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THE OCCURRENCE OF '*CANDIDATUS* PHYTOPLASMA ASTERIS' IN *FRAXINUS EXCELSIOR* TREES IN POLAND

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Abstract

The presence of '*Candidatus* Phytoplasma asteris' in *Fraxinus excelsior* plants with shoot proliferation, leaf malformation and diminishing symptoms was demonstrated by nested polymerase chain reaction (PCR) using universal primers pairs that amplified phytoplasma rRNA gene fragment. Amplified cDNA of isolates J1 and J2 were sequenced (GenBank accession number EU219611 and EU219612). The nucleotide sequences of the ash isolates were nearly identical and they showed more than 99% similarity to the corresponding fragments of sequence of '*Candidatus* Phytoplasma asteris', former group 16SrI.

Key words: ash, shoot proliferation, aster yellows phytoplasma

Introduction

The *Fraxinus* genus consists of about 65 species, growing mainly in temperate climate zone. The only native species in Poland is *F. excelsior*.

In the USA a severe disease of *Fraxinus* spp. is ash yellows, called also ash phloem necrosis, caused by ash yellows phytoplasma '*Candidatus* Phytoplasma fraxini' (Matteoni and Sinclair 1985, Dyer and Sinclair 1991, Griffiths et al. 1999, Sinclair and Griffiths 2000). In Europe the ash yellows phytoplasma has a status of quarantine organism and was not found till now.

At the end of 2005, symptoms of growth abnormalities including shoot proliferation, leaf rosetting and malformation of two-three years old ash trees (*F. excelsior*) used as rootstock, as well as on plants of cultivar 'Aurea', were observed in a commercial nursery in south part of Poland. The symptoms were similar to ash yellows disease in the USA.

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The objective of this paper was to report on the occurrence of shoot proliferation, leaf malformation and diminishing symptoms in ash trees in Poland and evaluation of their association with phytoplasma infection using molecular methods.

Materials and methods

Observations on trees were made on cultivated and naturally occurring plants of *F. excelsior* throughout Poland. The selected *F. excelsior* 'Aurea' trees with severe disease symptoms were analyzed.

Nucleic acids were extracted from shoot phloem and leaves of three symptomatic and two symptomless ash trees, and from leaves of healthy *Catharanthus roseus* plants, using a DNeasy Plant Mini Kit (Qiagen, USA). Samples of *C. roseus* leaves inoculated by grafting with the reference strains of aster yellows phytoplasma (AY1, 16SrI-B, kindly supplied by Dr. I.-M. Lee, Beltsville, USA) and the reference strain of apple proliferation phytoplasma (AP, 16SrX-A) and elm yellows phytoplasma (ULW, 16SrV), kindly supplied by Dr. A. Bertaccini, Bologna, Italy, were also included in this study. Samples for analyses were taken from ash trees in the beginning of May, July and October.

Polymerase chain reaction (PCR) was performed with phytoplasma universal primers: P1/P7 (Deng and Hiruki 1991, Kirkpatrick et al. 1994) in the first round, and R16F2n/R16R2 (Lee et al. 1993) or fA/rA (Ahrens and Seemüller 1992) in the second ones, as described by Kamińska et al. (2003) and Śliwa and Kamińska (2004). The amplification product (5 μl) was subjected to electrophoresis in 1% agarose gel and then observed with a UV transilluminator.

The amplification products obtained in the second round of PCR using primers R16F2n/R16R2 were digested with restriction enzymes *Hha*I, *Mse*I and *Rsa*I (Gibco BRL, Life Technologies, Poland), according to the producer's recommendation. Products of enzymatic hydrolysis were subjected to electrophoresis in 8% polyacrylamid gel and then, after staining in water solution of ethidium bromide, analyzed in UV light. The size of the obtained fragments was evaluated based on position of molecular marker Φ174 DNA/*Hinf*I (Promega, Symbios, Gdańsk, Poland).

16S rRNA gene fragments of phytoplasmas found in two *F. excelsior* trees (J1 and J2) were sequenced. Sequencing was performed in AbiPrism 3100 Genetic Analyzer apparatus (Applied Biosystems, USA), in Maria Skłodowska Memorial Cancer Center and Institute of Oncology, Warsaw, Poland.

