X-Disease Confirmation and Distribution in Chokecherry in North Dakota

Y. H. Guo, Graduate Student, Department of Plant Sciences; **J. A. Walla,** Research Associate, Department of Plant Pathology; **Z.-M. Cheng,** Assistant Professor, Department of Plant Sciences, North Dakota State University, Fargo 58105; and **I.-M. Lee,** Research Plant Pathologist, USDA-ARS, PSI BARC-West, Molecular Plant Pathology Laboratory, Beltsville, MD 20705

ABSTRACT

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A phytoplasma was observed in diseased chokecherry plants in North Dakota by electron microscopy and identified with a phytoplasma-specific polyclonal antibody and with restriction fragment length polymorphism (RFLP) analysis of 16S ribosomal DNA (16S rDNA) sequence amplified with the nested polymerase chain reaction (PCR). The phytoplasma was partially purified directly from infected chokecherry plants for use in raising a polyclonal antibody in mice. The specificity of the polyclonal antibody against phytoplasma was shown by its reaction with diseased, but not with healthy, chokecherries upon immunofluorescence staining. Immunofluorescence staining demonstrated the chokecherry phytoplasma in North Dakota to be serologically related to phytoplasmas associated with eastern X, western X, milkweed yellows, and pigeon pea witches'-broom disease. The antibody did not react with phytoplasmas associated with ash yellows, aster yellows, elm yellows, goldenrod yellows, spirea stunt, and palm lethal yellowing. The identity as X-disease phytoplasma was supported by RFLP analysis of the amplified products obtained from the nested-PCR by using two phytoplasma-universal primer pairs. The RFLP patterns from *Mse*I and *Hpa*II digestion indicated that the X-disease phytoplasma in chokecherry belongs to subgroup A in the 16S rRNA group III. The presence of X-disease symptoms, electron microscopic observation of phytoplasmas in symptomatic plants, detection with the polyclonal antibody, and detection and identification by PCR-RFLP analysis provided the first confirmation that chokecherry X-disease occurs in the Great Plains region. Chokecherry X-disease was found to be widespread in North Dakota based on positive serological detection of X-disease phytoplasma in chokecherry samples collected throughout North Dakota. X-disease in chokecherry was also confirmed in South Dakota and in Saskatchewan, Canada.

Additional keywords: phytoplasma purification, *Prunus virginiana*, serology

Chokecherry (*Prunus virginiana* L.) is an important native woody species that occurs in natural and planted stands in the northern Great Plains region of North America (30). Natural stands commonly occur in dense clonal clumps and provide important habitat and food for wildlife. About one million chokecherry seedlings are grown annually in the Great Plains for agroforestry plantings such as field and farmstead windbreaks, stream bank stabilization, and wildlife habitat. A disease of chokecherry, long assumed to be X-disease (e.g., 26), is considered to be the most limiting problem of chokecherry grown in the northern Great Plains. X-disease symptoms include red and stunted leaves and shoots, deformed and discolored fruit, and reduced winter hardiness of current-year shoots, followed by branch and stem dieback and plant mortality. After the disease was introduced into a planting, 80% of the chokecherry plants developed symptoms within 3 years and 50% died within 8 years (23).

X-disease is an economically important and geographically widespread disease of stone fruit trees in many areas of North America. It occurs most notably in peach (*P. persica* (L.) Batsch), sweet (*P. avium* (L.) L.) and sour (*P. cerasus* L.) cherries, chokecherry, and nectarine (*P. persica* (L.) Batsch var. *nucipersica* (Suckow) C.K. Schneid.) (4). The association of a phytoplasma, formerly known as a mycoplasmalike organism (MLO), with X-disease was first reported in 1970 from infected celery (*Apium graveolens* L.) and leafhoppers (21). Evidence that the X-disease phytoplasma occurs in chokecherry was first presented by Granett and Gilmer (5), who published micrographs of phytoplasmas present in infected chokecherry in the northeastern United States. The occurrence of X-disease symptoms in chokecherry has since been reported in most areas of the United States (23,25). Symptomatic plants were first reported in North Dakota in 1949 (26). Because they have not been cultured in vitro, phytoplasmas have usually been differentiated by symptomology, host range, and vector–pathogen relationships. The X-disease phytoplasmas are commonly divided into two strains, eastern and western, depending on the geographical region and leafhopper vector (25,29). Association of phytoplasmas with symptomatic chokecherries from the Great Plains has been assumed but not confirmed. Assuming such association, the expected identity would be X-disease phytoplasma, based on symptomology in this *Prunus* species. Currently available means to determine if the disease is associated with X-disease phytoplasma are serology and molecular biology. Polyclonal antibodies, a serological tool, have been produced against X-disease phytoplasma-enriched extracts derived from infected plants (28) and insect vectors (13). Their application in identification and detection of X-disease phytoplasma has been limited because those antibodies had substantial cross-reactivity with healthy host antigens. This problem can be avoided by the use of monoclonal antibodies or DNA probes from X-disease phytoplasma (10,14). Analysis of restriction fragment length polymorphisms (RFLPs) of 16S rDNA fragment derived from polymerase chain reaction (PCR) has been used to differentiate phytoplasmas from different hosts (6–9,17–20).

The purposes of this paper were to determine if phytoplasmas were associated with the described chokecherry disease in North Dakota by transmission electron microscopic observation, and to identify the phytoplasma by raising a polyclonal antibody against it and by analyzing the RFLP of the nested-PCR products (16S rDNA) amplified from infected chokecherries. The spatial distribution of the phytoplasma in North Dakota and temporal changes in titer of the phytoplasma in plant tissues were also examined.

