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Short communication

Electron microscopy and molecular characterization of phytoplasmas associated with strawflower yellows in the Czech Republic

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Abstract

Strawflower (*Helichrysum bracteatum*) with symptoms resembling those associated to phytoplasma infection were observed in several areas in the Czech Republic during the period 1994–2001. Plants with leaf bronzing, reddening and necrosis, proliferation of secondary shoots, flower abnormalities and dwarfing died in advanced stages of the disease. The disease incidence ranged from 2% to 70% and caused significant loss to the flower and seed production. Transmission electron microscopy showed phytoplasmas in sieve cells of affected plants, but not in healthy ones. Association of phytoplasmas with the disease was confirmed by polymerase chain reaction using phytoplasma universal ribosomal primers R16F2n/R16R2. An amplification product of the expected size (1.2 kb) was observed in all samples of the symptomatic strawflowers. The restriction profiles obtained following separate digestion with three endonucleases (*Alu*I, *Hha*I, *Mse*I) showed that phytoplasmas infecting strawflowers from different localities in the Czech Republic were uniform and undistinguishable from aster yellows (subgroup 16SrI-B). Sequence analysis of 1771 bp of the ribosomal operon amplified with primers P1/U3, R16F2n/R2 and 16R758/P7 indicated that the closest related phytoplasmas were those associated with '*Rehmannia glutinosa* var. *purpurea*', both originating from Bohemia. This is the first report on the occurrence of a phytoplasma-associated disease of strawflower in the Czech Republic.

Strawflower (*Helichrysum bracteatum*), a member of the family Asteraceae is a small acreage flower crop in the Czech Republic, grown primarily for cut flowers for dry bouquets. Strawflowers with flower abnormalities and stunted growth reminiscent of phytoplasma infection were repeatedly found at different locations in gardens in Southern Bohemia [localities (number of symptomatic/healthy looking plants examined): Hroznějovice near Poněšice (6/2), Jamné near Boršov nad Vltavou (12/1), Křemže (6/1)] during the period 1994–2001. In 1999, strawflower plants grown in fields for commercial seed production in the Central Moravia region [Veselíčko near Lipník nad Bečvou (6/1)] also showed yellows symptoms from July to October onwards; affected plants ranged from 2% to 70%.

Early symptoms consisted of leaf vein clearing with light green mosaic on some leaf blades of regular shape and size. New leaves were smaller and strongly narrowed. Subsequently, many secondary shoots with no buds or very small buds were formed. Pale green, filamentous leaflets on the shoots became downwardly curled towards the petioles. They revealed a corrugated surface with swollen veins. Mature leaves prematurely changed colour from green to bronze or brown with severe reddening and necrosis along the leaf margins and tips. Later in the season, flowers were malformed and consequently failed to produce seeds. Elongated and often necrotic styles in the disk tubular florets protruded from nondeveloped ray florets. Severely infected strawflowers were stunted and some

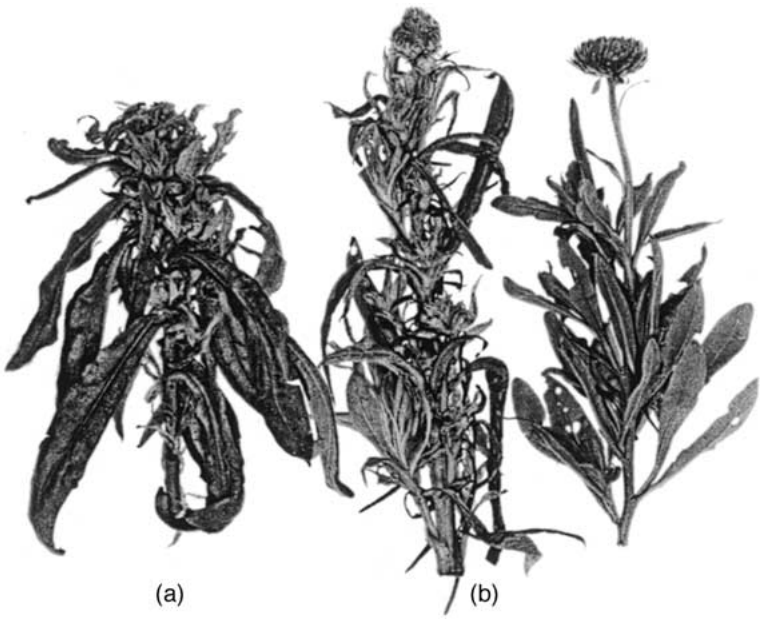

Figure 1. Strawflowers with abnormal production of secondary shoots, leaf necrosis, dwarf, flower absence (a) and flower malformation (b) (right-healthy control).

of them died in advanced stages of the disease, usually in September or October (Figure 1).

