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The first detection of '*Candidatus* Phytoplasma trifolii' in *Rhododendron* hybridum

Jaroslava Přibylová · Karel Petrzik · Josef Špak

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Abstract Four *Rhododendron* hybridum plants (from cvs Moravanka and Don Juan), all exhibited symptoms of shortened axillary shoots, reduced leaves with vein clearing and yellowing, undeveloped flowers, and general stunting in a rhododendron nursery garden in southern Bohemia in 2007. Electron microscopy examination of ultra-thin sections revealed the presence of numerous polymorphic phytoplasma-like bodies in the phloem tissue of leaf midribs and petioles. The phytoplasma etiology of this disease was further confirmed by polymerase chain reaction (PCR) using universal phytoplasma primers. Restriction fragment length polymorphism (RFLP) analysis of amplification products obtained with a R16F2/R16R2 primer pair from all symptomatic plants indicated the presence of phytoplasma from the 16SrVI-A subgroup. A detailed comparison of the amplified sequences and phylogenetic analysis confirmed that the phytoplasma belonged to the subgroup 16SrVI-A (clover proliferation phytoplasma group). This is the first report of the natural occurrence of '*Candidatus* Phytoplasma trifolii' in plants of *Rhododendron* hybridum.

J. Přibylová (✉) · K. Petrzik · J. Špak
Department of Plant Virology,
Institute of Plant Molecular Biology,
Biology Centre of Academy of Sciences
of the Czech Republic, v.v.i.,
Branišovská 31,
370 05 České Budějovice, Czech Republic
e-mail: pribyl@umbr.cas.cz

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Fungal and phytoplasmal pathogens are known to occur in genus *Rhododendron*. In particular, *Phytophthora* species (Schwingle et al. 2007), powdery mildew (Lebeda et al. 2007), *Exobasidum racemosum* (Li and Guo 2006) and *Rhizoctonia* species (Rinehart et al. 2007) are responsible for causing extensive losses of *Rhododendron* sp. plants. Phytoplasma disease of *Rhododendron* sp. plants was first observed in the Ukraine in azalea and showed symptoms of yellowing (Onishchenko et al. 1988). Witches'-broom disease was observed on the Kanehirai azalea (*Rhododendron kanehirai*) in Taiwan (Wang 1994). In both cases of affected azaleas, phytoplasmas were detected in sieve elements by electron microscopy examination. The first phytoplasma-like symptoms were observed in the rhododendron plants 'Cunningham's White' in the Czech Republic in 1997; these symptoms included leaf malformation and variegation. Phytoplasma was detected using nested PCR and identified by restriction fragment length polymorphism (RFLP) as stolbur-type phytoplasma (Mertelík et al. 2006).

In this paper, we describe the detection and molecular analysis of a phytoplasma in naturally infected rhododendron plants with symptoms of stunting and yellowing. The phytoplasma was identified as a member of clover proliferation phytoplasma group (16SrVI group)

(Lee et al. 1998) or ('*Candidatus* Phytoplasma trifolii') (Hiruki and Wang 2004).

Four plants of *Rhododendron* hybridum. (three cv. Moravanka [13, 14, 15], one cv. Don Juan) showing symptoms of phytoplasma infection were found in a rhododendron nursery garden in southern Bohemia in 2007. All of these plants exhibited shortened axillary shoots, reduced leaves with vein clearing and yellowing often combined with necrosis, undeveloped flowers and general stunting (Fig. 1). These symptomatic plants originated from meristem culture-propagules in 2000 at the Research Institute for Landscape and Ornamental Gardening (RILOG) Průhonice, v.v.i. (Czech Republic). Adult plants were then grown in peaty soil within the nursery garden located in a forest at Mažice in southern Bohemia. All four symptomatic plants were taken from the field and maintained under outdoor conditions in an insect-proof nethouse.

Examination of ultra-thin cross-sections of leaf midribs and petioles from axillary shoots of two symptomatic rhododendron plants (cvs Moravanka and Don Juan) with a Jeol 100 MB transmission electron microscope revealed a large number of structures resembling phytoplasmas in phloem tissue. Some phloem cells were entirely filled with these bodies (cv. Moravanka) (Fig. 2) or contained phytoplasma bodies in residual cytoplasm (cv. Don Juan). Bodies exhibited a large range of size, from 50 to 600 nm, with an average size of 260 nm. No other pathogenic organisms, bacteria, virus or virus-like structures, were observed.