MATERIALS AND METHODS

Chokecherry samples for examination of phytoplasma presence. Trees in chokecherry plantings with symptoms indicative of X-disease were identified near Fargo, North Dakota, in 1990. Two sample trees tested positive in 1990 with an X-disease phytoplasma-group-specific DNA probe, but negative with an X-disease phytoplasma-specific DNA probe (J. A. Walla, NDSU, and B. C. Kirkpatrick,

Corresponding author: Z.-M. Cheng
E-mail: zcheng@plains.nodak.edu

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UC-Davis, CA, *unpublished*). Samples were collected periodically in 1993 and 1994 from seven trees, including those sampled in 1990, to determine whether phytoplasmas were present and, if so, to examine seasonal fluctuation in presence and titer of phytoplasmas in them. These collections are described in Tables 1 and 2. Trees 1 and 2 were previously tested in 1990 and had typical X-disease symptoms. Trees 3 and 5 had symptoms like those expected of X-disease, as noted above, except that the diseased leaves were yellow rather than the typical red color. Trees 4 and 6 had an unusual growth pattern, but the symptoms were not typical of X-disease. Tree 7 had no detectable disease symptoms and was used as a healthy control.

Phytoplasmas for comparison with the phytoplasma from chokecherry in North Dakota. Phytoplasma strains used for comparison with chokecherry phytoplasma from North Dakota were kindly provided by other researchers. Ash yellows phytoplasma (AshY2), aster yellows phytoplasma (New Jersey strain), and western X-disease phytoplasma in infected periwinkles (*Catharanthus roseus* (L.) G. Don) and eastern X-disease phytoplasma in celery (*Apium graveolens* L.) were provided by T. A. Chen, Rutgers University, New Brunswick, NJ. A phytoplasma in periwinkle that was insect-transmitted from pear showing decline symptoms (24) and that is genetically and serologically similar to western X-disease phytoplasma (B. C. Kirkpatrick, *personal communication*), was provided by B. C. Kirkpatrick, University of California at Davis. Elm yellows phytoplasma (EY1), Canada peach X-disease phytoplasma (CX), milkweed yellows phytoplasma (MW1), goldenrod yellows phytoplasma (GR1), and spirea stunt phytoplasma (SP1), all in periwinkles, and eastern X-disease phytoplasma in chokecherry were provided by W. A. Sinclair, Cornell University, Ithaca, NY. Palm lethal yellowing phytoplasma in Manila palm (*Veitchia merrillii* (Becc.) H.E. Moore) and Florida pigeon pea witches'-broom (PPWB) phytoplasma in periwinkle were provided by N. A. Harrison, University of Florida, Fort Lauderdale.

Table 1. Confirmation of X-disease phytoplasma in chokecherry in North Dakota as determined by symptom presence, transmission electron microscope (TEM) examination, immunofluorescence (IF) staining, and restriction fragment length polymorphism (RFLP) analyses of the nested-polymerase chain reaction (PCR) amplified products from chokecherries

Tree accession[a]	X-disease symptoms[b]	Observation with TEM[c]	IF staining[d]	PCR-RFLP analysis[e]
Tree 1	T	+	+	+
Tree 2	T	+	+	NT
Tree 3	T	+	+	+
Tree 4	AT	–	–	NT
Tree 5	T	NT	+	+
Tree 6	AT	NT	–	–
Tree 7	None	NT	–	–

[a] Samples were collected during July and August, 1993 when symptoms were fully developed.
[b] T = typical X-disease symptoms, which include stunted red (or yellow) leaves, stunted shoots, deformed and discolored fruit, reduced winter hardiness of current shoots, and branch and stem dieback; AT = atypical symptoms, which include sparse and stunted foliage.
[c] + = phytoplasma present, – = phytoplasma absent, NT = not tested.
[d] + = positive; – = negative, immunofluorescence staining with a polyclonal antibody against the chokecherry phytoplasma.
[e] RFLP analysis of nested-PCR amplified products with four restriction enzymes (see description in text). + = RFLP profiles matched an X-disease phytoplasma group; – = no product was amplified in nested-PCR or RFLP profiles did not match an X-disease phytoplasma group.

Table 2. Seasonal fluctuation of X-disease phytoplasmas in chokecherry in North Dakota on different collection dates in 1993 as determined with a transmission electron microscope (TEM) and immunofluorescence (IF) staining with the polyclonal antibody against the chokecherry phytoplasma[a]

Collection date[b]	Phytoplasma presence and titer under TEM and IF					
	Tree 1		Tree 2		Tree 3	
	TEM[c]	IF[d]	TEM	IF	TEM	IF
4/?	–	–	–	–	NT	NT
5/27	L	?	L	+	L	+
6/18	L	?	H	++	H	++
7/27	M	+	H	++	H	++
8/11	NT	+	NT	++	NT	++

[a] The results of IF test in 1994 were similar to those from 1993, so were not shown.
[b] Growth stages and maximum symptom development at each collection date: mid-April, twigs dormant, buds not expanded; 27 May, new shoots 2 to 5 cm long, flowering, no leaf discoloration; 18 June, new shoots fully expanded, fruit ca. 1/3 full size, leaves stunted and yellowing; 27 July, fruit full size, leaves stunted with some reddening; 11 August, fruit ripe, leaves stunted and quite red.
[c] Presence as observed by transmission electron microscopy: – = phytoplasmas not observed; L, M, H = phytoplasmas present at a low, moderate, or high titer; NT = not tested.
[d] Immunofluorescence staining: +, ++ = presence and relative intensity of immunofluorescence; – = absence of immunofluorescence; ? = indeterminable; NT = not tested.

