19 plasmid DNA alone when membranes were subjected to moderate stringency (55° C) posthybridization wash conditions. By increasing the penultimate wash temperature to 60° C or higher, hybridizations between MLO DNA probe and pUC19 was virtually eliminated with little or no reduction in signal intensity from recombinant plasmids. Of the 70 apparently disease-specific recombinants, relatively strong hybridization signals were produced by 21 recombinants that were clearly evident after only 2 hr of autoradiography. Moderate signals were observed for 46 recombinants, and three gave only weak signals during this time.

Characterization of probes. Fourteen recombinant plasmids were selected to include a range of cloned DNA insert sizes. Each insert was apparently unique, based on size analysis following agarose gel electrophoresis of *Eco*RI-*Hin*dIII restriction endonuclease digests. To determine their specificity for detecting WB MLO DNA, each of the 14 recombinant plasmids or their cloned MLO DNA inserts was used as probes in dot hybridizations, with total undigested DNA extracts from plants affected by several other MLO-associated diseases. A summary of results from these hybridizations is given in Table 1. Results representative of dot hybridizations conducted with two of these probes are illustrated in Figure 2. No hybridizations were observed between any probe and DNA from healthy host plants, which included periwinkle, pigeon pea, palm, celery, or corn (data for only periwinkle and pigeon pea are given). Nine of the 14 probes hybridized to DNA extracts from WB-affected periwinkle and pigeon pea but not to extracts from any of the additional MLO-infected plants that were examined. Of the remaining probes, WBA13I, pW8A48, and WBB7I also hybridized to extracts containing DNA

