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Diagnosis of X-Disease Phytoplasma in Stone Fruits by a Monoclonal Antibody Developed Directly from a Woody Plant[1]

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Abstract

A monoclonal antibody was produced by using enriched antigen from an infected chokecherry plant, and was used to detect the X-disease phytoplasma using both ELISA and immunofluorescence staining. The monoclonal antibody reacted with five of seven tested phytoplasmas in the X-disease phytoplasma cluster, and one of four phytoplasmas (pigeon pea witches'-broom phytoplasma) outside that cluster. The efficiency of the monoclonal antibody for detection of X-disease phytoplasmas was verified through assays of infected stone fruit plants. The efficacy of the monoclonal antibody was confirmed by X-disease phytoplasma-specific nested-polymerase chain reaction. Development of the monoclonal antibody represents a substantial improvement in the potential for commercial diagnosis of X-disease phytoplasma infection in nurseries and orchards and for screening large numbers of stone fruit plants for X-disease phytoplasma infection in breeding programs.

Index words: diagnosis, monoclonal antibody, phytoplasma, polymerase chain reaction, stone fruits, X-disease.

Species used in this study: chokecherry (*Prunus virginiana* L.); peach (*P. persica* (L.) Batsch); sour cherry (*P. cerasus* L.); and sweet cherry (*P. avium* L.).

Significance to the Nursery Industry

X-disease is a serious disease in stone fruits in North America. Because the pathogen associated with the disease, X-disease phytoplasma, has not yet been cultured *in vitro*, and because the pathogen may be present without causing visual symptoms, timely diagnosis of X-disease is very difficult. Conclusive diagnosis with previously developed methods (such as electron microscopy and DNA analysis) is time-consuming and expensive. The monoclonal antibody produced here is specific to X-disease phytoplasmas and is convenient to use by either ELISA or immunofluorescence. This will assist nurseries to quickly and inexpensively diagnose stock plants and propagules of stone fruit trees for infection by the X-disease phytoplasma. This monoclonal antibody will also be valuable for use in screening large numbers of stone fruit trees in breeding programs to develop X-disease resistant trees.

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Introduction

X-disease is one of the most serious diseases of stone fruit trees in many areas of North America, and can cause severe economic damage in the fruit industry, especially in peach (*P. persica* (L.) Batsch), sweet cherry (*P. avium* (L.) L.), sour cherry (*P. cerasus* L.), nectarine (*P. persica* (L.) Batsch var. *nucipersica* (Suckow) K. C. Schneid.), and chokecherry (*P. virginiana* L.) (3). A phytoplasma, formerly known as a mycoplasmalike organism, is associated with diseased plants and is considered to be the causal agent (13, 14). The X-disease phytoplasmas are commonly divided into two strains, eastern and western, depending on the geographical region and leafhopper vectors (9, 13, 14). Currently, there are no effective measures to adequately control this disease in susceptible stone fruit species.

Because the X-disease phytoplasma, like all other phytoplasmas, has not yet been cultured *in vitro*, traditional diagnosis of phytoplasma-associated diseases has relied on symptomology, plant and insect host range, and electron microscopy. These methods are time-consuming and non-specific. Polyclonal antibodies, a serological tool, have been produced against X-disease phytoplasma-enriched extracts derived from an infected experimental host (15) and insect vectors (8), or chokecherry (5). Their application in identification and detection of X-disease phytoplasma has been limited because those antibodies had substantial cross-reactivity with healthy host antigens or required a relatively high phytoplasma titer for reliable detection. In addition, due to the method of their production, polyclonal antibodies may be available in limited quantities; specificity of different batches must be determined, and data obtained using different polyclonal antibody may not be comparable. These problems can be avoided by the use of monoclonal antibodies. A monoclonal antibody was developed against the eastern strain of the X-disease phytoplasma from celery (*Apium graveolens*), an experimentally infected plant host (7), but the monoclonal antibody is no longer available due to loss of the original cell lines.

Polymerase chain reaction (PCR) technology and analysis of restriction fragment length polymorphisms (RFLPs) of 16S rRNA gene fragment have proved to be specific and very sensitive for detecting the X-disease phytoplasma in a number of hosts (1, 3, 4). However, application of PCR on a large scale is limited by relatively expensive and time-consuming DNA extraction from woody plants.

The objectives of this research were to develop a monoclonal antibody to the X-disease phytoplasma by preparing an antigen directly from naturally infected chokecherry plants and to apply it for diagnosis of X-disease phytoplasma infection.