Electron microscopy. Leaves collected from mid-April to late July in 1993 from chokecherry trees with symptoms typical and atypical of X-disease near Fargo, North Dakota, (Table 1, trees 1 to 4) were prepared for electron microscopy to determine the presence and titer of phytoplasmas. Petioles and midveins were dissected from at least three freshly collected leaves of each plant and sectioned into 1- to 2-mm-long pieces. Three tissue sections from each midvein were fixed in 3% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) at 4°C overnight and then postfixed in 1% osmium tetroxide for 2 h at room temperature. After dehydration in a graded acetone series, sections were embedded in Spurr's resin. Multiple ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (TEM). Multiple sections from each specimen were observed. Occurrence of typical phytoplasma cells resulted in a positive rating for presence. If the maximum number of phytoplasma cells per sieve tube element in any section was five or less, the titer was considered to be low. If the maximum number was more than 50, the titer was considered to be high. A titer between these two extremes was considered moderate.

Production of polyclonal antibody. *Antigen preparation.* A chokecherry tree (tree 2 in Table 1) that showed typical X-disease symptoms and was observed to have a high titer of phytoplasmas by electron microscopy was used as the source of phytoplasmas for polyclonal antibody production. A modification of a method described by Lee and Davis (16) was used to prepare phytoplasma antigen. Symptomatic leaves were collected in August 1993. Leaf veins (10 g) were cut with a sharp forceps from fresh chokecherry leaves and washed with sterile distilled water. The veins were placed in 200 ml of enzymatic solution containing 15% (vol/vol) pectinase, 2% (wt/vol) cellulase, and 1% (wt/vol) hemicellulase (Sigma Chemical Co., St. Louis, MO) in an isolation solution containing 0.6 M mannitol, 30 mM 3-(4-morpholino)-propanesulfonic acid (MOPS), and 1 mM calcium chloride (pH 5.5), and incubated for 12 to 24 h at 25°C. After incubation, vascular bundles were teased from the surrounding macerated parenchymatous cell mass using a fine forceps and transferred to additional cool isolation solution (pH 7.2). Bundles were gently ground in a mortar with a pestle, and cell debris was removed from the resulting macerate by centrifugation at 8,000 × *g* for 10 min. The clarified supernatant was centrifuged again at 16,000 × *g*

for 30 min at 4°C to sediment the phytoplasmas. The resulting cell pellet was washed once by resuspension in isolation solution, followed by centrifugation at 16,000 × *g* for 30 min. The cell preparation was resuspended in 0.5 ml of PBS, homogenized by ultrasonication, and used as antigen to immunize mice. The same extraction procedure was used on leaf veins from a healthy chokecherry tree. The resulting extract was diluted 100 times with PBS.

Production of polyclonal antibody. About 8-week-old BALB/c mice were immunized to raise the polyclonal antibody with the phytoplasma preparation. The antigen preparation (100 µl) was mixed with an equal volume of Freundt's complete adjuvant and administrated by intraperitoneal injection. Each mouse received two injections with an interval of 1 week between injections. On days 30 and 31 after initial injection, an additional 100 µl of antigen preparation was used for intravenous injections, except that adjuvant was not added. Blood was initially taken from the tail of the mice on day 36. Each subsequent bleeding was done 5 days after additional intravenous injections with 100 µl of antigen preparation. The blood was diluted 10 times with PBS and centrifuged in a microcentrifuge for 10 min to remove red cells. The supernatant was saved, stored at –20°C, and used as polyclonal antibody.

Testing the polyclonal antibody. Before use, the polyclonal antibody was diluted with the healthy chokecherry antigen preparation in PBS for cross-absorption. The optimum reaction dilution was determined by a series of twofold dilutions. The optimum dilution was considered to be the highest dilution at which infected plant material could readily be distinguished from noninfected plant material. The specificity of the polyclonal antibody was examined by comparing immunofluorescence staining reactions of phytoplasmas in chokecherry from North Dakota with those of X-disease phytoplasma and other phytoplasmas in chokecherry and other hosts. Noninfected (healthy) host tissues were also tested. These materials are listed in Table 3.

Freehand cross sections of leaf midribs, leaf petioles, or fruit peduncles from both diseased and healthy chokecherries, periwinkles, or palms were fixed in acetone for at least 10 min. These fixed sections (and those of celery provided by T. A. Chen) were washed with PBS one to three times. Polyclonal antibody was diluted 10-fold with healthy chokecherry extract in PBS to absorb host-specific antibodies. The sections were incubated with the cross-absorbed polyclonal antibody overnight at 4°C. After incubation, the sections were washed three times with PBS and stained with 50 µl of anti-mouse IgG + IgM conjugated with fluorescein isothiocyanate (Sigma Chemical Co.) at a dilution of 1:1,000 (vol/vol) for 30 min at 37°C. The stained sections were washed three times in PBS and examined by epifluorescence microscopy under ultraviolet light with main wavelength of 495 nm (10). About 10 of the thinnest sections were examined for each plant sample. If immunofluorescence clearly occurred in phloem tissues, the sample was rated positive. If no immunofluorescence was observed in phloem tissues, the sample was rated negative. If fluorescence that occurred in phloem tissues was not more intense and brighter green than the autofluorescence that occurred in xylem tissues, the sample was classified as indeterminable. Photomicrographs were made with an automatic photomicrographic system using black-and-white film (Kodak Tri-X pan ASA 400). Samples from leaf midribs or petioles from healthy plants and blood from a nonimmunized mouse were used as controls.