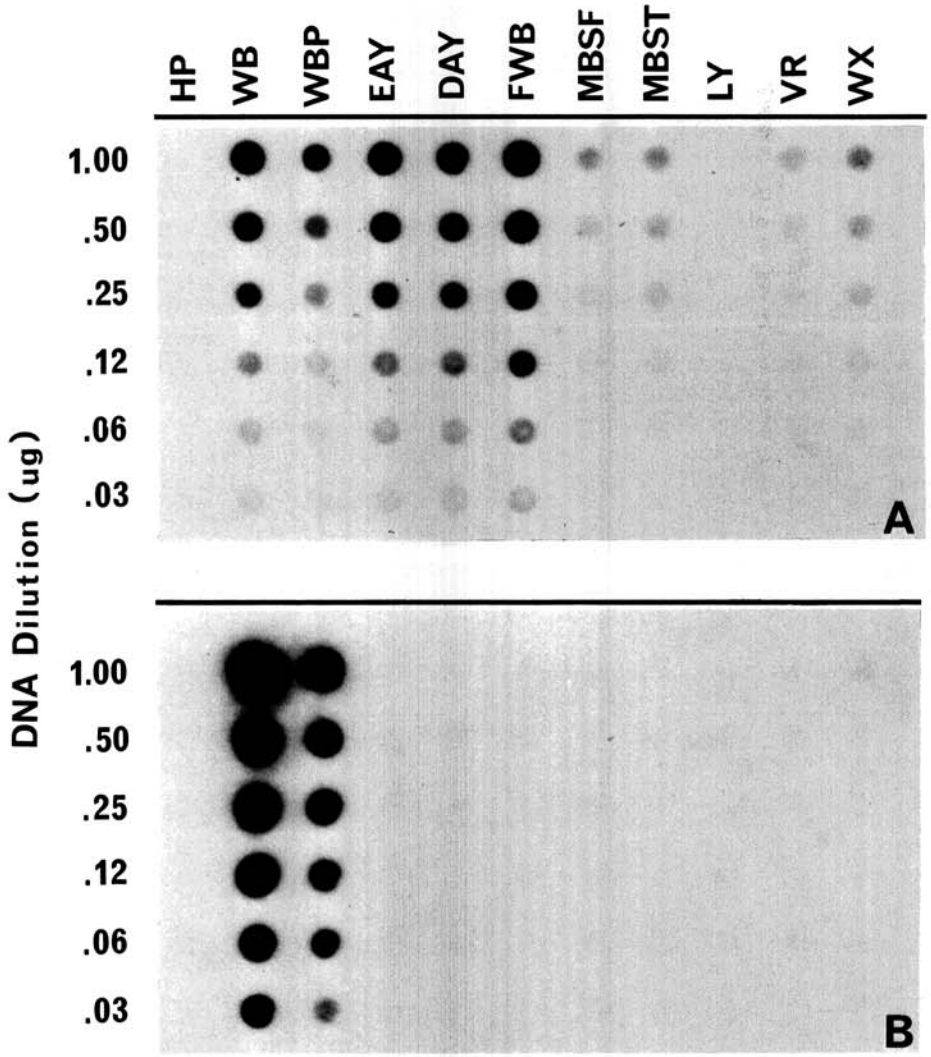

Fig. 2. Dot hybridizations of ^{32}P-labeled cloned DNA probe, **A,** WBA28I (a 1.3-kb insert of extrachromosomal WB MLO DNA), and, **B,** WBA12I (a 1.4-kb insert of chromosomal WB MLO DNA) to DNA extracts from healthy *Catharanthus roseus* (periwinkle) plants or from host plants affected by various mycoplasmalike organism (MLO)-associated diseases. HP, healthy periwinkle; WB, witches'-broom MLO-infected periwinkle; WBP, witches'-broom MLO-infected pigeon pea; periwinkle plants with: EAY, Eastern aster yellows; DAY, Western dwarf aster yellows; FWB, Florida periwinkle witches'-broom; MBSFL, Florida maize bushy stunt; MBST, Texas maize bushy stunt; LY, *Livistona chinensis* (Chinese fan palm) with lethal yellowing, VR, beet leafhopper-transmitted virescence agent; corn plants with: WX, celery with Western-X; MLOs.

of Eastern aster yellows or Florida periwinkle witches'-broom MLOs, producing discernible, albeit weak, hybridization signals. Only probes WBA12I and WBB2I gave signals with any of the additional MLOs examined that are not known to be indigenous to Florida. Both probes hybridized to DNA extracts from Western-X infected celery; probe WBA12I also hybridized to DNA from periwinkle infected with Western dwarf aster yellows and, to a much lesser degree, with similar extracts containing DNA of the beet leafhopper-transmitted virescence agent.

Each probe was also used in Southern hybridizations against blots of total undigested DNA extracted from healthy periwinkle plants, WB-affected periwinkle and pigeon pea, as well as extracts from various plant hosts with MLO-associated diseases indigenous to Florida (Fig. 3). Distinct differences in the patterns of hybridization between probes WBA12I and WBA28I and MLO DNA were evident. Probe WBA12I hybridized to blots of WB-affected periwinkle and pigeon pea, Florida periwinkle witches'-broom, and Eastern aster yellows DNA extracts at locations corresponding to the position of the visible undigested chromosomal DNA bands in the adjacent gel (Fig. 3A,B). By comparison, signals produced by probe WBA28I indicated that this probe hybridized to bands of lower molecular weight extrachromosomal (possibly plasmid) DNA, and that hybridizations were limited only to bands present in DNA from WB-affected periwinkle and pigeon pea (Fig. 3A,C).

Detection of the WB MLO in pigeon pea. Dot hybridizations were used to assess the utility of probes for detecting the WB MLO in field-collected pigeon pea samples. Figure 4 illustrates hybridization signals obtained by screening replicate membranes containing DNA samples from nine WB-affected plants by using either WBA28I or WBA12I as probes. Neither probe gave any discernible hybridization signal with DNA from healthy pigeon pea or periwinkle tissues. Both probes gave positive signals with all nine samples that were tested and with DNA from WB-affected periwinkle included as a positive control. Furthermore, the intensity of signals was observed to vary considerably from sample to sample and depended on the particular probe used.