Materials and Methods

Monoclonal antibody development and testing. A monoclonal antibody was developed using previously described techniques (7, 12). Preparation of phytoplasma antigen from an infected chokecherry tree and host antigen from a healthy chokecherry tree, partial purification of phytoplasmas, and immunization of an 8-week-old BALB/c mouse were described previously (5). Hybridomas that produced specific antibodies reacting with diseased, but not with healthy, chokecherry preparations were selected for further subcloning. Monoclonal cell lines were obtained by limited dilution (7), subcultured for monoclonal antibody production, and stored in liquid nitrogen for later use. The antibody class was determined by indirect ELISA with the mouse monoclonal antibody isotyping kit (Sigma Immuno Type™, Sigma Immuno Chemicals, MO) according to the manufacturer's instructions. The monoclonal antibody used in this study was the undiluted culture supernatants of hybridoma clones.

Sensitivity of the monoclonal antibody for detection of phytoplasmas was determined by indirect ELISA with a peroxidase system (12). Antigens from chokecherry infected by the X-disease phytoplasma and from periwinkle infected by the eastern X-disease phytoplasma were prepared (5) for hybridoma culture screening, except that periwinkle leaf veins were not subjected to acetone fixation. The optimum reaction dilution was determined by a series of two-fold dilutions. The optimum dilution was considered to be the highest dilution at which infected plant material could readily be distinguished from non-infected plant material. A dilution series for each preparation was made in a coating buffer (7). The antibody dilution limit was expressed as the highest dilution of antigen preparations at which the diseased preparations could be differentiated from the healthy preparations by their OD490 values. Immunofluorescence staining (IF) was used to evaluate the specific binding of the monoclonal antibody to phytoplasmas. Cross sections through phloem tissues of leaf midribs or young stems were made by hand with a razor blade and stained with the monoclonal antibody by a previously described method (5, 7). A Nikon Labophot-2 with a DS-Epifluorescence-2 attachment and 495 nm main wave length was used for IF. 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 undeterminable. Samples from leaf midribs or petioles from healthy plants were used as controls. Seven to ten sections from each sample were examined. Hybridoma culture medium was RPMI complete medium supplemented with HAT (Sigma Co., St Louis, MO).

Specificity of the monoclonal antibody was tested by indirect ELISA and IF staining of phytoplasma strains listed in Table 1. Ash yellows phytoplasma (AshY2), aster yellows phytoplasma (New Jersey strain), and western X-disease phytoplasma in infected periwinkle (*Catharanthus roseus* (L.) G. Don) were provided by T.A. Chen, Rutgers University, New Brunswick, NJ. Canada peach X-disease (= eastern X-disease) phytoplasma (CX), elm yellows phytoplasma (EY1), goldenrod yellows phytoplasma (GR1), milkweed yellows phytoplasma (MW1), and spirea stunt phytoplasma (SP1) in infected periwinkle were provided by W.A. Sinclair, Cornell University, Ithaca, NY. A phytoplasma in periwinkle that was insect transmitted from a declining pear tree and that is genetically and serologically similar to western X-disease phytoplasma was provided by B.C. Kirkpatrick, University of California-Davis, Davis, CA. Florida pigeon pea witches'-broom phytoplasma (PPWB) in periwinkle was provided by N.A. Harrison, University of Florida, Fort Lauderdale, FL. Walnut witches'-broom phytoplasma was provided by C.J. Chang, University of Georgia, Griffin, GA.

Sensitivity of the monoclonal antibody. The sensitivity of the monoclonal antibody for detecting X-disease phytoplasmas relative to the polyclonal antibody (5) and to the nested-PCR amplification was tested using samples from 11 diseased chokecherry trees from five sites across North Dakota, from one healthy chokecherry tree, and from one chokecherry seedling grown from seed in the greenhouse. The nested-PCR test used the phytoplasma-specific universal primer pair R16F2/R2 and the X-disease phytoplasma group-specific primer pair R16(III)F2/R1 (10). These primer pairs amplify the 16S rRNA gene sequences of phytoplasmas in the X-disease cluster. Procedures for extraction of total nucleic acids from chokecherry and conditions for the nested-PCR were described previously (5).

Table 1. Specificity reactions of monoclonal antibody 14-D11 with various phytoplasmas.

