A New Disease in *Limonium* hybrids. I. Molecular Identification

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Abstract. Yellows diseases in ornamental crops have become more common in Israel, and phytoplasmas have been detected in several crops. Recently, symptoms typical of a phytoplasma infection were observed on a large number of *Limonium* hybrids grown in commercial fields in Israel. Examination of samples from diseased plants by electron microscopy revealed the presence of pleiomorphic membrane-bound bodies in the phloem cells. Diseased plants were additionally analyzed by PCR using universal and nested primers and revealed upon sequencing products of 1143, 788 and 722 bp (Li-IL, Li-b2-IL, Li-v3-IL, respectively). Analysis of the PCR products, associated with infected *Limonium*, clustered within two major groups of phytoplasmas (16SrV and 16SrIX), elm yellows and almond witches broom. This is the first published record of these phytoplasmas in Israel.

Although many species of *Limonium* (Plumbaginaceae Juss.), herbaceous perennials, are native to the Mediterranean region, seedlings for commercial production are usually imported from Europe, North America or Asia. Two species and two hybrids are grown in commercial production in the Arava valley (located between the Dead Sea and Red Sea); these include *Limonium altaica* ('Emile'), *L. sinuatum* (annual statice), *L. latifolium* × *L. caspium* ('Beltlaard'), and *L. latifolium* × *L. belidifolium* ('Misty', 'Supreme' and 'Sunglow'). In any one year there are a total of ≈70 ha; ≈15 ha are in *L. sinuatum* production and the remainder in *L. altaica* and *Limonium* hybrids. These plants are grown primarily for export to Europe as cut flowers for floral arrangements. Flowers are grown for 1 to 10 years in tunnels (7 × 100 m) covered with plastic; both ends are open and ventilation holes are cut about every 2 m. For the first 15 years of commercial production in the Arava, the crops were not affected by yellows disease. In October 2000 diseased plants were first observed in the northern Arava.

Only *L. latifolium* hybrids were observed to be infected. Symptoms included: small and/or deformed/discolored flowers; small, narrow basal leaves, often yellow in color; excessive leaf growth (witches broom or 'asparagus fern'); and eventual plant death. Over the course of that season up to 60% of the plants were affected in some areas. Symptoms resembled those caused by phytoplasma infections.

The present paper reports the outbreak of phytoplasma in *Limonium* hybrids grown in commercial fields in Israel and the molecular identification and phylogenetic relationships of diverse phytoplasmas associated with the disease.

Materials and Methods

Mechanical inoculation. Leaf samples of *Limonium* grown in commercial plastic tunnels were examined for virus particles using electron microscopy and mechanical inoculation. Symptomatic samples were homogenized in 1% K₂HPO₄, and the sap was inoculated to *Capsicum annuum* L., *Chenopodium quinoa* Willd., *C. amaranticolor*, *Cucumis sativus*, *Cucurbita pepo*, *Datura stramonium*, *Gomphrena globosa*, *Lycopersicon esculentum*, *Nicotiana benthamiana* Domin, *N. clevelandii* Glay, *N. glutinosa*, *N. tabacum* 'NN', *N. tabacum* 'Samsun', *N. tabacum* 'Samsun NN', *N. tabacum* Xanthi nc, *N. rustica*, *N. sylvestris*, *N. tabacum* 'White Burley', *Petunia hybrida* and *Physalis floridensis* Rydb., predusted with

carborundum. These plants were maintained in the greenhouse for one month and observed for symptom development.

Electron microscopy. Crude plant extracts in 0.1 M phosphate buffer were negatively stained with 20 g·L⁻¹ uranyl acetate and examined for virus in a electron microscope (model 100CX II; Joel Ltd., Tokyo, Japan). For ultrathin sections, pieces of petioles and/or leaf veins (1 to 2 mm²) excised from healthy and symptomatic leaves of *Limonium* hybrids, were fixed in 2.5% glutaraldehyde buffered with 75 mM potassium phosphate, pH 7.0, for 2 h. The samples were then rinsed in the same buffer and postfixed in 1% OsO₄ for 3 h. After a buffer rinse, the samples were dehydrated in a graded acetone series, embedded in an Epon-Araldite resin mixture, and polymerized as described by Orion and Franck (1990). Sectioned material was stained with uranyl acetate followed by lead nitrate and examined by electron microscopy.

Polymerase chain reaction and sequence analysis. DNA was prepared from leaf midribs and petioles as described by Tanne et al. (2000). DNA fragments were amplified using the universal phytoplasma primers P1/P7 (Schneider et al., 1993). The product was further amplified by nested PCR with the r16SF2/r16SR2 and fU3-fR5 primer pairs (Lee et al., 1995). The DNA template in all PCR assays was 20 ng in a 50 µL assay. Standard PCR conditions were as in Tanne et al. (2000). Amplified samples were electrophoresed in a 1.2% agarose gel, stained with 0.5 (g·mL⁻¹) ethidium bromide, and photographed under UV illumination (Sambrook et al., 1989). DNA extracted from symptom-free *Limonium* and naturally infected periwinkle plants served as negative and positive controls, respectively.