DISCUSSION

In previous reports on probe development, strategies for obtaining sources of MLO DNA suitable for molecular cloning have included preparation of host concentrates enriched with intact MLOs (Davis *et al.* 1988a; Lee and Davis 1988), buoyant density separations of MLO DNA from crude DNA extracts (Kollar *et al.* 1990), or strategies that combine both of the above (Kirkpatrick *et al.* 1987; Sears *et al.* 1989). Using the latter approach, our methods for extraction of plant tissues and subsequent isolation of total DNA from MLO-enriched host fractions were essentially the same as those developed by Kirkpatrick *et al.* (1987). Separations of MLO DNA from mixtures with plant DNA by CsCl-bisbenzimide density gradient centrifugation were achieved by the procedure of Garber and Yoder (1983) for fractionation of *Cochliobolus*

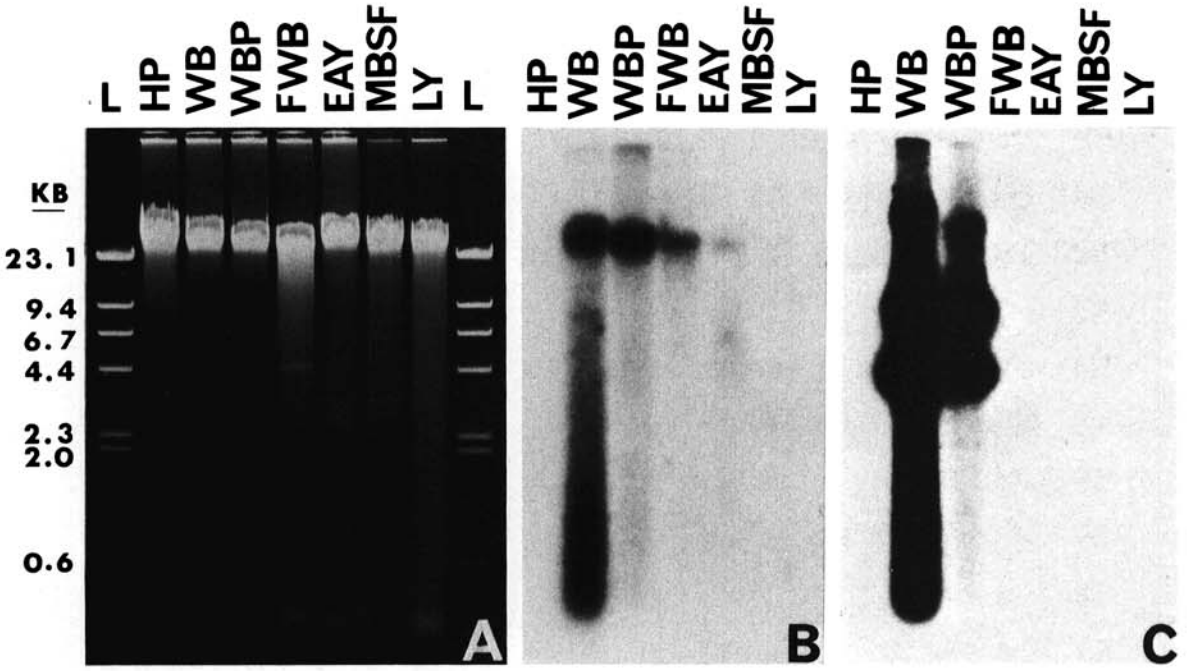

Fig. 3. Southern blot analyses of total undigested DNA extracted from healthy *Catharanthus roseus* (periwinkle) plants or from various host plants affected by mycoplasmalike organism (MLO)-associated diseases indigenous to Florida. Undigested DNA extracted from plants, **A,** electrophoresed and stained with ethidium bromide; **B,** then hybridized with ^{32}P-labeled cloned DNA probe WBA12I (a 1.4-kb insert of chromosomal WB MLO DNA); and, **C,** WBA28I (a 1.3-kb insert of extrachromosomal WB MLO DNA). L, lambda DNA-*Hin*dIII fragments; DNA extracts from: HP, healthy periwinkle; WB, witches'-broom MLO-infected periwinkle; WBP, witches'-broom MLO-infected pigeon pea; periwinkle plants with: FWB, periwinkle witches'-broom; EAY, Eastern aster yellows; MBSF, corn plants with maize bushy stunt; LY, *Livistona chinensis* (Chinese fan palm) with lethal yellowing; MLOs.

