DETECTION OF '*Candidatus* Phytoplasma asteris' IN ASHLEAF MAPLE TREES WITH SHOOT PROLIFERATION AND DECLINE

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A B S T R A C T

In the late 1990s, a new and devastating disease was noticed among ashleaf maples in nurseries and urban areas of southern and central Poland. Symptoms include severe growth abnormalities, shoot proliferation and decline, which may indicate that the disease in Poland is caused by a phytoplasma. To test the hypothesis, we tested diseased ashleaf maples for the presence of phytoplasma. PCR and RFLP revealed that one particular strain of phytoplasma, the aster yellows phytoplasma, was present in diseased ashleaf maple trees.

To further test this hypothesis, we inoculated *Catharanthus roseus* test plants with bud grafts from infected trees, and then inoculated young, healthy maple seedlings with shoot grafts from symptomatic periwinkles. Although the infection rate was low and the course of the disease was milder, or even latent, the presence of the aster yellows phytoplasma in both symptomatic and asymptomatic plants was present. The results suggest that the aster yellows phytoplasma is connected with the ashleaf maple disease. The aster yellows phytoplasma has been assigned to group 16SrI, subgroup B (16SrI-B). Now this group is reclassified as *'Candidatus* Phytoplasma asteris'.

Key words: maple, decline, phytoplasma, aster yellows

INTRODUCTION

The genus *Acer* consists of about 110 species of trees and shrubs, which naturally occur all over the world. Among the species which naturally occur in Poland are: *A. campestre* L., *A. platanoides* 'Globosum' and *A. pseudoplatanus* L. Hybrids and (Schultz and Manion, 1980; Fostad and cultivars of the ashleaf maple, *A. negnudo* are also very common.

In the late 1990s, a new and devastating disease was noticed among ashleaf maples in nurseries and urban areas of southern and central Poland. A decline in the population of the ashleaf maple has also been observed in the United States and Norway Pedersen, 1997). However, unlike in

the United States and Norway, the trees in Poland showed symptoms of shoot growth abnormalities, including shoot proliferation, which may indicate that the disease in Poland is caused by a phytoplasma. Similar symptoms had been observed in phytoplasma associated diseases in other tree species, including *Alnus, Populus, Crategus*, *Ulmus, Fraxinus, Quercus*, and *Cordyline* (Lederer and Seemüller, 1991; Valiunas et al., 2001; Seemüller and Lederer, 1988; Berges et al., 1997; Marcone et al., 1997; Griffiths et al., 1999; Matteoni and Sinclair, 1985; Sinclair and Griffiths, 2000; Ahrens and Seemüller, 1994; Valiunas, 2003; Andersen et al., 2001).

Therefore, the aim of the present study was to investigate whether the disease among ashleaf maples in Poland is also caused by a phytoplasma. We found that one strain of phytoplasma, the aster yellows strain, was present in diseased ashleaf maples. This phytoplasma is known to infect a wide range of hosts (Seemüller et al., 1998b; Lee et al., 2004).

The aster yellows phytoplasma has been assigned to 16SrI group, subgroup B (16SrI-B). Now this group is reclassified as *'Candidatus* Phytoplasma asteris'.

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MATERIAL AND METHODS

Plant material

We examined cultivated and naturally occurring ashleaf maple trees all over Poland, including three field-grown hybrids with symptoms of advanced disease, and four cultivated specimens of the cultivar Flamingo with milder symptoms. As a reference, we used asymptomatic two-year-old seedling and older field-grown trees growing in central Poland.

Transmission experiments

Two graft transmission experiments were carried out.

In the first experiment, twenty one periwinkle (*Catharanthus roseus* L.G. Don) seedlings, three or four months old, were each inoculated with two bud chips from two severely affected ashleaf maple trees. The bud chips were collected in either April or September of 2001.

In the second experiment, fifteen ashleaf maple saplings, five months old, were each inoculated with two buds from experimentally infected periwinkle plants which had developed symptoms of severe disease. The maples were grafted in May, 2003.

