

A New Disease in *Limonium latifolium* Hybrids. II. Investigating Insect Vectors

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Abstract. Symptoms typical of a phytoplasma infection were observed on a large number of *Limonium* hybrids in Israel in October 2000. Phytoplasma infection was confirmed by electron microscopy and polymerase chain reaction analysis. To identify the agent of pathogen spread surveys were made of candidate leafhoppers vectors in *Limonium* hybrid crops: one known phytoplasma vector (*Orosius orientalis* (Matsumura)) was present in very large numbers, ≈10-fold greater than any other species; three other known vectors were present in low to moderate numbers (*Circulifer haematoceps* complex (Mulsant et Rey), *C. tenellus* complex (Baker) and *Exitianus capicola* Stål); individual specimens of other vectors were occasionally found. Field collected specimens of these four species were shown to vector phytoplasma to healthy *Limonium* hybrid seedlings; this is the first time *E. capicola* has been shown to be a phytoplasma vector. A laboratory colony of *O. orientalis* was additionally shown to be able to acquire the phytoplasma from infected *Limonium* hybrids and subsequently vector it to healthy seedlings.

Two species and two hybrids of *Limonium* 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. bellidifolium* ('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 walk-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. There was little need for pest management as there were no major insect pests, weeds were rogued out by hand, and mildew was treated chemically, when necessary. In October 2000 diseased plants were first observed in the northern Arava and then the disease quickly spread to other areas. Only *L. latifolium* hybrids were observed to be infected. 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); and eventual plant death. Over the course of that season up to 60% of the plants were affected in some areas. Diseased plant tissue was examined by electron microscopy, and the presence of phytoplasma was confirmed. Management practices now include scheduled insecticide treatments in an attempt to control leafhoppers.

Phytoplasmas may be transmitted to healthy plants through the parasitic plant *Cuscuta pentagona* (dodder), by grafting or vegetative propagation, and, most effectively, by insects. The primary phytoplasma vectors are leafhoppers and planthoppers, although a psyllid has recently been proven to be a vector (Font et al., 1999). The objective of this study was to survey insects and determine the vector(s) of phytoplasma in *Limonium* hybrids.

Materials and Methods

Leafhopper survey. Surveys of leafhoppers began in December 2000 by placing yellow sticky traps in the tunnels with *Limonium* hybrids at three privately owned sites in the region. Yellow sticky traps (Jewnin-Joffe Industry Ltd., Israel) (14 × 20 cm) were placed vertically just above the canopy and replaced weekly throughout the year 2001. Leafhoppers were carefully removed from the traps, washed in two changes of technical grade hexanes to remove the glue, sorted to species, and then stored in 95% EtOH at room temperature. Samples, 25 to 100 leafhoppers per month (depending on trap catches) were analyzed by polymerase chain reaction (PCR) for the presence of phytoplasma DNA.

PCR and sequence analysis. Because phytoplasmas are phloem-limited, DNA was prepared from leaf midribs and petioles as described by Tanne et al. (2000), and from leafhoppers as described by Maixner et al. (1995). Phytoplasma DNA fragments were amplified using the universal phytoplasma primers P1/P7 (Schneider et al., 1993). The product of the first PCR reaction was further amplified by nested PCR with the r16SF2/r16SR2 and fU3-fR5 primer pairs (Lee et al., 1995), and used to amplify 1143, 788, and 722 bp fragments of phytoplasma DNA. The DNA template in all PCR assays was 20 ng in a 50-μL assay. Standard PCR conditions were as described in Tanne et al. (2000). Amplified samples were electrophoresed in a 1.2% agarose gel, stained with 0.5 μg·mL⁻¹ ethidium bromide, and photographed under UV illumination (Sambrook et al., 1989). DNA extracted from symptom-free *Limonium* and naturally infected periwinkles plants served as negative and positive controls, respectively.

Direct transmission studies. Vacuum samples of insects on *Limonium* hybrids were taken from the canopy, in the morning hours, in April, May, June, and November 2001. Leafhoppers were sorted to species and placed, in groups of two to five insects, on healthy *Limonium* hybrid seedlings in the laboratory for a minimum of 48 h. Plants were held in an insect-free growth chamber for at least 1 month before being analyzed by PCR for presence of phytoplasma DNA. Plants were symptom-free at the time of analysis.

Phytoplasma acquisition and transmission studies. A colony of *Orosius orientalis* (Matsumura), the predominant leafhopper found in the survey, had been previously established from individuals collected in another region of Israel and tested negative for the presence of phytoplasma DNA by PCR. Subsamples of this colony were removed and PCR analyzed routinely. This phytoplasma-free colony was established and reared on sesame (*Sesamum indicum*) and common bean (*Phaseolus vulgaris*) plants. On three separate occasions (in January, April, and May) symptomatic *Limonium* hybrids were brought to the laboratory. Groups of 20 adult leafhoppers were confined to these plants for a minimum of 3 d, and then transferred to sesame plants for 2 to 4 weeks before groups of 2 to 5 were placed on healthy *Limonium* hybrid seedlings. Inoculation access *Limonium* plants were held in an insect-free growth chamber for at least 1 month before being analyzed by PCR for presence of phytoplasma DNA. Plants were symptom-free at the time of analysis.