PCR-RFLP analysis. PCR-RFLP analysis was used to confirm the presence of X-disease phytoplasma in North Dakota and support the efficacy of the polyclonal antibody. Total nucleic acid from the leaves of chokecherry trees was extracted by the method described by Lee et al. (17). The nested-PCR assay (19) was used to detect X-disease phytoplasma DNA. A universal primer pair, R16F1/R0 (15), designed based on the 16S rRNA gene sequence was employed to initially amplify DNA from the extracted DNA. The

Table 3. Reactions of chokecherry phytoplasma polyclonal antibody with various phytoplasmas

Phytoplasma strains	Immunofluorescence[a]
Chokecherry phytoplasma from North Dakota	+
Healthy chokecherry	–
Eastern X-disease phytoplasma in celery	+
Eastern X-disease phytoplasma in chokecherry	+
Western X-disease phytoplasma in periwinkle	+
Canada peach X-disease phytoplasma in periwinkle	+
Phytoplasma in periwinkle isolated from pear in California	+
Milkweed yellows phytoplasma in periwinkle	+
Pigeon pea witches'-broom phytoplasma in periwinkle	+
Ash yellows phytoplasma in periwinkle	–
Aster yellows phytoplasma in periwinkle (New Jersey strain)	–
Elm yellows phytoplasma in periwinkle	–
Goldenrod yellows phytoplasma in periwinkle	–
Spirea stunt phytoplasma in periwinkle	–
Palm lethal yellowing phytoplasma in palm	–
Healthy celery	–
Healthy periwinkle	–
Healthy palm	–

[a] + = positive reaction; – = negative reaction

Table 4. Collections of chokecherry and plum from North Dakota, symptoms on those plants, and results of immunofluorescence (IF) staining with a polyclonal antibody

Sample number	Locations by county	Planting type	Possible X-disease symptoms[a]	IF staining[b]
Chokecherry samples with typical X-disease symptoms				
1	Cass	Landscape planting	1,2,3,4,5,6,7	+
2	Cass	Windbreak planting	1,2,3,4,5,6,7	+
3	Cass	Landscape planting	1,2,3,4,5,6,7	+
4	Ransom	Natural stand	1,4,5,6,7	+
5	Pembina	Natural stand	1,2,3,4,5,6,7	+
6	Barnes	Natural stand	1,4,5,6,7	+
7	Logan	Windbreak planting	1,4,6,7	+
8	Burleigh	Windbreak planting	1,4,5,6,7	+
9	Burleigh	Experimental planting	1	+
10	McKenzie	Natural stand	1,5,6,7	+
11	McKenzie	Natural stand	1,4,5,6,7	+
12	Morton	Natural stand	1,4	–
13	Stark	Experimental planting	1,4,5,6,7	–
14	Stark	Experimental planting	1,6,7	–
15	Billings	Natural stand	1,2,4,5,6,7	–
16	Mountrail	Natural stand	1,2,3,4,5,6,7	–
17	Ward	Natural stand	1,4,5,6,7	–
18	Billings	Natural stand	1,2,3,4,5,6,7	?
19	McHenry	Natural stand	1,4,5,6,7	?
Chokecherry samples with atypical X-disease symptoms				
1	Logan	Windbreak planting	2,4,5	?
2	McKenzie	Windbreak planting	1,2,3,4,5,6,7	–
Schubert chokecherry (= Canada red cherry) with natural purple leaves				
1	Burleigh	Landscape planting	4,5	–
2	Stark	Experimental planting	1	+
American plum				
1	Burleigh	Seed orchard	1,4,5	–

[a] Disease symptoms on plants tested for X-disease: 1 = red leaves; 2 = red berries; 3 = deformed berries; 4 = twig dieback; 5 = branch dieback; 6 = stunted leaves; 7 = stunted shoots.
[b] Immunofluorescence staining: + = positive; – = negative; ? = indeterminable.

amplified products were diluted 40-fold and used as DNA template for the subsequent PCR using a second universal primer pair, R16F2/R2 (20). The conditions for the nested-PCR and the detection of the amplified products was described by Lee et al. (19). RFLP analysis of the nested-PCR products (about 1.2 kb) was carried out by digestion with four restriction enzymes, *Hha*I, *Hpa*II, *Kpn*I, and *Mse*I (GIBCO BRL). The resulting fragments were separated by electrophoresis in a 5% polyacrylamide gel and visualized by staining with ethidium bromide. The gel was photographed on a UV transilluminator.

Survey of X-disease distribution in chokecherries in North Dakota and the surrounding area. Samples were collected 11 to 20 August 1993 from chokecherries with putative typical (19 plants) and atypical (two plants) symptoms of X-disease distributed throughout North Dakota. Samples were also collected from two trees of the naturally purple-leaved Schubert chokecherry (= Canada red cherry), a commercial cultivar, and from one American plum (*P. americana* Marsh.) that had some red leaves and had twig and branch dieback. These materials and their collection sites are listed in Table 4. For the overall collection, trees were sampled from natural stands and from windbreak, landscape, seed orchard, and experimental plantings. Symptoms on each plant collected that indicated possible X-disease infection were recorded and are also given in Table 4. All samples consisted of current-year twigs with live symptomatic leaves and/or fruit attached. The samples were placed in plastic bags with an identification tag and stored on ice in a cooler until transfer to a refrigerator (4°C) within 3 days of collection. Within 2 weeks after collection, thin sections of leaf petioles and/or fruit peduncles were made and placed in acetone for later immunofluorescence observations.