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Plant samples and the reference strains

From 2000 to 2004, tissue samples were collected in early spring, summer and autumn. Leaf and shoot phloem samples were taken from four symptomatic and two asymptomatic ashleaf maple trees growing in the field. Leaf samples were collected from five of the inoculated periwinkles

and four of the inoculated ashleaf maples. The first samples were collected three or four months after inoculation, and the last samples were collected ten to twelve months after that. Plants which tested negative were tested again.

Four strains of phytoplasma were included as a reference: aster yellows phytoplasma (AY1, 16SrI-B); apple proliferation phytoplasma (AP, 16SrX-A); X disease phytoplasma (CX, 16III-A); and elm yellows phytoplasma (ULW, 16SrV). Reference material was kindly provided by Dr. I.M. Lee of Beltsville, USA, and Dr. A. Bertac-cini of Bologna, Italy.

Phytoplasma detection and identification

DNA was isolated from approximately one gram of fresh leaf or stem phloem tissue using the phytoplasma enrichment procedure described by Ahrens and Seemüller (1992). Nested PCR amplification was performed using P1/P7 as first primer, followed by either universal phyto-plasma or group-specific primers **(see table below)**.

Each 50 µl PCR mixture contained: 1 µl of total nucleic acid extract from plant tissue; 1.25 µl of a 10 mM solution of the four dNTPs; 0.1 µl of 100 µM forward and reverse primers; 1xDNA polymerase buffer; and 1U of HotStarTaq DNA polymer-ase (Qiagen, Syngen Biotech, Wrocław, Poland). The following parameters were used:

- for direct PCR with primers P1/P7 and nested PCR with primers R16F2n/R16R2 – 35 cycles: 1 min at 94°C, 2 min at 60°C, 3 min at 72°C;
- for nested PCR with primers R16(I)F1/R16(I)R1 – 35 cycles: 45 s at 94°C, 1 min at 55°C, 2 min at 72°C;
- for nested PCR with primers fA/rA and fAT/rAS – 25 cycles: 1 min at 95°C, 1 min at 55°C, 1 min at 72°C.

During the last cycle, elongation time was extended to 9 min.

PCR products (5 µl) were analyzed by 1% agarose gel electrophoresis in 0.5 X TBE buffer, stained with ethidium bromide (0.5 µg/ml), and visualized under UV light.

RFLP analyses were performed after digestion of 10 µl of the products of PCR primed with R16F2n/R16R2 with the single restriction endonucleases *Alu*I, *Mse*I and *Rsa*I in accordance with the manufacturer's instructions (Gibco BRL, Life Technologies,

Primers	Specificity	Expected base pairs	Reference
P1/P7	universal phytoplasma	~1800	Schneider et al., 1995
fA/rA	universal phytoplasma	~560	Ahrens and Seemüller, 1992
R16F2n/R16R2	universal phytoplasma	~1250	Lee et al., 1998
R16(I)F1/R16(I)R1	aster yellows group	~1100	Lee et al., 1994

fAT/rAS	apple proliferation group	~500	Smart et al., 1996

Warsaw, Poland). The digested DNA was resolved by electrophoresis on 6% polyacrylamide gel, stained with ethidium bromide and visualized under UV light. The lengths of DNA fragments were estimated with the help of molecular weight markers (φX174DNA/*Hinf*I, Promega Symbios, Gdańsk, Poland). Phytoplasma 16S rRNA group designations were based on RFLP analysis (Lee et al., 1998).

RESULTS

Symptoms

Two forms of the disease were observed, differing in severity.

A particularly severe form of the disease was observed on several year-old ashleaf maple trees growing in urban areas. Apical shoot growth was reduced, leaf size was subnormal, and autumn coloration was premature. Leaves were sparse, severely malformed, and tufted at the tips of the single, slowly growing shoots. Sometimes the leaves were even necrotic (Fig. 1 and 2). As the disease progressed, twigs and branches died back, and witches' brooms formed. The disease usually started on one or a few branches and could spread over the whole tree in two or three years. Infected trees died within a few years after the first symptoms appear.