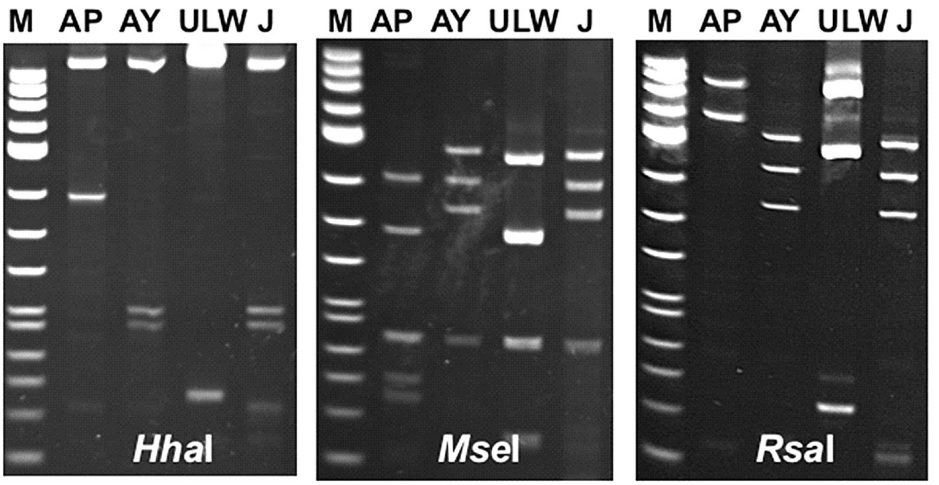
Sequences were analyzed using Lasergene 7.1 software (DNASTAR, USA) and then compared to sequences available in GenBank, with the algorithm BLAST, available in http://www.ncbi.nlm.nih.gov/BLAST/ (Altschul et al. 1997).

Results

Disease symptoms were observed on young two-three years old *F. excelsior* seedlings and on ash trees 'Aurea' growing in a nursery in southern Poland. The growth of affected trees was inhibited and they produced numerous side shoots. Their chlorotic leaves, with severely reduced leaf blades, often formed rosettes. Shoot proliferation was observed only in the first year while chlorosis, leaf diminishing and rosette forming were present during several years of experiment.

Molecular analysis showed that the trees with shoot proliferation and leaf symptoms were infected with a phytoplasma. After amplification of DNA isolated from the tested plants using universal primers P1/P7, specific DNA fragments (~1800 bp) were obtained only for reference isolates AY1, AP and ULW.

In the second round of PCR with universal primers fA/rA or F16F2n/R16R2 amplification product was observed for two out of three samples taken in October from phloem of three ash trees (Phot. 1). Neither in ash phloem sampled in May and July nor in samples from symptomless ash trees, any amplification product was found. It was not found either in any ash leaf sample.



Phot. 1. Electrophoresis of *Hha*I, *Mse*I and *Rsa*I digestion products obtained for phytoplasma detected in ash plant (J) and phytoplasma reference strains (AY, AP, ULW). M – molecular marker Φ174 DNA/*Hinf*I (Promega, Symbios) (photo by H. Berniak)

Preliminary identification of phytoplasmas was performed based on RFLP analysis. Restriction patterns obtained for tested cDNA were characteristic of the aster yellows group phytoplasmas, 16SrI (Phot. 1).

Nucleotide sequences of 16S rRNA gene fragment of phytoplasmas from ash trees were deposed in GenBank (Acc. No. EU219611 and EU219612). The similarity of analyzed sequences was as high as 99.7%. BLAST analysis revealed that those sequences were most similar (ca 99%) to sequences of '*Candidatus* Phytoplasma asteris', group 16SrI-B (Fig. 1).

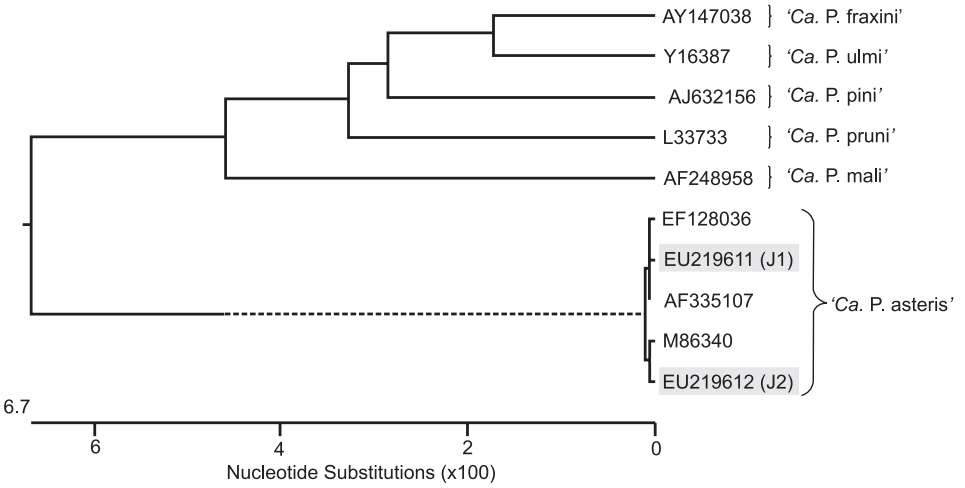


Fig. 1. Similarity dendrogram of sequence of 16S rRNA gene of phytoplasma isolated from ash plants (Acc. No. EU219611 and EU219612) and sequences of selected phytoplasmas from GenBank

