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EPIDEMICS OF GROUP 16SRI-A PHYTOPLASMAS IN A GARDEN OF VILNIUS REGION IN LITHUANIA

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Abstract

Jomantienė R., Ivanauskas A., Valiūnas D., Urbanavičienė L., Šneideris D., 2016: Epidemics of group 16SrI-A phytoplasmas in a garden of Vilnius region in Lithuania [16SrI-A, pogrupio fitoplazmų sukelta epidemija sode Vilniaus rajone Lietuvoje]. – Bot. Lith., 22(1): 16–22.

Here we report on a plant disease caused by insect-transmitted unculturable plant pathogenic bacteria detected in a private garden in Vilnius region. Samples of symptomatic peas (*Pisum sativum* L.), nasturtium (*Tropaeolum majus* L.), strawberry (*Fragaria* × *ananassa* Duchesne) and zucchini (*Cucurbita pepo* var. *giromontina*) plant tissues were collected. Based on the molecular technique, the Internet tools and phylogenetic analysis, these pathogens were identified as phytoplasmas and classified in phytoplasma RFLP (restriction fragment length polymorphism) group 16SrI, subgroup A. Because this pathogen may be spread by insect-vector that comes from the wild nature, the phytoplasmas could cause a problem in agriculture of Lithuania.

Keywords: agriculture, nasturtium, pathogen, pea, 16S rDNA, strawberry, symptoms, zucchini.

INTRODUCTION

Diseased plants exhibiting symptoms such as yellowing of leaves, shoot proliferation, stunting, phyllody may be noticed in any garden or in the wild nature. Such symptoms are similar to those caused by bacteria named phytoplasmas. Phytoplasmas exhibit numerous features (they are uncultivable organisms, reside in a plant phloem, contain small A+T rich genomes, etc.), which makes their investigation rather difficult (Lee et al., 2000). Often, when diseased plant samples are collected from the wild nature and phytoplasma titer is low, it is difficult to amplify phytoplasmal DNA. Low G+C content in the genomes complicates the design of primers for amplification of genes and also the investigation of the phytoplasma genomes. Classification of these pathogens is based on the sequence analyses of the genetic markers as 16S rRNA, rp (ribosomal protein), sec Y genes (Gundersen et al., 1994; Lee et al., 1998; Martini et al., 2007, Lee et al., 2010). Accumulating data on biodiversity of phytoplasmas revealed more than 35 16Sr groups and more than hundred subgroups worldwide (Wei et al., 2007; Davis et al., 2013). Phytoplasmas infect the angiosperms and the gymnosperms. Phytoplasmas are transmitted by insect-vectors that feed on plants mainly leafhoppers and psyllids (Weintraub & Beanland, 2006). The diseases can cause big economic losses related to grapevine (Davis et al., 1993), strawberry (Jomantiene et al., 1998), stone fruit (Valiunas et al., 2009b) also vegetable (Lee et al., 1998) production. Phytoplasmas can cause a serious damage in the wild nature: numerous diseased pine trees infected by phytoplasma were detected in the Curonian Spit, a unique and UNESCO protected region of Lithuania (Valiunas et al., 2015), also the wild blueberry proliferation has spread in the forest of Vilnius region (Valiunas et al., 2004), diseased oak trees grow near Alytus (Jomantiene et al., 2002).

The aim of this study was to reveal the pathogens in the diseased plants in the garden surrounded by

the forest. We expected the results to indicate what similar damages could be possible in the cultivated plants of agriculture in Lithuania.