In 1994, samples from symptomatic chokecherry trees were collected in South Dakota and Saskatchewan, Canada. The South Dakota samples were collected and sent by R. Dorset (South Dakota Division of Forestry, Pierre) in August from a natural stand in Custer County (southwest South Dakota) and from a planted chokecherry in Beadle County (east central South Dakota). The Saskatchewan sample was collected and sent by D. Raynard and B. Neill (PFRA Shelterbelt Centre, Indian Head, Saskatchewan) in August from a planted black chokecherry (*P. virginianae* var. *melanocarpa* (A. Nels.) Sarg.) in south central Saskatchewan. These samples were also analyzed by immunofluorescence staining as previously.

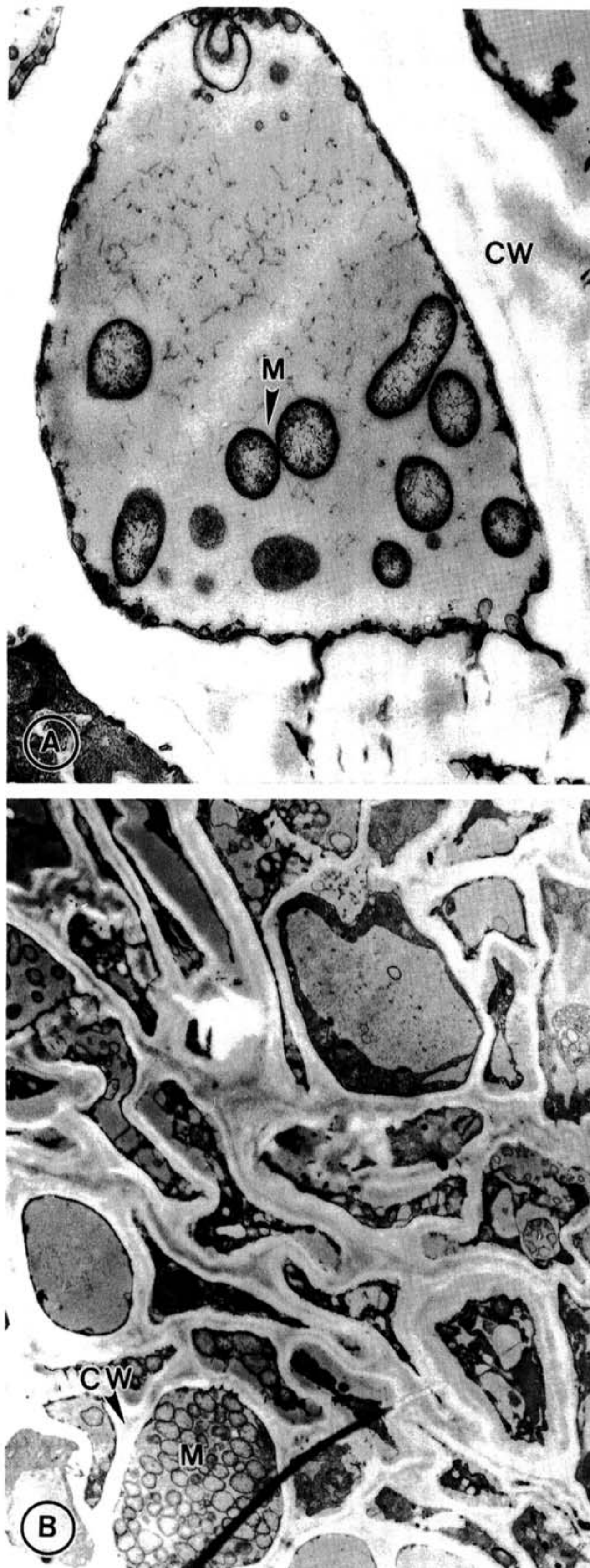

Fig. 1. Electron micrographs of leaf petiole cross sections from infected chokecherry. **(A)** Phloem element with a moderate number of phytoplasmas; **(B)** phloem elements with a high number of phytoplasmas. CW = cell wall; M = phytoplasma. Magnification: **A**, 24,000×; **B**, 6,500×.

RESULTS

Electron microscopy. Phytoplasmas were observed by TEM in chokecherry

samples collected during 1993 near Fargo, North Dakota (Fig. 1) and found only in samples from chokecherry trees showing X-disease symptoms (Table 1). The phytoplasmas appeared pleomorphic with spherical, ovate, and filamentous forms. These cells exhibited a typical unit plasma membrane, ribosomal granules, and a fibrous network of DNA. Phytoplasmas were observed in sieve elements of diseased samples, but never in surrounding parenchyma cells and xylem elements.

Serology. The polyclonal antibody was evaluated by immunofluorescence staining of tissues from both diseased and healthy chokecherry trees. Under ultraviolet light, diseased and healthy sections treated with healthy mouse blood appeared a dull green color, typical of autofluorescence, in xylem areas only (Fig. 2A). When incorporated with polyclonal antibody, the fluorescein isothiocyanate-specific bright green color, typical of immunofluorescence, was observed in phloem areas of sample sections from trees with X-disease symptoms (Fig. 2B, Table 1). No immunofluorescence was seen in sections from healthy trees and trees with atypical X-disease symptoms (Table 1) or in blood samples from a nonimmunized mouse. Optimal results were obtained when the sections were incubated with polyclonal antibody diluted 100-fold in healthy chokecherry preparation in PBS.