Phytoplasma strains	X-disease cluster[z]	ELISA[y]	IF[x]
Chokecherry X-disease phytoplasma	Yes	0.414*	+
Eastern X-disease phytoplasma	Yes	0.874*	+
Western X-disease phytoplasma	Yes	0.893*	+
Phytoplasma from declining pear	Yes	NT[w]	+
Milkweed yellows phytoplasma	Yes	NT	+
Goldenrod yellows phytoplasma	Yes	NT	–
Spirea stunt phytoplasma	Yes	0.002	–
Walnut witches'-broom phytoplasma	No	NT	–
Pigeon pea witches'-broom phytoplasma	No	NT	+
Ash yellows phytoplasma	No	0.087	–
Aster yellows phytoplasma	No	NT	–
Elm yellows phytoplasma	No	0.068	–
Healthy chokecherry	–	0.063	–
Healthy periwinkle	–	0.002	–

[z]X-disease cluster separation according to Gunderson et al. (3).

[y]OD490 values; monoclonal antibody was undiluted culture supernatant. The OD values with an asterick are high enough (at least 2× the negative control value) to be considered positive.

[x]IF = immunofluorescence staining; + = positive reaction; – = negative reaction.

[w]NT = Not tested.

Diagnosis of X-disease phytoplasmas in stone fruit trees. The value of the monoclonal antibody for diagnosis of the X-disease phytoplasma in various stone fruits from outside North Dakota was determined by IF. The samples tested included three samples of peach from Michigan (provided by U. Rahardja, Michigan State University, East Lansing, MI), four samples of sweet cherry from California (provided by B.C. Kirkpatrick, University of California at Davis, Davis, CA), 11 samples of sweet cherry and four samples of sour cherry from New York and one sample of chokecherry from Iowa (provided by R.L. Andersen, Cornell University, Geneva, NY), and one sample of chokecherry from Nebraska. The samples were shipped in moist paper towels in plastic bags. Freehand cross sections through phloem tissues of leaf midribs, leaf petioles, or fruit peduncles were made with a razor blade and stored in acetone until assayed.

Results and Discussion

Monoclonal antibody development and testing. Two stable hybridoma clones, 14-D11 and 7-F2, secreting antibodies specific to infected chokecherry but not to healthy chokecherry were obtained from two independent fusions. Both monoclonal antibodies belong to antibody class IgM. Because clone 14-D11 produced a higher antibody titer in its culture supernatant, the monoclonal antibody 14-D11 was used throughout this study.

The specific reactions of monoclonal antibody (MA) 14-D11 with various phytoplasmas are shown in Table 1. The monoclonal antibody reacted with chokecherry X-disease, eastern X-disease, western X-disease, pear decline, milkweed yellows phytoplasmas, and Florida pigeon pea witches'-broom, but not with ash yellows, aster yellows, elm yellows, goldenrod yellows, or spirea stunt phytoplasmas.

Undiluted culture supernatant of clone 14-D11 could be used to detect the chokecherry X-disease phytoplasma when the infected chokecherry preparation was diluted up to 64 times (Table 2). MA 14-D11 allowed detection of the eastern X-disease phytoplasma when infected periwinkle preparation was diluted up to 1024 times.

MA 14-D11 allowed ready determination of the location of chokecherry X-disease phytoplasmas in infected tissues in immunofluorescence staining. FITC-specific fluorescence was observed only in phloem areas of diseased sections. No immunofluorescence was seen in sections of healthy tissues treated with MA 14-D11 or diseased sections treated with RPMI-1640 complete medium. Stronger immunofluorescence was observed in infected periwinkle phloem than in phloems of *Prunus* spp. (data not shown).

X-disease phytoplasma detection from chokecherry and other Prunus plants by the monoclonal antibody. Of 13 chokecherry trees sampled from five sites in North Dakota, 10 were positive for the X-disease phytoplasma using the monoclonal antibody, 7 using the polyclonal antibody and 11 using the nested-PCR amplification (Table 3). Both healthy plants examined were negative for the X-disease phytoplasma using all three detection methods.

Among 24 stone fruit samples from outside of North Dakota that were tested with the monoclonal antibody, 18 were positive, six were negative and two were questionable (Table 4). The X-disease phytoplasma was detected with the monoclonal antibody in some samples representing each host species and each geographic source. All the five samples tested with nested-PCR were positive, confirming that the X-disease phytoplasma was present in those samples.