MATERIALS AND METHODS

Plant samples, PCR, RFLP and sequence analysis, and phytoplasma classification

Samples of symptomatic peas (*Pisum sativum* L.), nasturtium (*Tropaeolum majus* L.), strawberry (*Fragaria* × *ananassa* Duchesne) and zucchini (*Cucurbita pepo var. giromontina*) plant tissues, including leaves and petioles**,** were collected in a private garden in Vilnius region, Lithuania. DNA was extracted from the collected tissues using a Genomic DNA purification Kit (Thermo Fisher Scientific, Vilnius, Lithuania) and used as template in polymerase chain reactions (PCRs) for amplification of 16S ribosomal (r) RNA and ribosomal protein gene sequences. In semi-nested and nested PCRs for amplification of rRNA gene sequences the first reaction was primed by primer pair P1/16S-Sr (Deng & Hiruki, 1991; Lee et al., 2004). Products obtained in the first PCR were diluted 1:50 with sterile water and used as template in the second (semi-nested) PCR primed by primer pair P1A/16S-Sr and in nested PCR primed by R16F2n/R16R2n (F2n/R2n) (Lee et al., 1998, 2006). Amplifications were conducted under the same conditions (94°C for 1 min, 55°C for 2 min, 72°C for 3 min) for 35 cycles (first denaturation was at 94°C for 10 min, and extension in final cycle was at 72°C for 10 min) in Perkin Elmer buffer, 0.25 mM dNTPs, 0.4 μM of each primer, and 1 unit of HotStart AmpliTaq Gold DNA polymerase (Applied Biosystems Inc., Foster City, CA) per 50 μl of reaction mixture. Annealing temperature for amplification of rp sequences was 50°C. 16S rDNA products (1.2 kbp, F2n/R2n segment) of the nested PCR were subjected to enzymatic restriction fragment length polymorphism (RFLP) analysis using restriction endonucleases *Alu*I, BfaI, *Mse*I, *Rsa*I, *Hae*III, *Hha*I, HpaII, HinfI **(**Thermo Fisher Scientific, Vilnius, Lithuania). Digested products were analysed using electrophoresis through 5% acrylamide gel for rDNA. DNA bands were stained with ethidium bromide and visualized using a UV transilluminator. Phytoplasmas were classified in subgroups through comparison of RFLP patterns with the patterns previously published in accordance with the classification scheme of Lee et al. (1998). The amplified from nasturtium infecting phytoplasma 16S rDNA ann *rp* gene fragments were cloned in *Escherichia coli*, using InsT/Aclone™ PCR Product Cloning Kit (Thermo Fisher Scientific, Vilnius) and sequenced. For phylogenetic analyses, nucleotide sequences were aligned using Clustal X 1.63 b (Thompson et al., 1997), and trees were viewed using TreeViewPPC (Page, 1996**).** Internet tools BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and *i*PhyClssifier (http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) were used for phytoplasma identification, too.

RESULTS

Symptoms of the diseased plants

In the private garden of Vilnius region, we detected numerous diseased plants showing symptoms similar to phytoplasma infection. The five nasturtium plants were stunted and yellowing, also exhibited an abnormal proliferation of axillary shoots (Fig. 1, A). The 11 diseased plants of strawberry showed symptoms of green bell growths on the stem and fruit phyllody (Fig. 1, B). The numerous pea plants exhibited symptoms of witches broom (proliferation of stems) and yellowing (Fig.1, C). The diseased seven zucchini plants had smaller yellowing leaves with the laciniated edge (Fig.1, D).

Amplification of 16S rDNA and identification of pathogen

Total DNA extracted from the diseased plants was used as template in direct and nested PCR for amplification of 16S rDNA. First, the 16S rDNA was amplified from each symptomatic plant separately. The positive results of nested PCR indicated that the plants may be infected by phytoplasma (Fig. 2).

RFLP (restriction fragment length polymorphism) analysis of PCR products amplified in nested PCR using eight restriction endonucleases revealed phytoplasma of group 16SrI. All investigated plants were infected by the same phytoplasma (data not shown). The key enzyme *Hha*I indicated that 16S rDNA amplified from NasPr phytoplasma strain belongs to subgroup A in group 16SrI (Fig. 3).

Fig. 1. Nasturtium (A), strawberry (B), pea (C), and zucchini (D) exhibiting disease symptoms associated with infection by phytoplasma

Nucleotide sequence analyses

16S rDNA from nasturtium was cloned and sequenced. BLAST search using this 16S rDNA as query confirmed that the 16S rDNA belongs to phytoplasma of group 16SrI-A. *i*Phy classifier also classified the 16S rDNA amplified from nasturtium to subgroup 16SrI-A. The phylogenetic analysis confirmed nasturtium 16S rDNA similarity with the other 16S rDNAs from phytoplasma strains of subgroup 16SrI-A detected in plants (Fig. 4). Analysis of *rp* gene sequences also confirmed phytoplasma classification to subgroup I-A (data not shown).