A milder form of the disease was observed on young nursery specimens of the cultivar Flamingo, which were one, two or three years old. The leaves were chlorotic or reddened, shoot proliferation was present, and newly expanding leaves were malformed. Overall growth was also reduced.

Phytoplasma detection and identification

After amplification of DNA with the universal primer pair P1/P7, a specific DNA band was obtained only with severely infected periwinkles and with samples of the reference strains: AY1, AP, ULW and CX. No specific band was seen with any of the ashleaf maples or the less severely infected periwinkles.

After nested amplification with the two universal phytoplasma primer pairs (fA/rA and R16F2n/R16R2) and the aster yellows specific primers (R16(I)F1/R16(I)R1), seven out of nine ashleaf maple samples collected in August and October tested positive, whereas none collected in February and May tested positive (Fig. 3ab). Two out of three samples collected from asymptomatic periwinkles also tested positive, as did two out of four of samples collected from the inoculated maples.

Products of PCR primed by R16F2n/R16R2 primer pair from naturally infected *A. negundo* and inoculated periwinkle and maple seedlings were subjected to RFLP analysis using three restriction enzymes. The RFLP band patterns indicated that the plants were infected with a phytoplasma belonging to the aster yellows subgroup B (Fig. 4). This phytoplasma was detected in all naturally infected maples and in all inoculated maples and periwinkles.

Transmission experiments

Three to four months after inoculation, chlorosis and slow growth were observed in 11 out of 21 of the

Figure 1 and 2. Severe leaf malformation and necrosis, and abnormal shoot growth at the base of the trunk of ashleaf maple infected with the aster yellows phytoplasma

Fig. 2

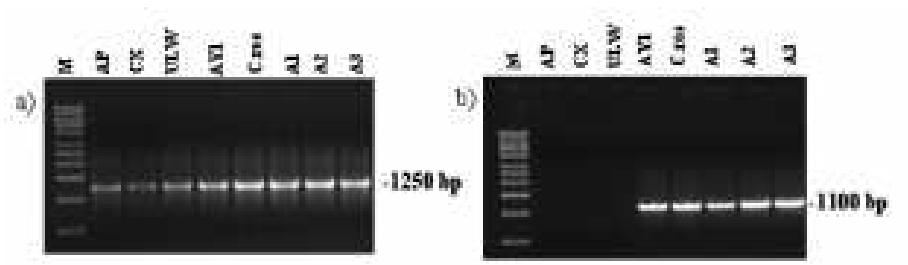

Figure 3. Nested polymerase chain reaction (PCR) products obtained with DNA from *C. roseus* plants experimentally infected by grafting (C ros), affected maple hybrids with disease symptoms (A1) and symptomless (A2) and maple 'Flamingo' (A3) using the following primer pair combinations:

(a) P1/P7 followed by R16F2n/R16R2,
(b) P1/P7 followed by R16(I)F1/R16(I)R1;
M – 1 kb DNA molecular marker (Sigma – Aldrich). The phytoplasma reference strains were: AP, CX, ULW and AY1.