Because the X-disease phytoplasma, like other phytoplasmas, has not been purified and cultured in an artificial medium, conclusive identification of infection by a specific phytoplasma has relied on serological and PCR-based technologies. Since development of the first monoclonal antibody against a phytoplasma (12), monoclonal antibodies against several phytoplasmas have been developed and widely utilized for phytoplasma detection. The monoclonal antibody reported here can be similarly used for X-disease phytoplasma diagnosis and research. The monoclonal antibody is more sensitive than a previously developed polyclonal antibody (5) and can be used in both indirect ELISA and IF. Because there is no need for absorption of the monoclonal antibody with healthy antigen, as is required for use of the polyclonal antibody (5), application of the monoclonal antibody is faster and easier. Another advantage is that the unlimited supply of the monoclonal antibody allows ready ap-

Table 2. ELISA reaction of monoclonal antibody 14-D11 with serial dilutions of antigen preparations from chokecherry and periwinkle plants[z].

Dilution times	Chokecherry preparation[y]		Periwinkle preparation[x]	
	Diseased	Healthy	Diseased	Healthy
0	0.417*	0.121	0.667*	0.041
8	0.252*	0.116	0.711*	0.000
16	0.214*	0.041	0.723*	0.009
32	0.196*	0.088	0.795*	0.037
64	0.138	0.090	0.799*	0.031
128	0.114	0.120	0.610*	0.081
256	0.118	0.104	0.488*	0.001
512	0.106	0.009	0.425*	0.049
1024	NT[w]	NT	0.302*	0.027

[z]ELISA reaction OD_{490} values; monoclonal antibody was undiluted culture supernatant. The OD values with an asterisk are high enough (at least 2× the negative control value) to be considered positive.

[y]X-disease phytoplasma-infected chokecherry.

[x]Eastern X-disease phytoplasma-infected periwinkle.

[w]Not tested.

Table 3. Detection of chokecherry X-disease phytoplasma from North Dakota with monoclonal antibody 14-D11[z] and comparison with a polyclonal antibody and nested-PCR amplification.

Samples (site #/tree #)	Monoclonal antibody[y]	Polyclonal antibody[y]	Nested-PCR[x]
1/1	+	–	+
1/2	+	+	+
2/1	+	+	+
3/1	–	–	+
4/1	+	–	+
4/2	+	+	+
4/3	+	+	+
4/4	+	–	+
4/5	+	+	+
5/1	+	+	+
5/2	+	+	+
5/3 (healthy)	–	–	–
GH-(germinated from seed)	–	–	–

[z]undiluted culture supernatant of 14-D11.

[y]immunofluorescence: + = presence; – = absence.

[x]+ = product amplified; – = no product amplified.

Table 4. X-disease phytoplasma detection from *Prunus* plants with monoclonal antibody 14-D11 and nested-PCR[z].

Samples	Monoclonal antibody	Nested-PCR
Chokecherry:		
NY	+	+
NE	+	NT
IA	+	NT
Peach:		
MI-1	+	NT
MI-2	+	NT
MI-3	+	NT
Sour cherry:		
NY	+	NT
Sweet cherry:		
CA-1	+	+
CA-2	+	+
CA-3	+	+
CA-4	+	+
NY-13791-T28	–	NT
T29	+	NT
T30	+	NT
T31	+	NT
Q50R16T15	+	NT
NY-13688	–	NT
NY-Hebros-T45	–	NT
T46	+	NT
NY-Sam—North	–	NT
South	+	NT
NYSAES-low9-132	+	NT
-165	?	NT
-50-1-3	?	NT

[z]+ = positive reaction; – = negative reaction; NT = not tested; ? = questionable.

plication of ELISA for detection of the X-disease phytoplasma, thus permitting a large number of samples to be processed. As expected, the monoclonal antibody was less sensitive than nested-PCR amplification. However, the relatively low cost and faster application make it superior for initial screening to detect the X-disease phytoplasma, especially when a large number of samples is handled.

To our knowledge, this is the first monoclonal 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 allow development of diagnostic capabilities that were previously unavailable. The procedure used produced a relatively specific monoclonal antibody, both in terms of discriminating between healthy and diseased hosts and in detecting the X-disease phytoplasma in infected plants. The relatively quick and inexpensive production of this antibody and its ready application to large-scale diagnosis for infected plants indicate the utility of such a procedure. Results of PCR amplification analysis indicated that the monoclonal antibody provides good reliability for detecting X-disease phytoplasmas from chokecherry and sweet cherry.