Amplified products from several *Limonium* samples were cleaned using QIAquick purification kit (Qiagen, Hilden, Germany) and sequenced by the dideoxynucleotide chain termination method, using r16SF2 or fU3 primers at the Weizmann Institute of Science, Rehovot, Israel. Sequence analysis was performed with the DNAMAN package, version 4 (Lynnon Biosoft, Canada). Phytoplasma-related sequences were initially identified using the BLASTN search program of the GenBank (Altschul et al., 1990). The nucleotide sequence of the phytoplasma infecting *Limonium* was compared to the following: almond witches' broom phytoplasma (AlmWB1-4, AF390136-9), american aster yellows phytoplasma (AAY, X68373), australian grapevine yellows phytoplasma (AUSGY, L76865), aster yellows phytoplasma (AY1, AF322645), aster yellows phytoplasma (SAY, AJ271323), *Candidatus fraxini* phytoplasma (AshY1, AF092209); *Candidatus fraxini* phytoplasma (AshY, X68339); *Candidatus fraxini* phytoplasma (AshY1-NY, AF092209); *Candidatus fraxini* phytoplasma (AshY3, AF105315); *Candidatus fraxini* phytoplasma (AshY5, AF105316); clover yellow edge phytoplasma (CYE-C, AF175304), clover yellow edge phytoplasma (CYE-Or, AF189288), Clover yellow edge phytoplasma (CYE-L, AF173558), elm yellows phytoplasma (EY-

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Fig. 1. Symptoms of phytoplasma in naturally infected *Limonium*. (A) Healthy plant with blue flowers on left, diseased plant center right. (B) Asparagus fern. (C) Leaflets instead of flowers. (D) Narrow, yellow basal leaves developing above old broad healthy leaves.

Arl, AF268895); elm yellows phytoplasma (EY1-NY, AF189214); elm yellows phytoplasma (EY1-WVEY, AF122911); elm yellows phytoplasma (EY1-ULMUS, AF122910), elm yellows phytoplasma (ULW, X68376); mollicutes from *Saccharum officinarum* L. (SCWL, X76432), pigeon pea witches' broom phytoplasma (PPWB, AF248957); periwinkle little leaf phytoplasma (PLL-Bd, AF228053); brinjal little leaf phytoplasma (BLL-Bd-brinjal, AF228052); *Candidatus mali* phytoplasma (AT1, AJ542542), *Candidatus pyri* phytoplasma (PearD, U54989), *Candidatus aurantifolia* phytoplasma (WBDL, U15442), *Candidatus phoenicium* phytoplasma (Can-PPH-21, AF515637), coconut lethal yellowing phytoplasma (LY-C2, AF498309); phytoplasma sp. (ACLR, X68338), mycoplasma-like

organism (PPER, X68374), mollicutes from *V. myrtillus* (VAC, X76430), mollicutes from *C. roseus* (STOL-Cros, X76427), mollicutes from *V. vinifera* (VK, X76428), phytoplasma sp. (LfY5, AF500334); *Paulownia* witches' broom phytoplasma (PauWB, AF279271), peanut witches' broom phytoplasma (PnWB, AY139868), rice yellow dwarf phytoplasma (RYD, AY139873), stolbur phytoplasma (STOL-Lily, AY169309), stolbur phytoplasma (STOL, AF248959), sweetpotato witches' broom phytoplasma (SPWB, AY139866), *Stylosanthes* little leaf phytoplasma (SLI-AUS, AJ289192); tomato big bud phytoplasma (BB, AF222064), tsuwabuki witches' broom phytoplasma (TWB, D12580), *Ziziphus jujube* witches' broom phytoplasma (JWB-Ch, AF305240); *Ziziphus jujube* witches' broom phytoplasma (JWB-Ko, AB052879); loofah witches-broom phytoplasma (LfWB1, AF353090); loofah witches-broom phytoplasma (LfWB2, AY139871); phytoplasma sp. LfY5-Oaxaca (LfY5, AF500333); western X phytoplasma (WX, L04682).

Results

A typical naturally infected *Limonium* is illustrated in Fig. 1. Symptoms included small and/or deformed or discolored flowers; small, narrow basal leaves, often yellow in color; excessive leaf growth (witches broom or asparagus fern). Over the course of the season up to 60% of the plants were affected in some areas. None of the 20 herbaceous test plant species listed in the materials and methods exhibited any symptoms related to viral infection up to one month following mechanical inoculation.