Serological relationships between chokecherry phytoplasma in North Dakota and other phytoplasmas are indicated by the reactions shown in Table 3. The polyclonal antibody strongly reacted with eastern X-disease phytoplasma, western X-disease phytoplasma, milkweed yellows phytoplasma, a phytoplasma from pear that is putatively similar to western X-disease phytoplasma, and PPWB phytoplasma. The polyclonal antibody did not react with phytoplasmas associated with ash yellows, aster yellows, elm yellows, goldenrod yellows, spirea stunt, and palm lethal yellowing.

PCR-RFLP analysis. When DNAs from chokecherry trees were amplified by nested-PCR with primers designed based on the 16S rRNA gene, a 1.2-kb fragment was detected from the DNA of trees 1, 3, 5, and 7, but not from tree 6 (Fig. 3A). For tree 1, 3, and 5, the RFLP profiles of this 1.2-kb fragment resulting from digestion with four restriction endonucleases were identical to those observed in subgroup A in 16S rRNA group III, which is the X-disease phytoplasma group (19,20) (Fig. 3B). A 1.2-kb fragment was also amplified from the DNA of tree 7, which showed no X-disease symptoms. However, its RFLP profiles with four restriction endonucleases were different from those in trees 1, 3, and 5. The patterns matched those in 16S rRNA group I-A, which is the eastern type of aster yellows phytoplasma (20).

Detection of X-disease phytoplasma from chokecherry and distribution of X-disease in North Dakota and the surrounding area. Of seven chokecherry trees sampled periodically during 1993 and 1994, positive reactions in immunofluorescence staining confirmed the presence of X-disease phytoplasma in phloem elements of trees 1, 2, 3, and 5, which had typical X-disease symptoms (Table 1). No immunofluorescent reactions occurred in trees 4, 6, and 7, which had atypical or no

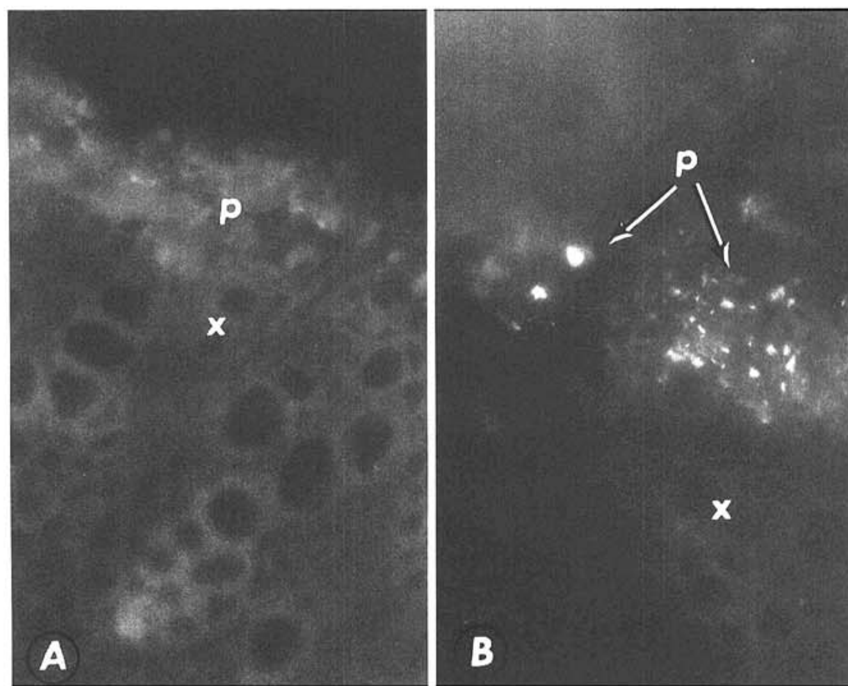

Fig. 2. Immunofluorescence of chokecherry petiole cross sections stained with X-disease phytoplasma polyclonal antibody. **(A)** Healthy chokecherry section showing faint autofluorescence in the phloem area; **(B)** diseased chokecherry section showing bright immunofluorescence in the phloem. X = xylem; P = phloem.