In addition to reaction with the X-disease phytoplasma in chokecherry, the monoclonal antibody reacted with four other phytoplasmas in the X-disease cluster and with the Florida PPWB phytoplasma, indicating that a serological relatedness exists between the X-disease phytoplasma in chokecherry and these five phytoplasma strains. A serological relationship among these phytoplasmas was also shown in studies using a polyclonal antibody (5), other monoclonal antibodies (7), and DNA hybridization (6). Recent analyses of the conserved 16S rRNA and ribosomal protein genes in the X-disease phytoplasma genome indicate that the X-disease phytoplasma in chokecherry is closely related, but not identical, to eastern and western X-disease phytoplasmas (3, 4).

The monoclonal antibody did not react with the goldenrod yellows phytoplasma or the spirea stunt phytoplasma, two strains in the X-disease phytoplasma cluster (2). The epitopes detected by the monoclonal antibody are apparently absent in these phytoplasmas. Notably, these same two phytoplasmas did not react with a polyclonal antibody that had a relatively broad sensitivity to phytoplasmas in the X-disease phytoplasma cluster (5).

The reaction with PPWB phytoplasma was interesting. This reaction indicates that the monoclonal antibody is not entirely specific to phytoplasmas in the X-disease phytoplasma cluster as currently recognized. Notably, this phytoplasma was the only one tested outside the X-disease cluster that reacted with a polyclonal antibody developed against X-disease phytoplasmas from chokecherry (5). The PPWB phytoplasma has been reported to be relatively similar to, but distinct from, phytoplasmas in the X-disease cluster (2). Clearly these phytoplasmas have epitopes in common that are absent in more distantly related phytoplasmas.

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An Inoculation Technique for Dogwood Anthracnose[1]

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Abstract

Researchers have been generally unable to infect dogwood foliage (*Cornus florida* L.) with dogwood anthracnose (*Discula destructiva* Redlin) in artificial environments. We tested the influence of four factors on development of *D. destructiva* lesions in intact *C. florida* leaves wounded with a pin-prick device: (1) propagule (conidia or vegetative hyphae), (2) isolate origin (Catoctin Mountain, MD or Great Smoky Mountains National Park, TN), (3) controlled environment (growth room or air conditioned, humidified chamber in a greenhouse), and (4) period of time leaves were enclosed in humidified bags following inoculation. Of the manipulated factors, time spent in moistened bags and experimental environment were most important in determining lesion size. Lesions of similar size resulted from 0, 2 and 4 days in humidified bags, but leaves enclosed for 7 days had lesions over 5 times as large. Lesions that formed on trees in the greenhouse chamber were about 15 times larger than those in the growth room. Neither fungal propagule nor isolate origin affected lesion size. Based on this information, we have successfully infected both *C. florida* and *Cornus kousa* with *D. destructiva* in subsequent studies.

Index words: dogwood anthracnose; Flowering dogwood; *Cornus florida* L.; *Discula destructiva* Redlin.

Significance to the Nursery Industry

Attempts to study dogwood anthracnose on flowering dogwood have been hampered by difficulties in reliably producing the disease under experimental conditions. This report summarizes a technique for inoculating intact dogwood leaves and consistently obtaining infection with dogwood anthracnose. We have used this information to help us test the resistance of various *Cornus* cultivars to dogwood anthracnose, and the technique should also be of interest to others investigating various aspects of the biology and control of this disease.

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Introduction

Flowering dogwoods (*Cornus florida* L.) are attractive, valuable landscape trees common in the southeastern United States that can generate gross returns of up to $60,000 per harvested acre (3). Since the late 1970s, trees of various *Cornus* species have suffered health decline and sometimes death due to a fungal disease, dogwood anthracnose (5), caused by *Discula destructiva* Redlin (13).

The nursery industry would benefit from trees which are resistant to dogwood anthracnose. Since the disease has devastated native *C. florida* trees in some areas of the eastern United States (15), resistant germplasm must be discovered in order to overcome the economic impact the disease has had on the businesses selling dogwoods in those areas. Efforts to study disease progression and resistance have been hindered by the lack of a reliable inoculation technique that consistently results in diseased foliage.

Although some researchers have succeeded in obtaining severe disease development in forested areas using natural inoculum (4), many attempts at producing the disease in laboratories and greenhouses have not been successful. Various inoculation techniques have been tried, most with only limited success (1, 12, 16). Grand et al. (11) wounded detached leaves with a pin-prick device and rubbed agar plugs of inoculum into wounds. After inoculation, leaves were enclosed