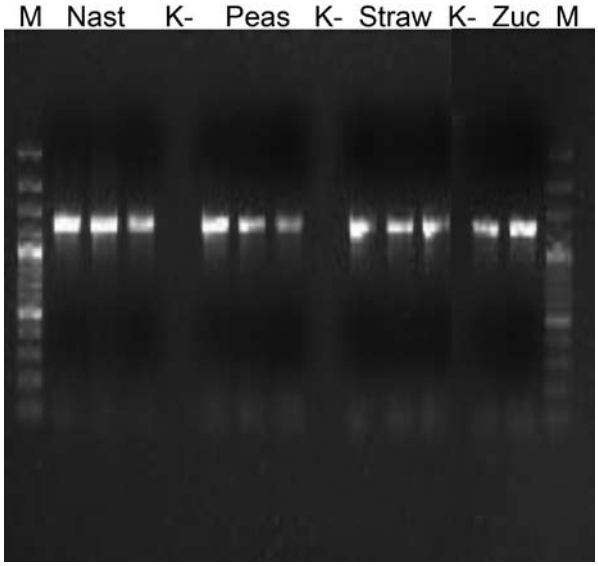

Fig. 2. Nested PCR primed by primers 16S F2n/R2n from nasturtium, pea, strawberry and zucchini plants. M, GeneRuler™ 100 bp DNA Ladder Plus (Thermo Fisher Scientific, Vilnius, Lithuania), fragment sizes: 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100; K^-, negative water control

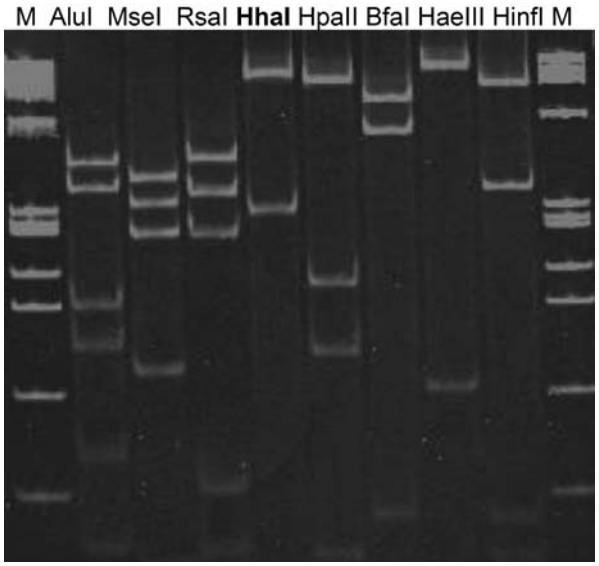

Fig. 3. RFLP analysis of 16S rDNA amplified from diseased nasturtium plant. M, DNA Ladder ØX174 DNA/*Bsu*RI (*Hae*III) (Thermo Fisher Scientific, Vilnius, Lithuania), fragment sizes: 1353, 1078, 872, 692, 310, 281, 271, 234, 194, 118, 72 bp