Discussion

The young plants of *F. excelsior* with symptoms of growth inhibition, leaf diminishing and shoot proliferation were infected with a phytoplasma. PCR-RFLP and 16S rRNA gene sequence analyses suggest that the phytoplasma belongs to aster yellows phytoplasma group (AY, 16SrI), recently reclassified as a candidate species '*Candidatus* Phytoplasma asteris'. Phytoplasmas from the 16SrI group are the most common phytoplasmas in Europe and North America and have a very broad host range (Seemüller et al. 1998, Lee et al. 2004). Phytoplasmas of 16SrI group were found in many poplar species with witches' broom symptoms and shoot dieback and even entire tree mortality in France, Germany, Hungary and The Netherlands (Sharma and Cousin 1986, Seemüller and Lederer 1988, Berges et al. 1997). Recently, aster yellows phytoplasma was found in Poland in magnolia, rose and maple plants with growth inhibition, proliferation, shoot dieback and leaf symptoms (Kamińska et al. 2001 a, 2001 b, 2003, 2004, Kamińska and Śliwa 2006).

Phytoplasma in ash was present in such a low concentration that it could be detected only in the second round of PCR. Ash tree examination showed also that it was possible to found the phytoplasma in phloem in autumn but not in spring or summer. Low titer and seasonal changes in phytoplasma level in the host plants must probably have great effect on the false negative results and on symptom development. The difficulties in phytoplasma detection seem characteristic of woody plants, e.g. stone trees (Schaper and Seemüller 1984, Seemüller et al. 1998, Jarausch et al. 1999), rose and magnolia (Kamińska et al. 2001 a, 2001 b, 2003, Śliwa

and Kamińska 2004). Jarausch et al. (1999) claim that the difficulties in phytoplasma detection in *Prunus* spp. plants result from limited movement of phytoplasmas from old to new phloem, due to fast degeneration of both phloem elements and the phytoplasmas in it.

The results of observations and experiments point to the existence of a new severe ash disease in Polish nurseries. Detection of a phytoplasma in trees displaying growth disorders and leaf malformation may suggest that the symptoms are connected with tree infection with '*Candidatus* Phytoplasma asteris'. Considering the fact that the diseased ash trees were infected with a phytoplasma, and that '*Candidatus* Phytoplasma asteris' is the most often occurring phytoplasma in Poland, we assume that aster yellows phytoplasma is the major cause of ash proliferation of *F. excelsior* trees in Poland.

Conclusions

1. For the first time in Poland a *Fraxinus excelsior* disease causing inhibition of tree growth, shoot proliferation, leaf diminishing and malformation was described.
2. The disease described on ash in Poland resembles, from the point of view of symptoms, the ash yellows caused by '*Candidatus* Phytoplasma fraxini', occurring in USA.
3. PCR-RFLP and sequence analysis point to the fact that ash proliferation in Poland is connected with plant infestation by aster yellows phytoplasma '*Candidatus* Phytoplasma asteris', which is probably the disease causing agent.

Streszczenie

WYSTĘPOWANIE FITOPLAZMY '*CANDIDATUS* PHYTOPLASMA ASTERIS' W DRZEWACH *FRAXINUS EXCELSIOR* W POLSCE

Stwierdzono występowanie fitoplazmy '*Candidatus* Phytoplasma asteris' w młodych drzewach *Fraxinus excelsior* z objawami proliferacji pędów, zdrobnieniem i deformacją liści. Obecność fitoplazmy w łyku chorych jesionów wykazano w drugiej rundzie łańcuchowej reakcji polimerazy (PCR) ze starterami uniwersalnymi dla fitoplazm – P1/P7 w pierwszej rundzie oraz fA/rA lub R16F2n/R16R2 w drugiej rundzie. Zamplifikowane cDNA izolatów fitoplazm J1 i J2 zsekwencjonowano (GenBank Acc. No. EU219611 i EU219612). Sekwencje fitoplazm z jesionów były prawie identyczne (99,7%). Na podstawie analizy BLAST wykazano, że były one w ponad 99% podobne do sekwencji '*Candidatus* Phytoplasma asteris', dawniej grupa 16SrI.

Literature

Ahrens U., Seemüller E., 1992: Detection of plant pathogenic mycoplasmalike organisms by a polymerase chain reaction that amplifies a sequence of the 16S rRNA gene. Phytopathology 82: 828–832.

Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Zhang Z., Miller W., Lipman D.J., 1997: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acids Res. 25: 3389–3402.

Berges R., Cousin M.-T., Rouxy J., Mäurer R., Seemüller E., 1997: Detection of phytoplasma infection in declining *Populus nigra* 'Italica' trees and molecular differentiation of the aster yellows phytoplasmas identified in various *Populus* species. Eur. J. For. Pathol. 27: 33–43.