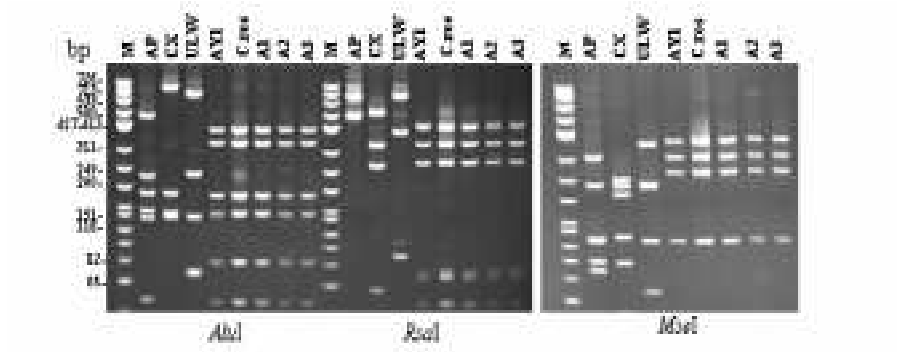

Figure 4. Restriction profiles after *Alu*I, *Rsa*I and *Mse*I digestion of the nested PCR products (primed by R16F2n/R16R2 primer pair) from *C. roseus* experimentally infected by grafting (C ros), affected maple hybrids with disease symptoms (A1) and symptomless (A2) and maple 'Flamingo' (A3). M – molecular weight marker PhiX 174 DNA/*Hin*fI (Promega Symbios); The phytoplasma reference strains were: apple proliferation phytoplasma (AP), X-disease phytoplasma (CX), elm yellows phytoplasma (ULW) and aster yellows phytoplasma (AY1).

inoculated periwinkles. However, one year after inoculation, severe leaf and shoot chlorosis, increased production of secondary shoots, and small, pale flowers were observed in only 2 out of 21 of the inoculated plants (Fig. 5).

None of the other inoculated periwinkle plants had developed symptoms by the end of the two-year observation period. Direct PCR testing revealed the presence of the aster yellows phytoplasma only in some inoculated plants with pronounced symptoms. Nested PCR testing also revealed the presence of this phytoplasma in asymptomatic inoculated plants. None of the control periwinkles or maples showed symptoms or tested positive for the aster yellows phytoplasma.

By the end of the sixteen-month observation period, only one out of fifteen inoculated maples had developed signs of chlorosis and retarded growth. PCR testing revealed the presence of the phytoplasma in this plant, as well as in one of the asymptomatic inoculated maples.

DISCUSSION

The disease affecting ashleaf

maple trees in Poland is probably caused by a phytoplasma. PCR and RFLP showed that a phytoplasma of the aster yellows group was present in diseased maples growing in the field, as well as in inoculated periwinkles and maples. The aster yellows group, 16SrI-B, now is reclassified as '*Candidatus* Phytoplasma asteris'. Members of this group have a wide host range and are encountered in Europe and North America (Seemüller et al., 1998b; Lee et al., 2004). Phytoplasmas of this group were detected in several species of poplar with witches' broom formation, shoot dieback and dieback in France, Germany, Hungary and the Netherlands (Sharma and Cousin, 1986; Berges et al., 1997; Seemüller and Lederer, 1988). The aster yellows phytoplasma has also been recently detected in willows and oaks with shoot proliferation in Lithuania, and in stunted magnolias and roses with shoot proliferation and dieback in Poland (Valiunas, 2003; Kamińska et al., 2001ab; 2003).

In our study, the aster yellows phytoplasma was present in such low titer that it could be detected only with nested PCR. The phytoplasma could be detected in stem phloem at the end of the summer but not in the winter or in the spring. The low titer, seasonal fluctuations, and uneven distribution of phytoplasmas in the host plant possibly play a role in the development of symptoms and also contribute to false negative diagnostic testing. It is sometimes difficult to detect phytoplasmas in the springtime in many woody plants, including stone fruits, roses and magnolias (Schaper and Seemüller, 1984; Seemüller et al., 1998a; Jarausch et al., 1999; Kamińska et al., 2001ab, 2003; Śliwa and Kamińska, 2004). Difficulties in detecting phytoplasmas in *Prunus* have been attributed to limited movement of the pathogen from old phloem to new phloem, because a considerable amount of the phyto-plasma biomass is lost when the old phloem breaks down (Jarausch et al., 1999).