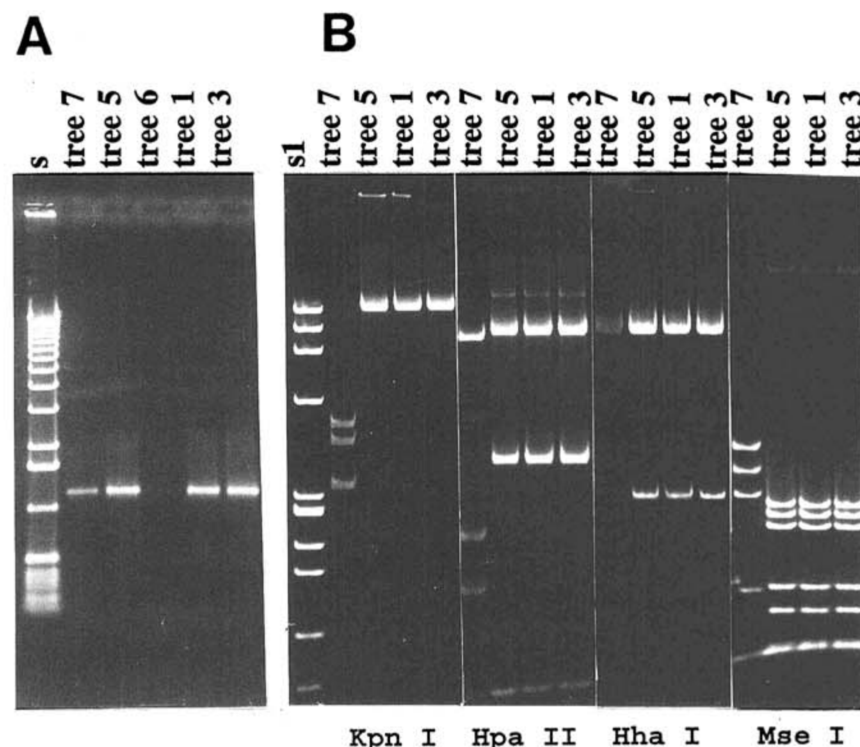

Fig. 3. (A) Nested-polymerase chain reaction (PCR) amplification from chokecherry DNAs using the universal primer pair R16F1/R0 followed by R16F2/R2; **(B)** restriction fragment length polymorphism analyses of the nested-PCR amplified products with restriction enzymes *Hha*I, *Hpa*II, *Kpn*I, and *Mse*I. Lanes: S = 1-kb DNA ladder (GIBCO BRL); S1 = ϕx174 RF I DNA *Hae*III digest, fragment sizes in base pairs from top to bottom are 1,353, 1,087, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

symptoms (Table 1). Particularly high numbers of phytoplasmas were observed in samples from trees 2 and 3 collected on June 18 and July 27 (Table 2). Phytoplasmas were seen in collections made in each sampling period except April. A strong immunofluorescence response was observed in the same trees in which high phytoplasma numbers were observed with TEM (Table 2). No phytoplasmas were detected with either TEM or IF in samples collected in April before growth began. The immunofluorescence staining data in 1994 were similar to those in 1993, so were not shown.

Of 19 sampled chokecherry trees showing typical X-disease symptoms collected throughout North Dakota, 11 had a positive reaction with the polyclonal antibody in immunofluorescence staining, six had a negative reaction, and two were indeterminable (Table 4). Two samples from trees with atypical disease symptoms and one American plum sample were negative or undeterminable for immunofluorescence. Samples from one of the two Schubert chokecherry trees had a positive reaction. X-disease phytoplasma was found in each of the four quadrants of North Dakota. All chokecherry samples received from South Dakota and from Saskatchewan reacted positively with the polyclonal antibody in immunofluorescence staining.

DISCUSSION

In chokecherry plants in the Great Plains that show X-disease-like symptoms, the symptoms have been assumed to be caused by western X-disease (22,23). However, the actual causal pathogen has not been determined. In this study, phytoplasmas in diseased chokecherry trees in North Dakota were observed under TEM. The structural and morphological characteristics of the phytoplasmas were like those previously reported for X-disease phytoplasma in chokecherry (2,5,12,21). The presence of phytoplasmas in diseased trees strongly suggests that the observed symptoms on chokecherry in North Dakota are caused by phytoplasmas.

The similarity of X-disease phytoplasma in chokecherry in North Dakota to eastern and western X-disease phytoplasmas is unknown. Chokecherry in North Dakota is geographically and ecologically isolated from chokecherry in eastern and western North America. The cross-reactivity of the polyclonal antibody against chokecherry X-disease phytoplasmas in North Dakota with the eastern and western X-disease phytoplasmas indicates that the chokecherry disease in North Dakota is caused by a phytoplasma similar to X-disease phytoplasma in other areas. Based on cross-reactivity with various phytoplasmas, this polyclonal antibody is active against antigens common to several X-disease phytoplasma strains; it may be useful for screening samples for X-disease phytoplasma. Our results are consistent with those from recent serological and molecular studies (10,18) in which eastern and western X-disease phytoplasmas were found to be closely related.

Results of our PCR-RFLP analysis provide further evidence that the phytoplasma associated with the X-disease-like chokecherry disease is X-disease phytoplasma. The match of RFLP patterns of the PCR-amplified 1.2-kb fragment with those in 16S rRNA group III, subgroup A suggests that the phytoplasma from chokecherry in North Dakota is X-disease phytoplasma. However, the relative similarity of the X-disease phytoplasma in chokecherry in North Dakota with western or eastern X-disease phytoplasma needs further examination. The consistent co-occurrence of typical X-disease symptoms with positive serological and PCR-RFLP analyses confirms that the polyclonal antibody can detect the chokecherry X-disease phytoplasma. The usefulness of the polyclonal antibody for detecting chokecherry X-disease phytoplasma is further supported by the negative results from tree 6 with atypical symptoms and tree 7, which was infected with aster yellows but not X-disease phytoplasma. Therefore, the polyclonal antibody was found to be useful for studying the incidence of chokecherry X-disease.

The reaction between the antibody and the phytoplasmas associated with milkweed yellows and with a phytoplasma found in pear (24) provides further evidence that the milkweed yellows phytoplasma is in the X-disease phytoplasma strain cluster (6), and that the phytoplasma from pear is similar to western X-disease phytoplasma (B. C. Kirkpatrick, *personal communication*). However, it is difficult to determine how much common antigen these phytoplasmas share based on immunofluorescence staining. Our polyclonal antibody also reacted with the phytoplasma associated with PPWB, indicating that the PPWB phytoplasma has antigens in common with X-disease phytoplasma. Some similarity between western X-disease phytoplasma and PPWB phytoplasma was also found by Harrison et al. (9), who cloned two PPWB-phytoplasma DNA probes, which had a weak reaction with western X-disease phytoplasma. Further study is needed to explain what common antigens are shared by X-disease phytoplasmas and PPWB phytoplasma.