heterostrophus (Drechs.) DNA components. Although this procedure incorporated considerably more bisbenzimide than was recommended by Kollar *et al.* (1990) for density gradient separations of the apple proliferation agent, and formed visible precipitates upon addition to gradients, the final observed DNA separations we achieved were quite similar to those described for apple proliferation and other MLOs from periwinkle. Whether precipitate formation represented a loss of A+T rich DNA from solution as a result of the quantity of fluorochrome used was not determined. Nevertheless, the estimated percent yield of WB MLO DNA from our gradient separations of total DNA extracts was within the range described for the apple proliferation agent.

Our primary concern in screening DNA probes for detection of the WB MLO was their relative sensitivity and specificity. Our results from several probes indicate that they are able to consistently detect DNA of the pathogen in 1 μg of host DNA, a quantity extracted from the equivalent of 200 mg of host tissue. However, probe detection sensitivity varied according to the plant sample tested; this was particularly evident after screening field-collected samples of naturally infected pigeon pea. Between sample variation was interpreted to reflect differences in MLO titer in infected plants, possibly caused by an uneven distribution of the MLO in host tissues, differences in age of plant infections, or both. Detection sensitivity also varied according to the particular probe used. Thus, hybridization signals produced by probes consisting of extrachromosomal DNA of the WB MLO were generally stronger than those produced by chromosomal DNA probes, which may be attributed to the multiple copy number of extrachromosomal DNA in the MLO, as has been reported previously for other MLOs (Davis *et al.* 1988a; Sears *et al.* 1989; Davis *et al.* 1990a). The apparent specificity of nine of the 14 probes characterized thus far to discriminate the WB MLO from other MLOs, particularly those that are indigenous to south Florida, should provide for accurate disease diagnosis. It should also facilitate the further analysis and identification of possible insect vectors and plant hosts of the pathogen.

After the discovery of extrachromosomal DNA in an MLO (Davis *et al.* 1988a), the occurrence of similar DNA (apparently plasmids) in numerous other MLOs has been described (Davis *et al.* 1990a; Kuske *et al.* 1990; Lee and Davis 1988; Lee *et al.* 1990). Furthermore, nucleotide sequence similarities between these DNAs appears to be widespread among MLOs, especially those MLOs comprising the aster yellows cluster (Davis *et al.* 1990a; Lee and Davis 1988; Lee *et al.* 1990). However, extrachromosomal DNA of the beet leafhopper-transmitted virescence agent reportedly exhibits little or no sequence