Leaf midribs of young shoots from the four symptomatic and three asymptomatic (normal with healthy appearance) rhododendron plants were obtained for DNA extraction. DNA was isolated from 3 g of fresh tissue according to the method proposed by Lee et al. (1991). Nucleic acid pellet was resuspended in 40 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA) to a final concentration of 20 ng μl^{-1}. DNA extracts were used as template for direct PCR specifically amplifying a portion of 16S ribosomal RNA (rRNA) gene using the universal phytoplasma primers R16F2 and R16R2 (Gundersen and Lee 1996). Tubes with the reaction mixture devoid of DNA templates were included in each experiment as negative controls. Six microlitres of each PCR product were analysed by running through a 1% agarose electrophoretic gel, followed by staining in

Fig. 1 Rhododendron shrub of cv. Moravanka affected by '*Candidatus* Phytoplasma trifolii', showing healthy-appearing flowers as well as symptoms of yellowing, shortening of internodes and general stunting

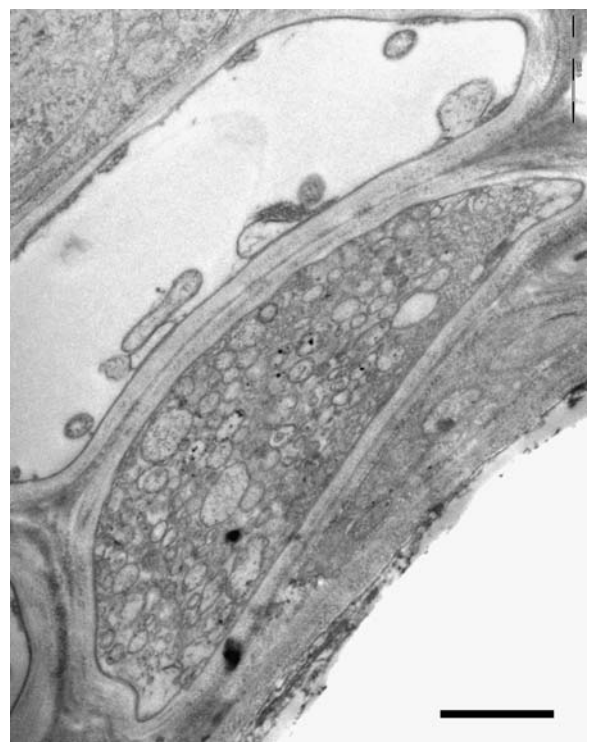

Fig. 2 Transmission electron micrograph of an ultra-thin section of the sieve element of the leaf midrib of cv. Moravanka showing a large number of phytoplasmas (*bar*, 500 nm)

SYBR Green I and visualisation of DNA bands under a UV transilluminator. PCR products of about 1.2 kb localised between 152 and 1,397 bp from the 16SrRNA gene were obtained in direct PCR with universal phytoplasma primers R16F2/R16R2 in all four infected rhododendron plants. No PCR product was obtained from asymptomatic samples. To identify phytoplasmas infecting rhododendron plants, PCR products of infected rhododendron samples amplified with R16F2/R16R2 primers were digested with 10 U each of restriction endonucleases *AluI*, *BsuRI*, *HhaI*, *HpaII* and *RsaI* (Fermentas, Lithuania) in 20 µl total volume (10 µl PCR products) at 37°C overnight. The digests were resolved on an 8% polyacrylamide gel followed by ethidium bromide staining and photographed under UV at 312 nm using a transilluminator. The approximate molecular weights and the sizes of the resulting DNA bands were estimated using a 100 bp DNA Ladder (NE Biolabs, Beverly, MA, USA) (Fig. 3). The digestion of amplified fragments showed patterns practically identical among the digested PCR products of four rhododendron isolates, suggesting that all isolates originated from the same phytoplasma. When compared with the standard restriction pattern, the rhododendron-infecting phytoplama was classified as a member of the 16SrVI-A subgroup of the revised classification scheme of phytoplasmas (Lee et al. 1998).