Severe histological changes were observed on ultrathin sections of the vascular tissues of affected plants under Jeol 1010 transmission electron microscope (TEM): sieve tube elements were sometimes compressed and narrows. Some had extended cell walls and reduction of the lumen (Figure 2a). This phenomenon was mostly observed on ultrathin sections from leaflets of secondary shoots but rarely on samples from stalks. Swollen and degenerated chloroplasts with less amount of granal thylakoids were found in many parenchymatic cells of phloem tissues. Phytoplasma-like organisms were detected in both degenerated and phloem tissues of regular shape and size. They were observed in mature and immature phloem sieve tubes. Cells filled by the micro-organisms were also seen (Figure 2b). The majority of particles were ovoid or spherical with size ranged from 80 × 80 to 125 × 1200 nm in diameter (average 330 × 380 nm), some were irregular in form or elongated. The prokaryotes were always found in diseased plants; no other microorganisms, bacteria or viruses, were noted. No phytoplasmas or tissue degeneration were present in asymptomatic plants.

Tissues of flowers, axillary shoots and leaf midribs from 5 plants of healthy and 30 diseased strawflowers and of reference phytoplasma strains [aster yellows (subgroup 16SrI-B), clover phyllody (subgroup 16SrI-C), apple proliferation (group 16SrX-A), grapevine yellows-stolbur (group 16SrXII-A)] were subjected to DNA extraction according to Lee et al. (1991). Nucleic acid pellets were resuspended in 50 µl of TE buffer [10 mM Tris–HCl, 1 mM EDTA (pH 8.0)] to give a final concentration 20 ng μl^{-1}. DNA extracts were used in direct polymerase chain reaction (PCR) to amplify part of the 16S ribosomal RNA (rRNA) gene using the phytoplasma universal primers R16F2n and R16R2 (Schaff et al., 1992). Tubes with the reaction mixture devoid of DNA templates or containing DNA from symptomless samples were included in each experiment as negative controls. Six microlitres of each PCR product were analysed by electrophoresis through a 1% agarose gel followed by staining in ethidium bromide (EB) and visualisation of DNA bands using an UV transilluminator. Using primer pair R16F2n/R16R2, DNA fragments of about 1.2 kb from the 16S rRNA gene were detected in all 30 samples of symptomatic strawflowers from different areas in the Czech Republic, regardless of which plant organ was tested. No PCR product was obtained from asymptomatic plants or from the water control. Amplification of the reference phytoplasma strains resulted in a product of the same size as those in the diseased strawflowers.