The chokecherry X-disease phytoplasma polyclonal antibody did not show activity against ash yellows phytoplasma, aster yellows phytoplasma, elm yellows phytoplasma, goldenrod yellows phytoplasma, spirea stunt phytoplasma, or palm lethal yellowing phytoplasma. These results suggest that those phytoplasmas are different from chokecherry X-disease phytoplasma from North Dakota. However, goldenrod yellows phytoplasma and spirea stunt phytoplasma have been found to be in the X-disease phytoplasma cluster (6). Our polyclonal antibody, therefore, may not be useful for detecting all subclusters in the X-disease phytoplasma cluster. Other factors could result in negative reactions. Phytoplasmas were assumed to be present in the samples tested based on prior testing or on the presence of disease symptoms. The plant samples that were received for goldenrod yellows and spirea stunt both had disease symptoms. The plants on which the sample containing spirea stunt phytoplasma were grafted also developed disease symptoms. (Grafts with the sample containing goldenrod yellows phytoplasma were not successful.) These observations indicate that the samples contained phytoplasmas. Thus, it is possible but unlikely that the tested samples did not contain phytoplasmas. If the phytoplasma titer in the tested samples was too low, a negative reaction could occur. Palm lethal yellowing phytoplasma is known to occur at low titers in the tested hosts (N. A. Harrison, *personal communication*). We cannot judge whether the phytoplasma titer was a problem in the antibody tests.

Since phytoplasmas were recognized as a possible etiological agent of yellows diseases, many attempts to purify the phytoplasmas to enhance development of polyclonal antibodies to phytoplasmas have been made (1,3,11,13,27–29). All polyclonal antibodies that have been reported were produced against antigens partially purified from herbaceous or experimental hosts, and they often exhibited prohibitively low specificities (13,28). The data in our study indicate that the procedure used for antigen purification directly from chokecherry plants is effective and reliable for producing antigen of sufficient quality to raise phytoplasma-specific polyclonal antibodies. To our knowledge, this is the first polyclonal antibody successfully produced for phytoplasma detection by using antigen directly extracted from a woody plant. This procedure, with little modification, could be suitable for preparing antigen from other phytoplasma-infected woody plants, so transmission of phytoplasmas from woody plants to herbaceous hosts would not be necessary. Because transmission from woody plants to herbaceous plants is sometimes technically very difficult, this procedure may provide diagnostic capabilities that were previously unavailable. The procedure used provided a relatively specific polyclonal antibody, both in terms of discriminating between healthy and diseased hosts and in detecting X-disease phytoplasma in infected plants. The relatively quick and inexpensive production of this antibody and its ready application to large-scale screening for infected plants indicate the utility of such an antibody.

One of the key steps in this procedure, we believe, is to increase antigen purity, which is considered to be a critical prerequisite for the production of phytoplasma-specific polyclonal antibody. The concentration of phytoplasmas in the antigen preparation was increased by collecting the vascular bundles from leaf veins digested with enzymes. Another important step appears to be the absorption of plant antigens, because it is possible that polyclonal antibody against an antigen obtained by partial purification of phytoplasmas from diseased plants may contain anti-plant antibody (1,28,29). This problem can be reduced by using healthy plant preparations to absorb the polyclonal antibody. This absorption method was used in this study, and the serological data show that the absorption gave a polyclonal antibody with no detectable host reaction.

Different assay methods may have an influence on the validity of conclusions drawn from assay results (1). Immunofluorescence staining was chosen in this study for two reasons. First, this staining procedure is simple and rapid. Second, the use of other assay methods, such as enzyme-linked immunosorbent assay (ELISA), may be difficult, because the tissues of chokecherry, like other woody plants, are hard to grind. In addition, many plant chemicals, such as phenolic compounds, would be released once the tissues are ground, which might block the antigen coating or the antigen-antibody reaction and thus increase background in ELISA.

Previous observations that X-disease of chokecherry was widespread in North Dakota were confirmed by the results from immunofluorescence tests, with positive samples found in each part of North Dakota (SE, SW, NW, NE). Prediction of the result from Schubert chokecherry samples based on the presence of expected symptoms could not be made because the cultivar is a naturally purple-leaved selection. There was no association between the presence of certain possible X-disease symptoms and the finding of positive immunofluorescence; six of 19 trees rated as having typical X-disease symptoms showed a negative reaction. Failure to detect X-disease phytoplasma in these samples could be due to several causes. The polyclonal antibody may not be active against all X-disease phytoplasma strains. This seems unlikely, given the expected broad spectrum of polyclonal antibodies and the strong activity against the divergent X-disease phytoplasma collections from other areas in the United States. Another possible cause is that symptoms previously associated with X-disease phytoplasma in chokecherry may not necessarily be diagnostic of X-disease. There may be other causes of reddened or stunted leaves and shoots, including herbicides, drought, and other stress factors. A third possibility is that the titer of the X-disease phytoplasma in the negative samples was too low for detection by immunofluorescence staining.

TEM observation of chokecherry plant materials collected at different dates in 1993 and the immunofluorescence results from collections during various parts of the growing season of two consecutive years indicated that phytoplasmas can occur at high numbers whenever plants are actively growing. This result provides an indication of the best time to collect diseased scion wood for graft inoculations, and to collect disease-free vegetative materials for propagation of such commercial cultivars as Canada red cherry.

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