In our study, the aster yellows phytoplasma was detected in both symptomatic and asymptomatic maples, which indicates latent infection. Asymptomatic infection by phytoplasmas has been reported in many woody plants, including *Prunus* spp., *Alnus spp.*, *Celtis australis*, *Fraxinus excelsior, Magnolia* spp. *and Rosa* spp. (Kirkpatrick et al., 1990; Uyemoto et al., 1992; Lederer and Seemüller, 1991; Bertaccini et al., 1996; Jarausch et al., 2001; Kamińska et al., 2001ab). Asymptomatic infection plays an important role in the ecology and epidemiology of phytoplasma associated diseases. Infected plants serve as a reservoir of inoculum which can spread to healthy plants. Detection of phytoplasmas in both symptomatic and asymptomatic plants is therefore essential in clarifying the role that phytoplasmas play in the development of diseases of unknown etiology.

As confirmed by the appearance of symptoms and by PCR technique, we successfully transmitted the aster yellows phytoplasma from diseased maples to periwinkles, and back again to maples. The transmission rate was low. The inoculated periwinkles had fewer and smaller leaves and flowers.

The leaves were often malformed or discolored. This is typical of phytoplasma infections (Hibben and Wolanski, 1971). However, our results were not completely in accordance with Koch's postulates; the inoculated maples developed only mild symptoms or remained asymptomatic, which indicates latent infection.

Our results suggest that a new and devastating maple disease is spreading through nurseries and urban areas in Poland. The presence of phytoplasma in diseased maples strongly suggests that the disease is associated with phytoplasma infection. However, the etiology of the maple disease has not been clearly elucidated. Other potential agents include eriophyid mites, such as *Shevtchenkella brevisetosus,* which has also been suspected of causing leaf abnormalities in maples (Hodgkiss, 1913; Ripka and Lillo, 1997). However, it is not very likely that mites can cause widespread disease characterized by extensive formation of witches' brooms and tree death. On the other hand, phytoplasmas are known to cause formation of witches' brooms and death in woody plants (Marcone et al., 1999). The presence of the aster yellows phytoplasma in diseased ashleaf maples strongly suggests that this phytoplasma is associated with the disease affecting ashleaf maple trees in Poland.

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WYSTĘPOWANIE ‘C*adidatus* PHTOPLASMA ASTERIS’ W DRZEWACH KLONU JESIONOLISTNEGO Z OBJAWAMI PROLIFERACJI I ZAMIERANIA

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S T R E S Z C Z E N I E

Pod koniec lat 90. stwierdzono występowanie nowej bardzo, groźnej choroby klonu jesionolistnego na terenie szkółek i terenów zurbanizowanych w Polsce centralnej i południowej. Objawy chorobowe obejmowały zaburzenia we wzroście, proliferację i zamieranie pędów sugerujące, że choroba ta jest powodowana przez fitoplazmę. Aby wykazać słuszność tego założenia przeprowadzono badania na obecność fitoplazm w drzewach klonu z objawami chorobowymi. Analiza PCR-RFLP wykazała, że w testowanych drzewach występuje fitoplazma żółtaczki astra.

W dalszym ciągu badań siewki rośliny testowej *Catharanthus roseus* zakażano za pomocą pąków pobranych z chorego drzewa, a następnie, młode siewki klonu zakażano za pomocą pędów pobranych z kataranta, który został zakażony fitoplazmą żółtaczki astra i miał objawy chorobowe. Występowanie fitoplazmy żółtaczka astra stwierdzono w zakażanych roślinach, zarówno wykazujących objawy chorobowe, jak i bez objawów. Wyniki te sugerują, że fitoplazma żółtaczki astra jest związana z zamieraniem klonu jesionolistnego. Fitoplazma żółtaczki astra należy do grupy 16SrI, podgrupy B (16SrI-B). Obecnie grupa ta została przeklasyfikowana jako gatunek kandydacki ‘*Candidatus* Phytoplasma asteris’.

Słowa kluczowe: klon, zamieranie, fitoplazma, żółtaczka astra