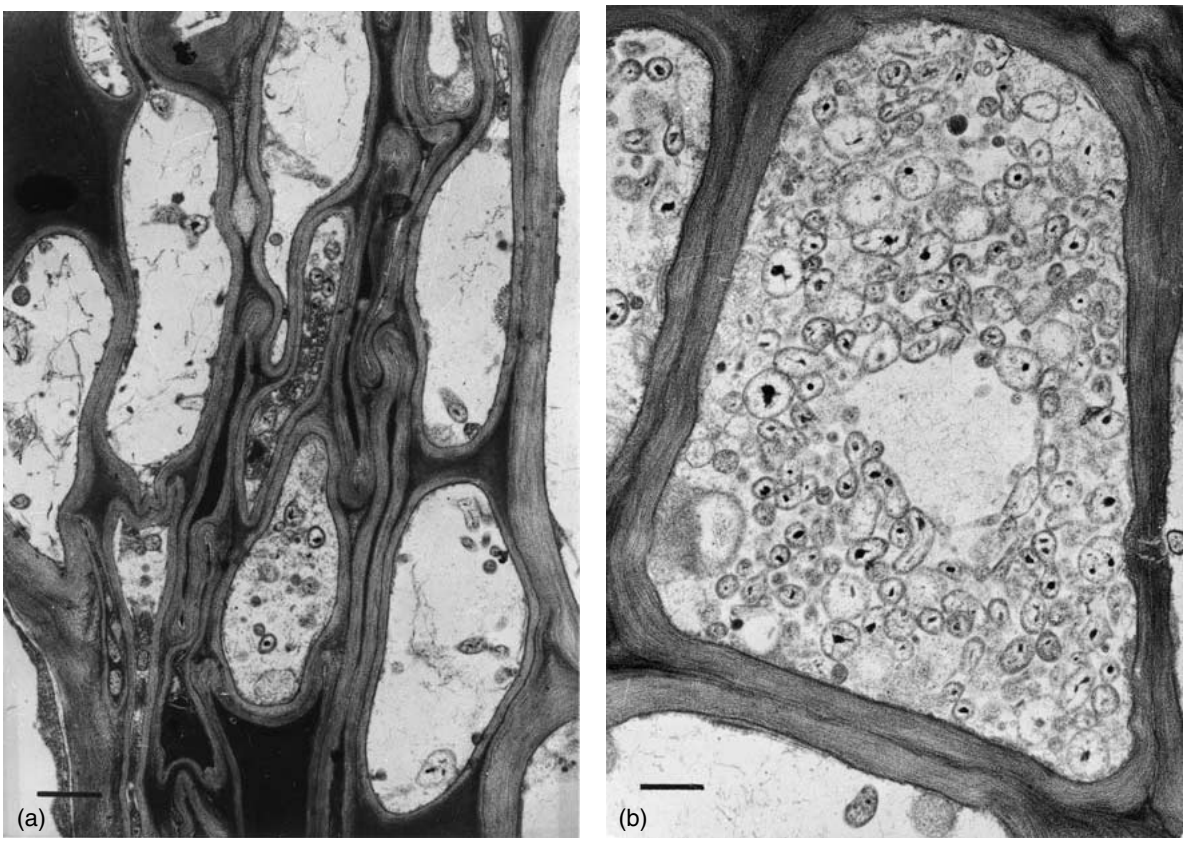

Figure 2. Ultrathin sections of the sieve elements of strawflowers with yellows symptoms: (a) phytoplasma particles in malformed sieve tube cells of the leaf midrib from secondary shoot. Bar = 1 μm. (b) nondamaged phloem cell of flower stalk filled with phytoplasma bodies. Bar = 500 nm.

DNA of PCR products (200 ng) were digested with 3 U each of restriction endonucleases *Alu*I, *Hha*I, *Mse*I (NE Biolabs, Beverly, MA, USA) in 20 μl volume at 37 °C overnight. The digest was resolved on 1.5% agarose gel, stained with EB and followed by visualisation under an UV transilluminator. The gel was photographed by Kodak digital camera and red filter. All phytoplasma positive samples from strawflowers showed the same restriction profiles with each of *Alu*I, *Hha*I, *Mse*I enzymes. These profiles were identical to those of aster yellows phytoplasma (16SrI-B) (Lee et al., 1998).

DNA isolated from one strawflower plant originating from a private garden in Jamné near Boršov nad Vltavou was used for sequencing. This plant exhibited light green mosaic on primarily infected leaves, abnormal production of secondary shoots, flower malformation and prematurely reddening and necrosis of mature leaves (Figure 1b). A set of overlapping PCR products from infected strawflower was generated by amplification with primers P1/U3 (position 6–1230), R16F2n/R16R2 (position 152–1397), 16R758/P7 (position 758–1818) (Gibb et al., 1995; Gundersen and Lee, 1996; Lorenz et al., 1995). PCR products were sequenced using a BIG DYE sequencing terminator kit (PE Biosystems, Warrington, UK), each one from both directions to cover the whole length of 16S rDNA and the 16/23S rDNA spacer region. Sequencing was performed in a ABI PRISM 310 sequencer (PE Applied Biosystems, Foster City, CA, USA). The sequence was aligned with sequences of phytoplasmas available in the GenBank using World Wide Web service ClustalW (http://www.ddbj.nig.ac.jp). The aligments were used as input data to construct phylogenetic trees with the Neighbour-Joining method implemented in ClustalW; each time the bootstrap option was run with 1000 resampling.

The tree was visualised with TreeView v. 1.6.1 program.