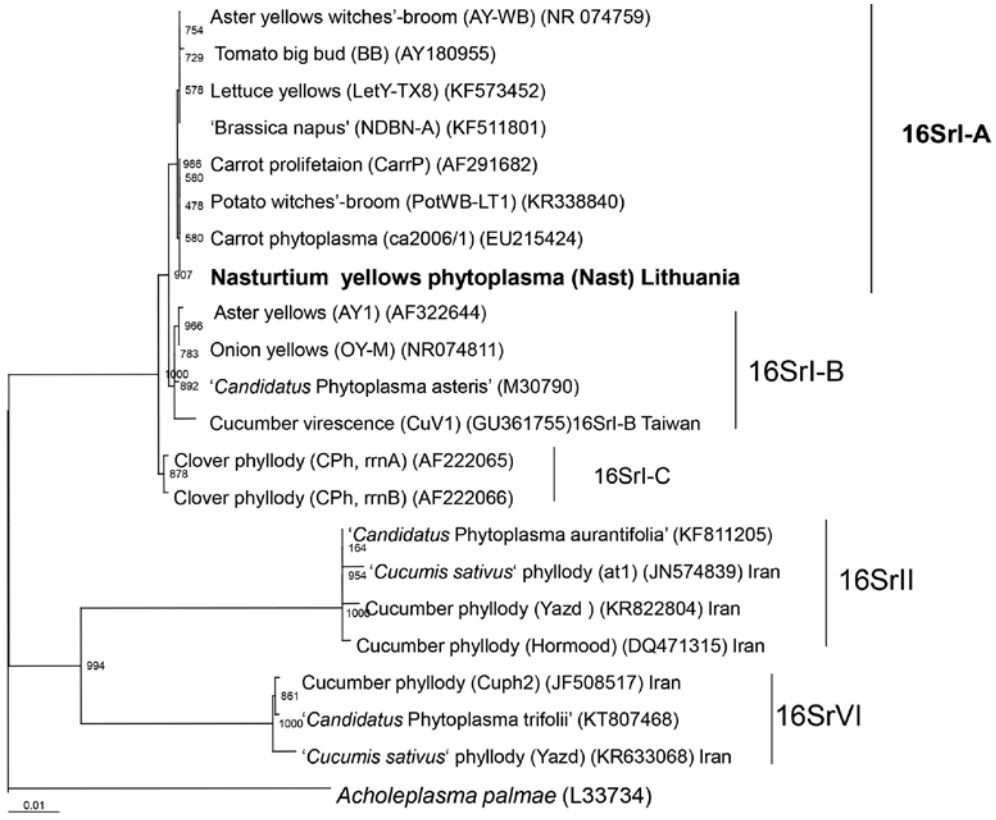

Fig. 4. Phylogenetic tree of phytoplasmas constructed by the Neighbour-Joining method of 16S rRNA gene sequences from phytoplasma strains and *Acholeplasma palmae*, employing *A. palmae* as the out-group. Length of bar is proportional to the number of inferred character state transformations. Nasturtium phytoplasma is in bold

DISCUSSION

In this study, we describe the investigation of plant infections caused by phytoplasmas. PCR, RFLP analysis, sequence analysis of 16S rDNA amplified from the diseased nasturtium plants using the Internet tools and phylogenetic screening indicated that plant-damaging phytoplasma strain belongs to group 16SrI subgroup A. Phytoplasma strains belonging to this subgroup are distributed in North America (Seemüller et al., 1994), however, they are rare in Europe (Valiunas et al., 2001). It is interesting that phytoplasmas of this subgroup cause plant infections in Lithuania relatively often (Valiunas et al., 2001). Recently, 16SrI-A phytoplasmas have been detected in pine trees in the Curonian Spit (Valiunas et al., 2015) and in potato plants (Ivanauskas et al., 2016). For the first time in Lithuania, the phytoplasma infection was detected in peas, nasturtium and zucchini. Phytoplasma infection in strawberry has been detected in Lithuania previously; however, the infection was caused by different phytoplasma strain classified in subgroup 16SrXII-E (Valiunas et al., 2006).

As phytoplasma infection was detected in numerous symptomatic plants in the garden, we can describe it as a small 16SrI-A phytoplasma "epidemics". The epidemics could be explained by the abundance of the insect vectors distributed in the surroundings.

The results of almost 15 years of investigation on phytoplasma diseases in Lithuania have not revealed a significant diversity of the pathogen. We have described only five diverse groups: 16SrI, 16SrIII, 16SrV, 16SrXII, 16SrXXI. The epidemics of phytoplasma disease caused by subgroup 16SrIII-T phytoplasma strain in sweet cherries have previously been reported in Kaunas region (Valiunas et al., 2009a). The situation in the Curonian Spit, where phytoplasma infected pine trees make 25%, can also be called "epidemics". This study has revealed a "small epidemics" caused by phytoplasma of subgroup 16SrI-A in the private garden. As there are no real ways how to fight phytoplasmas, the only one is a control of insect – vectors together with the distribution of phytoplasma diseases in the country. The phytoplasma infections can become a serious problem for Lithuanian agriculture.

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16SRI-A POGRUPIO FITOPLAZMŲ SUKELTA EPIDEMIJA SODE VILNIAUS RAJONE LIETUVOJE

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Santrauka

Straipsnyje yra pateikta informacija apie augalų ligas, kurias sukelia vabzdžių platinamos, nekultivuojamos, augalams patogeninės bakterijos, aptiktos privačiame sode Vilniaus rajone. Tyrimams buvo surinkti simptomatinių žirnių, nasturtų, braškių ir cukinijų augalų audinių pavyzdžiai. Molekuliniais metodais, internetiniais įrankiais ir filogenetine analize šie patogenai buvo identifikuoti kaip fitoplazmos ir klasifikuoti į 16SrI-A fitoplazmų pogrupį. Identifikuotos fitoplazmos yra platinamos vabzdžių, kurie plinta iš laukinės gamtos, todėl gali sukelti problemų Lietuvos žemės ūkiui.