Electron microscopy. Electron microscope (EM) observations of crude plant extracts revealed no virus or virus-like particles. EM of ultrathin sections of infected leaves of *Limonium* hybrids revealed the presence of pleomorphic membrane-bound bodies (Fig. 2) in the phloem cells. The size ranged from 200 to 1000 nm in diameter. No other types of bodies were observed. Similar bodies were not observed in samples of healthy *Limonium* hybrid plants.

Polymerase chain reaction and sequence analysis. Amplification of phytoplasma related sequences using the universal primers P1/P7 followed by a nested PCR with internal primers

indicated that all *Limonium* plants expressing symptoms were infected by phytoplasma, while symptom-less plants were phytoplasma-free (Fig. 3). The PCR fragments, 1143, 788 and 722 bp of several clones, designated Li-IL, Li-b2-IL, Li-v3-IL, respectively, were directly sequenced using r16SF2 or fU3 primers. Nucleotide sequencing of Li-IL amplified product have shown 99.4% identity with EY-Ar1, Li-b2-IL product 83% identity with BLL-Bd-Brinjal and Li-V3-IL 91.4% with PPWB phytoplasmas respectively. A comparison with 53 related sequences from the GenBank confirmed the classification of these phytoplasmas associated with *Limonium* disease to two major groups (16SrV and 16SrIX), elm yellows and almond witches' broom, respectively. Although, Li-IL and Li-b2-IL were classified to the same group they exhibit only 82.8% identity of nucleic acid sequence.

Discussion

A review of the literature revealed that phytoplasma infections are known from only a few species within the Plumbaginaceae: *L. sinuatum* in Korea (Hahm et al., 1998), Japan (Shiomi et al., 1999), Poland (Kaminska et al., 1999), and Lithuania (Valiunas et al., 2001), and *Goniolimon tataricum* (syns. *Limonium tataricum*) in Canada (Chang et al., 1996). Although *L. sinuatum* is grown in the same area of the Arava, and even immediately adjacent to *L. latifolium*, we have yet to find statics with symptoms of phytoplasma infection. *Limonium altaica* also remains symptomless.

The symptoms in *L. latifolium* hybrids are typical of those of a phytoplasma infection, and sequence analysis of amplification products of 1143, 788 and 722 bp (Li-IL, Li-b2-IL, Li-v3-IL, respectively) is consistent with elm yellows and almond witches' broom phytoplasmas classified into two major groups of 16SrV and 16SrIX according to Lee et al. (1998). These results emphasize that the use of molecular tools, especially sequencing amplified nucleic acid fragments of the 16SrRNA gene, provides tools to differentiate phytoplasmas that exist in mixed infection. These results show that

Fig. 2. Electron micrograph of a thin section through sieve cells of *Limonium* containing phytoplasma-like bodies indicated by arrows. Bar represents 200 nm.

Fig. 3. Gel electrophoresis of products from polymerase chain reaction (PCR) performed on DNA extracted from *Limonium* using nested-PCR with primers P1/P7 followed by primers r16SF2/r16SR2. Lane 1, DNA size markers, lane 2, healthy *Limonium*, lane 3-5, symptomatic *Limonium*.

Fig 4. Relationship dendrogram of *Limonium* infecting phytoplasma 16 s rRNA fragment with related sequences of phytoplasmas. The nucleic acid sequence was compared first by a BLASTN search of GenBank. Tree descriptions were generated using the neighbor-joining algorithm and based on calculations from pairwise nucleotide sequence distances derived from the multiple alignment format (not shown). Horizontal scale indicates sequence divergence, and vertical scale is arbitrary.

mixed phytoplasma infections associated with *Limonium* disease is not uncommon.

This is the first time an elm yellows disease and almond witches' broom have been recorded in Israel. Elm yellows phytoplasma has not been reported in Israel, but it has been present for years in North America (Baker, 1948), Europe (Maurer et al., 1993) and China (Griffiths et al., 1999). The disease was detected in a diverse group of woody or herbaceous plants: *Eucalyptus* (Marcone et al., 1996), *Apocynum* (Sunn hemp), *Prunus* (stone fruit), *Rubus* (raspberry, blackberry), *Ulmus* (elm), *Vitis* (grape), *Ziziphus* (jujube) (Griffiths et al., 1999), and, more recently, *Parthenocissus quinquefolia* (virginia creeper) (Harrison et al., 2001). However, almond witches' broom was recently detected in Lebanon (Abou-Jawdah, et al. 2002).

In 2000 growers in South America also observed a phytoplasma-type disease in 'Supreme' (*L. latifolium* x *L. bellidifolium*) plants grown in greenhouses. These seedlings, like those in Israel, were imported from California; most probably, this was the source of the phytoplasma. Phytoplasmas are vectored to plants by leafhoppers and planthoppers; in the following article we discuss the vectors found in *Limonium*.

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