The sequence of the phytoplasma infecting strawflower in the Czech Republic was deposited in the GenBank database under accession number AF515771. The detailed sequence comparison revealed the closest relationship with the '*Rehmannia glutinosa* var. *purpurea*' phytoplasma, both originating from Bohemia (Přibylová et al., 2001). The dendrogram in Figure 3 shows the phylogenetic position of

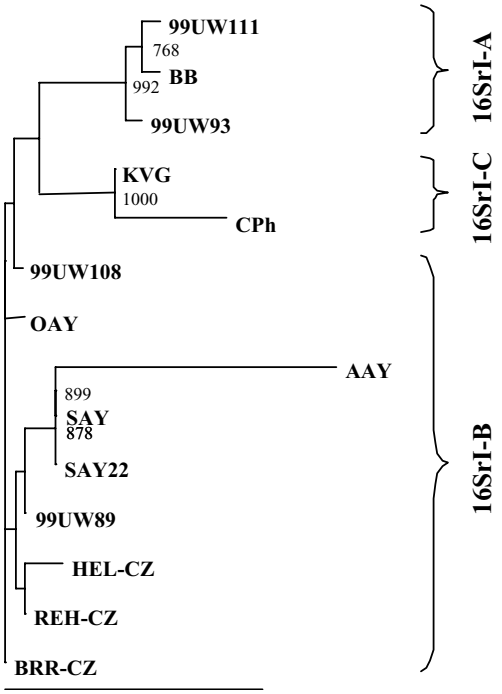

Figure 3. Phylogenetic dendrogram of aster yellows phytoplasma isolates. The analysis was carried out using the neighbour-joining tree option of ClustalW. The scale bar represents a phylogenetic distance of 1%. The numbers on the branches are bootstrap values. Phytoplasmas and GenBank accession numbers are as follows: HEL-CZ: '*Helichrysum bracteatum*' phytoplasma (AF515771), REH-CZ: '*Rehmannia glutinosa* var. *purpurea*' phytoplasma (AF335107), BRR-CZ: '*Brasica napus* var. *arvensis*' phytoplasma (U89378), 99UW108: Aster yellows phytoplasma B (AF268409), SAY: Severe strain of western American aster yellows (M86340), SAY22: Aster yellows phytoplasma strain AY1 (AF222063), AAY: American aster yellows (X68373), 99UW89: Aster yellows phytoplasma B (AF268407), OAY: *Oenothera* aster yellows (M30790), 99UW111: Aster yellows phytoplasma A (AF268408), 99UW93: Aster yellows phytoplasma A (AF268406), BB: Tomato big bud (L33760), CPh: Clover phyllody (L33762), KVG: Clover phyllody/Germany (X83870).

the Czech isolate of phytoplasma from *H. bracteatum* (HEL-CZ) in the aster yellows phytoplasma group. The aster yellows phytoplasma isolates are a phylogenetically closely related group. All Czech isolates (HEL-CZ, BRR-CZ, REH-CZ) can be classified as members of the aster yellows 16SrI-B subgroup.

There have been numerous reports of phytoplasma diseases in the family Asteraceae (McCoy et al., 1989). Phytoplasmas infecting some of these plants have been classified on the base of PCR/RFLP analyses on 16S rDNA gene as members of aster yellows subgroup 16SrI-A, 16SrI-B, 16SrI-H; 16SrII-C, 16SrIII-A, 16SrIX-A, Italian alfalfa witches'-broom and cirsium phyllody groups (see review in Seemuller et al., 1998; Khadhair et al., 1999; Schneider et al., 1999). However, there are only a limited number of reports about phytoplasmas on *H. bracteatum*. Severin and Freitag (1934) observed yellows symptoms on naturally affected plants in California (USA). Very recently, Wang and Hiruki (2001) differentiated aster yellows subgroup I by DNA heteroduplex mobility assay in phytoplasma isolates from *H. bracteatum* collected from the field in Canada. The only report about detection and molecular identification of phytoplasma disease on *H. bracteatum* in Europe is from Poland (Kaminska et al., 1996). In this case, an organism related to the American aster yellows was identified on the basis of RFLP analysis of a PCR-amplified 876 bp rDNA fragment using *Alu*I.

The characterisation of Czech strawflower phytoplasma by sequencing indicates that the sequence of 16S DNA can be considered identical to the '*R. glutinosa* var. *purpurea*' phytoplasma and confirmed the RFLP classification in the ribosomal subgroup 16SrI-B. However, only the 16S DNA sequence do not justify identity among phytoplasmas: other genes as well as biological features must be studied before affirming a generic phytoplasma identity. Vector(s) monitoring and sequencing of phytoplasma DNA isolated also from other plant species and insects will be important for a more precise characterisation of these pathogens and to clarify the epidemiology of phytoplasma diseases in the Czech Republic.

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