A set of overlapping PCR products from four symptomatic rhododendron plants was generated by amplification with primers P1/U3 (position 6–1,230), R16F2/R16R2 (position 152–1,397), 16R758/P7 (position 758–1,818) (Gibb et al. 1995, Lorenz et al. 1995). With use of a BIG DYE sequencing terminator kit (PE Biosystems, Warington, UK), each PCR product was sequenced from both directions to cover the whole length of 16S rDNA and the 16/23S rDNA spacer region. Sequencing was performed in an ABI PRISM 310 sequencer (PE Applied Biosystems, Foster City, CA, USA). The sequences were aligned with sequences of the 16SrVI group phytoplasmas available in the GenBank and a phylogenetic tree was constructed using the MEGA software (Kumar et al. 2004). The phylogenetic analysis grouped all four samples closely with other members of the '*Candidatus* Phytoplasma trifolii' (Fig. 4). The three sequences of cv. Moravanka were identical; they differed from the sequence of cv. Don Juan in one position (deletion at nucleotide 1747). The sequences of the 16S rDNA and the 16/23S rDNA spacer region were deposited in the GenBank database under accession numbers EU543440 (cv. Moravanka) and EU543441 (cv. Don Juan). The detailed sequence comparison revealed the closest relationship of the phytoplasmas infecting rhododendrons with potato witches'-broom (PWB; GenBank Accession No. AY500818), potato witches'-broom (PWB; GenBank Accession No. DQ256089) and clover proliferation (CP; GenBank Accession No. AY500130) and confirmed the classification in the '*Candidatus* Phytoplasma trifolii'. Five differences were found between the three sequences mentioned above and the sequences from rhododendrons: The deletions at positions 73, 74, 1,695 and 1,756 and the 'G' at position 1,021 clearly discriminate rhododendron

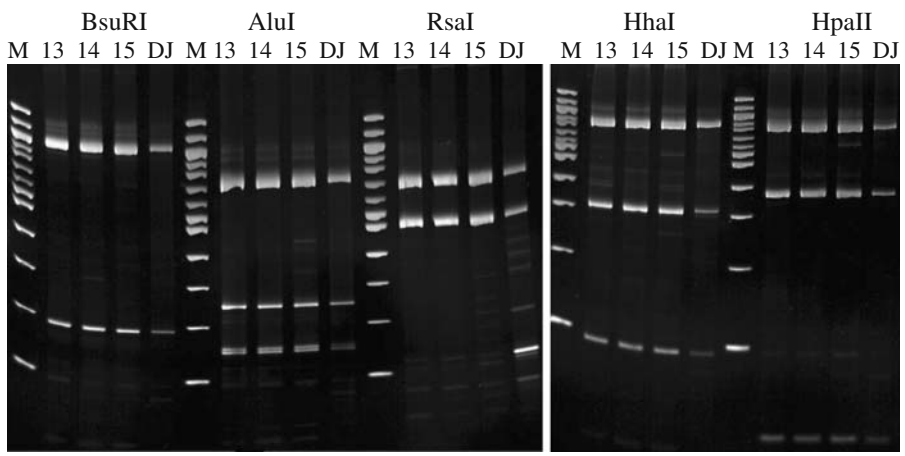

Fig. 3 Restriction fragment length polymorphism analysis of 1.2 kbp PCR products of Moravanka (*M*) *13*, *14*, *15* and Don Juan (*DJ*) digested with *BsuRI*, *AluI*, *RsaI*, *HhaI* and *HpaII*. *Lane M*, 100 bp DNA Ladder (NE Biolabs, Beverly, MA, USA)

