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Potential leafhopper vectors of phytoplasma in carrots

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Abstract. Phytoplasmas are insect-vectored pathogens that cause characteristic and destructive diseases in carrots and other vegetables. A phytoplasma disease was first observed in Israeli carrot fields in 1995. Analysis of infected carrots showed the presence of aster yellows and western-X phytoplasmas. In this study, commercial and experimental fields in the western Negev region of Israel were monitored for three years using yellow sticky traps and vacuum sampling. Potential vectors of leafhoppers and planthoppers were analysed by PCR for the presence of phytoplasma DNA. Infected plants were also assayed for phytoplasma DNA. Extracted phytoplasma DNA was subjected to RFLP analysis to determine groups to which the phytoplasmas belonged. It was determined that carrots and leafhoppers from the experimental station were infected with a phytoplasma belonging to the Elm Yellows (EY) group; this is the first report of EY infecting carrots. Based on our findings, the two most probable insect vectors are *Circulifer haematoceps* complex (Mulsant & Rey) and *Neoaliturus fenestratus* (Herrich-Schäffer).

Key words: carrot, phytoplasma, vectors, *Circulifer haematoceps*, *Neoaliturus fenestratus*

Résumé. Les phytoplasmas sont des pathogènes transmis par des insectes vecteurs, responsables de maladies destructives et caractéristiques sur les carottes et d'autres légumes. Une maladie à phytoplasma a été observée pour la première fois en Israël en 1995 dans des champs de carottes. L'analyse des carottes infectées à révélé la présence des phytoplasmas aster jaunes et western-X. Dans cette étude, des parcelles expérimentales et commerciales situées dans la région Ouest du Negev en Israël ont été suivis pendant 3 ans en utilisant des pièges englués jaunes et des pièges à succion. Les vecteurs potentiels, des cicadelles et les jassides, ont été analysés par PCR afin de contrôler la présence du DNA de phytoplasma. Les plantes infectées ont également été contrôlées pour le DNA de phytoplasma. Le DNA extrait a été soumis à une analyse par RFLP afin de déterminer le groupe d'appartenance des phytoplasmas. On a ainsi pu déterminer que les carottes et les jassides de la station expérimentale étaient infectés par des phytoplasmas du groupe Elm Yellows (EY); Il s'agit du premier signalement de carottes infectées par des phytoplasmas du groupe EY. Sur la base de nos résultats, le complexe *Circulifer haematoceps* (Mulsant et Rey) et *Neoaliturus fenestratus* (Herrich-Schäffer) sont probablement les deux principaux vecteurs.

Mots clés: carotte, phytoplasma, vecteurs, *Circulifer haematoceps*, *Neoaliturus fenestratus*

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Introduction

Carrot (*Daucus carota* L.) is a major crop worldwide; in Israel, about 45,000 tons of carrots are produced annually. Carrots have been known to be susceptible to phytoplasma ('yellows disease') for more than 50 years (Ivanoff and Ewart, 1944), with phytoplasma infections reported in Australia, Europe, North America and the Middle and Far East. The severity of this disease has become a limiting factor for carrot production in several regions of the United States (Gabelman *et al.*, 1994). In Israel, symptoms resembling yellows disease were first observed in carrots in 1995. At that time, Orenstein *et al.* (1999) determined that the majority of the carrots sampled from Beit She'an (north), the Sharon region (central) and Kibbutz Sa'ad (south) were infected with aster yellows (AY) phytoplasma, a small percentage in Beit She'an and Sa'ad with western-X (WX) phytoplasma, and fewer with a mixture of both types of phytoplasmas.

The symptoms observed in Israel are typical of AY infections observed in the USA: leaf yellowing or reddening, witches' broom, excessive adventitious roots and abnormally shaped carrots. However, in Israel, unlike the situation in the USA (Burkness *et al.*, 1999; Goodwin *et al.*, 1999), lettuce and celery crops grown in proximity to infected carrot crops show no signs or symptoms of yellows disease. Furthermore, the disease in Israel is highly sporadic; in the western Negev region in 1997, Kibbutz Sa'ad was severely affected (up to 70% loss), while at another Kibbutz 8 km away, with no physical barriers, no losses occurred. The following year the situation was reversed *vis à vis* these two kibbutzim, and for the past three years the levels of observed symptoms (at time of harvest) have been well below 10% at both kibbutzim; however, at other locations in Israel higher infestations were periodically reported.