Table 1. Survey and total number of leafhoppers, known to be phytoplasma or spiroplasma vectors, captured in *Limonium* hybrid crops from December 2000 to December 2001.

| Species | Total caught (no.) |
|-------------------------------|--------------------|
| <i>Orosius orientalis</i> | 8279 |
| <i>Circulifer haematoceps</i> | 825 |
| <i>Circulifer tenellus</i> | 832 |
| <i>Exitianus capicola</i> | 883 |
| <i>Austroagallia sinuata</i> | 94 |
| <i>Psammodettix</i> spp. | 32 |

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Table 2. Transmission trials of field-collected leafhopper specimens to healthy *Limonium* hybrid seedlings and confirmation of infection by PCR.

| Species | Date | Test plants (no.) | Plants positive for phytoplasma (no.) |
|-------------------------------|---------|-------------------|---------------------------------------|
| <i>Orosius orientalis</i> | 2 Apr. | 6 | 6 |
| | 23 May | 2 | 0 |
| | 11 June | 1 | 1 |
| | 4 Nov. | 5 | 4 |
| | 19 Nov. | 8 | 8 |
| <i>Circulifer haematoceps</i> | 24 Apr. | 6 | 6 |
| | 21 May | 4 | 3 |
| <i>Circulifer tenellus</i> | 24 Apr. | 6 | 6 |
| | 21 May | 3 | 2 |
| <i>Exitianus capicola</i> | 21 May | 1 | 0 |
| | 3 June | 1 | 0 |
| | 11 June | 1 | 1 |
| <i>Psammotettix</i> sp. | 11 June | 1 | 1 |

Table 3. Acquisition of phytoplasma from phytoplasma-infected *Limonium* plants and transmission to healthy plants by *Orosius orientalis*.

| Month removed from field | Test plants (no.) | Plants positive for phytoplasma (no.) |
|--------------------------|-------------------|---------------------------------------|
| January | 6 | 6 |
| April | 6 | 5 |
| May | 1 | 1 |

Results

Leafhopper survey. Six known leafhopper vectors of phytoplasmas or spiroplasmas were captured on sticky traps during the course of the survey (Table 1); no planthopper species were caught. Few leafhoppers were caught in July and August. Because of their vector status and abundance, samples of *O. orientalis*, *Circulifer haematoceps* (Mulsant et Rey) and *C. tenellus* (Baker) were analyzed by PCR for the presence of phytoplasma DNA. About 73% of the *O. orientalis* tested positive for phytoplasma and ≈50% of the *Circulifer* spp. tested positive.

Transmission studies. Results from confining field-collected leafhopper species on healthy *Limonium* hybrid seedlings are shown in Table 2. The number of plants used in transmission trials was proportional to the number of candidate species captured. All species except *Exitianus capicola* Stål successfully vectored phytoplasma to 85% to 90% of the plants tested; *E. capicola* only transmitted phytoplasma to 33% of test *Limonium*. There was no apparent seasonal effect; there was no apparent difference in the ability of insects collected in the spring or fall to vector phytoplasma. Phytoplasma-free *O. orientalis* were able to acquire phytoplasma from infected plants and subsequently transmit the pathogen to healthy *Limonium* hybrid seedlings (Table 3). Leafhoppers could acquire phytoplasma from infected plants collected from the field in either winter or spring.

Discussion

Phytoplasmas are transmitted by leafhoppers and planthoppers. Transmission of pathogens by insects, including leafhoppers and planthoppers, depends on the abundance of the vector(s) and their interplant movement (Irwin and Ruesink, 1986; Power, 1987, 1992) and host plant density (Power, 1987, 1992). *Limonium* seedlings are planted 50 cm apart and quickly form a continu-

ous canopy in each bed, allowing leafhoppers to move easily from one plant to another. Although beds are 1.2 m apart, the effective distance between plants in adjacent beds is ≈50 cm or less; a distance easily traversed by leafhoppers. In our survey, leafhoppers were trapped throughout the year, except mid-summer. The Arava has a plant-free period in mid-summer when temperatures are about 40°C daily and all annual plants are removed; the combined effects of intense heat and reduced host plant numbers severely reduces insect populations.

The abundance of *O. orientalis* was ≈10 times that of any other species caught in this study. It is distributed from the eastern Mediterranean to Japan, and has long been known to vector phytoplasma diseases such as: sesame phyllody in Israel (Klein, 1977) and India (Regupathy and Jayara, 1973); witches' broom disease of legumes in Japan (Ishihara, 1982); and alfalfa witches' broom in Iran (Salehi et al., 1995).

Three other species of leafhopper were trapped in relatively low numbers compared to *O. orientalis*. *Circulifer haematoceps* is a known vector of sesame phyllody in Turkey (Kersting, 1993) and Iran (Salehi and Izadpanah, 1992). *Circulifer tenellus* is a well-known vector of *Spiroplasma citri* in the U.S. (Kaloostian et al., 1975) and Israel (Almeida et al., 1997), and of curly-top virus in the U.S. (Gardner and Cannon, 1972), although this is apparently the first record of it vectoring a phytoplasma. *Exitianus exitiosus* is a known vector of spiroplasmas (Nault, 1980), although this is apparently the first record of *E. capicola* transmitting a phytoplasma.

In 1996, as part of another project, a survey of insect fauna was made in the Arava. A review of that survey material, indicated that all species observed in this study were represented in the 1996 material; that is, the potential vectors were already present in the area before the disease. Close querying of the growers in the Arava revealed that they noticed some diseased plants in 1999, but since very few plants were affected and the disease was not previously known, no management strategies were implemented. Since potential leafhopper vectors were already present in the area, it is not surprising that the disease rapidly spread in 2000. Moreover, in 2000 growers in South America also observed a phytoplasma-type disease in 'Supreme' *Limonium* hybrids grown in greenhouses from which leafhoppers are excluded. These seedlings, like those in Israel, were imported from California;

most probably, this was the source of the phytoplasma. Now that insect vectors of limonium yellows have been identified, research into possible management strategies for the insects and the pathogen is underway.

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