Deng S., Hiruki C., 1991: Amplification of 16S rRNA genes from culturable and nonculturable mollicutes. J. Microbiol. Methods 14: 53–61.

Dyer A.T., Sinclair W.A., 1991: Root necrosis and histological changes in surviving roots of white ash infected with mycoplasmalike organisms. Plant Dis. 75: 814–819.

Griffiths H.M., Sinclair W.A., Smart C.D., Davis R.E., 1999: The phytoplasma associated with ash yellows and lilac witches' broom: '*Candidatus* Phytoplasma fraxini'. Int. J. Syst. Bacteriol. 49: 1605–1614.

Jarausch W., Lansac M., Dosba F., 1999: Seasonal colonization pattern of European stone fruit yellows phytoplasmas in different *Prunus* species detected by specific PCR. J. Phytopathol. 147: 47–54.

Kamińska M., Dziekanowska D., Rudzińska-Langwald A., 2001 a: Detection of phytoplasma infection in rose, with degeneration symptoms. J. Phytopathol. 149: 3–10.

Kamińska M., Śliwa H., 2006: First report of a decline of ashleaf maple (*Acer negundo*) in Poland, associated with '*Candidatus* Phytoplasma asteris'. Plant Pathol. 55: 293.

Kamińska M., Śliwa H., Malinowski T., Skrzypczak Cz., 2003: The association of aster yellows phytoplasma with rose dieback disease in Poland. J. Phytopathol. 151: 469–476.

Kamińska M., Śliwa H., Rudzińska-Langwald A., 2001 b: The association of phytoplasma with stunting, leaf necrosis and witches' broom symptoms in magnolia plants. J. Phytopathol. 149: 719–724.

Kamińska M., Śliwa H., Rudzińska-Langwald A., 2004: Choroby fitoplazmatyczne roślin ozdobnych. Progr. Plant Prot. / Post. Ochr. Rośl. 44: 776–779.

Kirkpatrick B.C., Smart C.D., Gardner S.L., Gao J.-L., Ahrens U., Mäurer R., Schneider B., Lorenz K.-H., Seemüller E., Harrison N.A., Namba S., Daire X., 1994: Phylogenetic relationships of plant pathogenic MLOs established by 16/23S rDNA spacer sequences. Int. Org. Mycoplasmol. Lett. 3: 228–229.

Lee I.-M., Gundersen-Rindal D.E., Davis R.E., Bartoszyk I.M., 1998: Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. Int. J. Syst. Bacteriol. 48: 1153–1169.

Lee I.-M., Gundersen-Rindal D.E., Davis R.E., Bottner K.D., Marcone C., Seemüller E., 2004: '*Candidatus* Phytoplasma asteris', a novel phytoplasma taxon associated with aster yellows and related diseases. Int. J. Syst. Evol. Microbiol. 54: 1037–1048.

Lee I.-M., Hammond R.W., Davis R.E., Gundersen D.E., 1993: Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasmalike organisms. Phytopathology 83: 834–842.

Matteoni J.A., Sinclair W.A., 1985: Role of the mycoplasmal disease, ash yellows, in decline of white ash in New York State. Phytopathology 75: 355–360.

Schaper U., Seemüller E., 1984: Einfluss des Besiedlungsverhaltens auf die fluoreszenz-mikroskopische Nachweisbarkeit der Erreger der Apfeltriebsucht und des Birnenverfalls. Nachrichtenbl. Dtsch. Pflanzenschutzdienst (Berl.) 36: 21–25.

Seemüller E., Lederer W., 1988: MLO-associated decline of *Alnus glutinosa, Populus tremula* and *Crataegus monogyna*. J. Phytopathol. 121: 33–39.

Seemüller E., Marcone C., Laurer U., Ragozzino A., Göschl M., 1998: Current status of molecular classification of the phytoplasmas. J. Plant Pathol. 80: 3–26.

Sharma A.K., Cousin M.-T., 1986: Mycoplasmalike organisms (MLOs) associated with the witches' broom disease of poplar. J. Phytopathol. 117: 349–356.

Sinclair W.A., Griffiths H.M., 2000: Variation in aggressiveness of ash yellows phytoplasmas. Plant Dis. 84: 282–287.

Smart C.D., Schneider B., Blomquist C.L., Guerra L.J., Harrison N.A., Ahrens U., Lorenz K.-H., Seemüller E., Kirkpatrick B.C., 1996: Phytoplasma-specific PCR primers based on sequences of the 16S rRNA spacer region. Appl. Environ. Microbiol. 62: 2988–2993.

Śliwa H., Kamińska M., 2004: Experimental transmission of phytoplasmas from diseased magnolias to *Catharanthus roseus* test plants by grafting. Phytopathol. Pol. 32: 21–31.

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