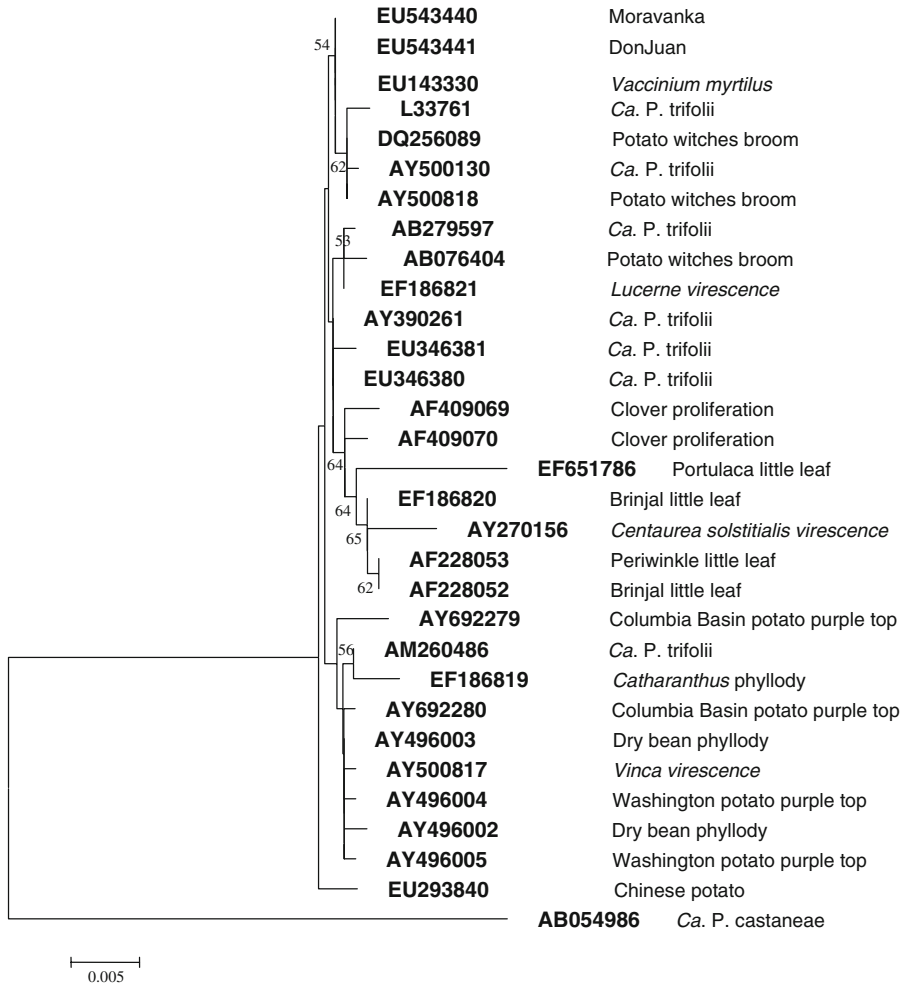

Fig. 4 Phylogenetic tree constructed using the analysis of partial 16S rRNA sequences. Neighbour-joining method with 1,000 replicates. Bar represents a phylogenetic distance of 0.5%. GenBank accession numbers and bootstrap values >50 are shown. '*Candidatus* Phytoplasma castaneae' was used as the outgroup

phytoplasmas. The homology search using CLUSTAW programme showed >99% similarity. (Nucleotide positions according to the AY500818 sequence).

'*Candidatus* Phytoplasma trifolii', a novel *Candidatus* species was designed for the clover proliferation phylogenetic group and is represented by the reference strain, clover proliferation phytoplasma (CP^R). Three subgroups have been classified by sequence homology and by the collective RFLP patterns of amplified 16SrRNA genes: CP-A, CP-B and CP-C (Hiruki and Wang 2004). Plants affected by 16SrVI phytoplasma group have been found in North America and Asia; few reports on wild plants have been published in Europe. Phytoplasmas belonging to 16SrVI group were identified in *Vaccinium myrtillus* in Austria (Borroto-Fernández et al. 2007). *Centaurea solstitialis virescence* phytoplasma found in Italy is considered a new subgroup 16SrVI-D of the clover proliferation group (Faggioli et al. 2004). This paper reports the first occurrence of the 16SrVI phytoplasma group in the Czech Republic. The detailed sequence characterisation of rhododendron phytoplasmas revealed the closest homology with the three above-mentioned sequences AY500818, DQ256089 and AY500130, members of '*Candidatus* Phytoplasma trifolii' (16SrVI-A), originating from Canada and found in clover and potato, respectively.

All rhododendron plants mentioned in this study (as in the study by Mertelík et al. 2006) originated from a breeding programme at the RILOG Průhonice

and from tissue cultures of Czech plant material. The phytoplasma diseases found in the Czech Republic differed from each other; they induced profoundly different symptoms. The stolbur-type rhododendron plants exhibited leaf malformation and variegation in contrast to the stunting, yellowing and absence of flowers of those infected by '*Candidatus* Phytoplasma trifolii'. The particularly detrimental effect of this disease on development of flowers and the leaf yellowing poses a serious problem for ornamental rhododendrons. Four 8-year-old seedlings infected by '*Candidatus* Phytoplasma trifolii' were found at the nursery garden at Mažice in southern Bohemia. Though it is probable these plants were not infected until their introduction to the field, the source of infection remains unknown. This is the first study of molecular detection and identification of the 16SrVI group in rhododendrons and the first report of CP group in the Czech Republic. Detection of the same phytoplasma in other plants and insect vectors is needed to determine the potential focus of infection and/or the way of introduction.

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