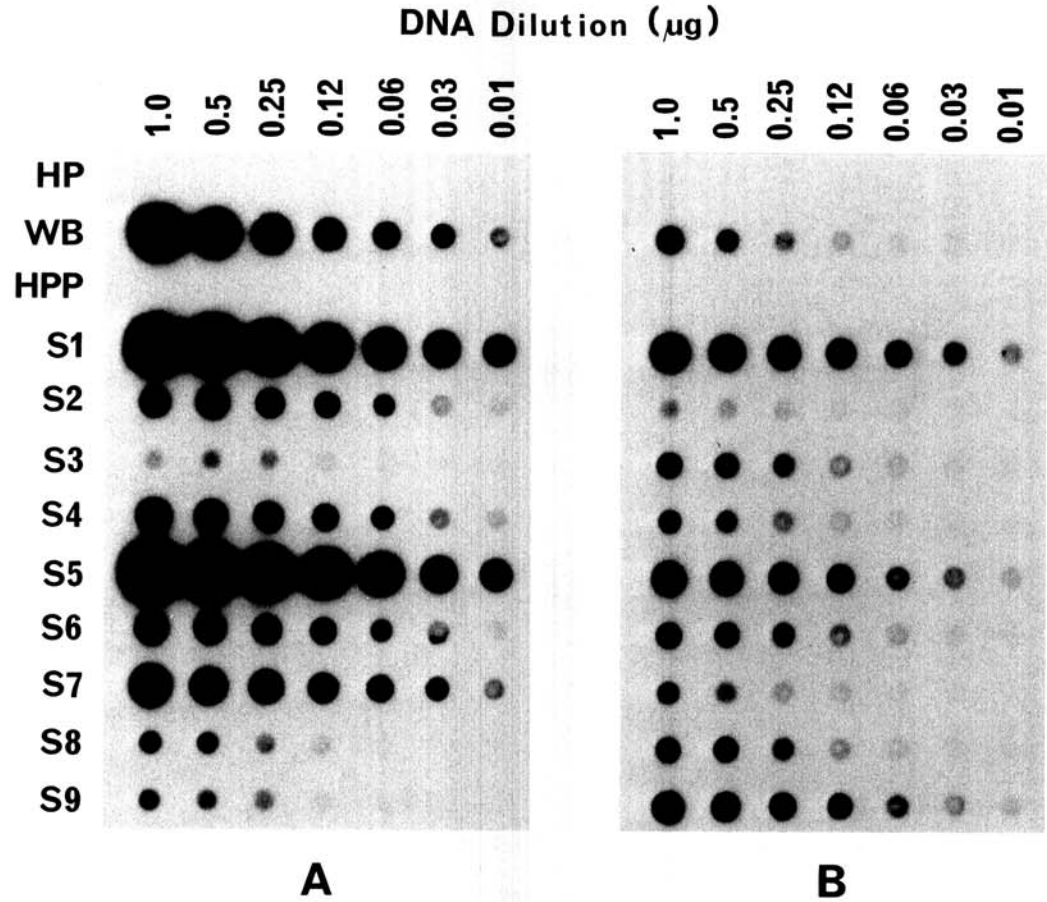

Fig. 4. Detection of witches'-broom (WB) mycoplasmalike organisms (MLOs) in field samples of *Cajanus cajan* (pigeon pea) plants from commercial fields in Dade County, Florida, by dot hybridization assays using cloned DNA probes. Blots were probed with ^{32}P-labeled cloned DNA probe, **A,** WBA28I (a 1.3-kb insert of extrachromosomal WB MLO DNA); **B,** WBA12I (a 1.4-kb insert of chromosomal WB MLO DNA). HP, healthy periwinkle; WB, witches'-broom MLO-infected periwinkle; HPP, healthy pigeon pea; S1-S9, witches'-broom affected pigeon pea plants.

similarity with extrachromosomal DNA of Western aster yellows MLOs (Shaw *et al.* 1990). In this study, Southern hybridization analyses provided the first evidence for the presence of extrachromosomal DNA in the WB MLO and confirmed that nine of the 14 probes that were examined consisted of fragments of this DNA. The hybridization data also revealed that each of these probes hybridized specifically to WB MLO DNA only. Thus, little or no nucleotide sequence similarity exists between these cloned fragments of extrachromosomal WB MLO DNA and extrachromosomal DNAs of maize bushy stunt, Eastern aster yellows, Western dwarf aster yellows, and the beet leafhopper-transmitted virescence agent.

The combined hybridization data revealed that five of the remaining probes, each consisting of cloned fragments of WB MLO chromosomal DNA, shared some nucleotide base pair similarity with DNA of other MLOs that occur in Florida and California, indicating that the MLOs may be genetically related. Signals were strongest between WB MLO probes and DNA of MLOs that occur in Florida; specifically, Eastern aster yellows and periwinkle witches'-broom. Interestingly, symptoms induced in periwinkle by the WB MLO were quite similar overall to symptoms of Eastern aster yellows and periwinkle witches'-broom in this host, but were also sufficiently distinct that this disease could be differentiated from the other two. Further work is needed to determine the true nature of the relationships among these various MLOs.

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