A number of leafhopper and one planthopper species known to be vectors of phytoplasmas, spiroplasmas or viruses occur in Israel. Among the known phytoplasma vectors, the only planthopper (Cixiidae) is *Hyalesthes obsoletus* Signoret. Leafhopper (Cicadellidae) vectors include *Neoaliturus fenestratus* (Herrich-Schäffer), *Circulifer haematoceps* (Mulsant & Rey), *C. tenellus* complex (Baker), *Macrosteles quadripunctulatus* (Kirschbaum), *M. sexnotatus* (Fallén) and *Orosius orientalis* (Matsumura) (Klein *et al.*, 2001; Orenstein *et al.*, 2003). Other cicadellids known to vector phytoplasmas and spiroplasmas in other locations, and which also occur in Israel, include *Anaceratagallia laevis* Ribaut, *Austroagallia sinuata* (Mulsant & Rey), *Exitianus capicola* Stål, and *Batracomorphus glaber* Haupt.

The goal of the research reported in this article was to monitor leafhopper and planthopper populations in carrot fields at Kibbutz Sa'ad (a known site of heavy phytoplasma infection) and Gilat Research Center, identify potential vector populations and analyse them for the presence of phytoplasma DNA, and identify the specific phytoplasma(s) found in infected insects and carrots.

Materials and methods

Kibbutz Sa'ad, 1999 and 2000

Two commercial carrot fields at Kibbutz Sa'ad were monitored for two consecutive years. During the spring season all fields were treated with insecticides (carbosulfan, cypermethrin and imidacloprid) to control leafhoppers, among other pests. For both the years, a total 5000 m^2 (25 rows of 100-m field width) of one field were left untreated for monitoring of potential phytoplasma vectors.

In 1999, seeds were planted on 11 February and monitoring commenced on 10 March, with 12 (15 × 17 cm) yellow sticky traps placed at plant height and evenly spaced around the field. Traps were changed once a week. Leafhoppers were carefully removed from the traps, washed in two changes of technical grade hexanes to remove the glue, and then stored in 95% ethanol until analysis by PCR. On the same day that the traps were changed, 50-m rows were sampled with a hand-held vacuum (Echo model No. PB 1000 leaf blower with air intake and exhaust ports reversed).

In 2000, seeds were planted on 1 March and monitoring commenced on 4 April, with 10 yellow sticky traps placed and changed as above, and vacuum samples taken. For both years, 400 plants were examined at time of harvest for phytoplasma symptoms, including leaf yellowing or reddening, crown-shoot proliferation, witches' broom growth, excessive adventitious roots and abnormally shaped carrots (Orenstein *et al.*, 1999).

Gilat, 2000

At the Gilat Research Center, two 0.2-ha fields were sown with Predor F1 hybrid carrot seeds, one on 9 March and the second on 30 March 2000; fields were 50 m apart. Plants were watered and cultivated according to standard agricultural practices for the region.

Sticky trap monitoring (eight 15 × 17 cm traps surrounding each field) commenced on 4 and 19 April, respectively, and ended on 25 July and 8 August, respectively. Traps were replaced at weekly intervals and returned to the laboratory for leafhopper identification. Leafhoppers were carefully removed and processed as described above. In 2000, the percent infection was evaluated by hand harvesting three areas of each field, scoring for

presence or absence of phytoplasma based on symptoms, and weighing each group. At harvest, carrot samples were returned to the laboratory and leaves were removed, stored individually in plastic bags and immediately frozen at −20°C.

DNA extraction

DNA was extracted from the leafhoppers following the procedure of Maixner *et al.* (1995). Samples of 5 leafhoppers were ground in 1.5 ml microcentrifuge tubes in 600 µl of extraction buffer (100 mM Tris–HCl pH 8.0, 2% cetyl trimethyl ammonium bromide (CTAB), 1.4 M NaCl, 20 mM EDTA, 0.2% β-mercaptoethanol). The slurry was incubated for 20 min at 60°C, and centrifuged for 10 min at 8000 *g*. The supernatant was collected and extracted with an equal volume of chloroform:isoamyl alcohol (24:1 v/v), followed by centrifugation and precipitation with one volume of isopropanol. After 20 min, at −20°C the preparation was centrifuged at 14,900 *g* for 20 min at 4°C. The pellet was washed twice with 70% ethanol, dried and re-suspended in 20 µl double-distilled water. For carrot samples, DNA was extracted from leaves, hairy roots and main roots. The procedure was as described above, except that 1 g of plant tissue was extracted in 8 ml of extraction buffer and the extraction buffer contained 3% CTAB.

Polymerase chain reaction (PCR)

A universal primer pair, rU3 and fU5, was synthesized according to the sequences published by Lorenz *et al.* (1995), amplifying a 1.2-kbp fragment of the conserved region of the 16S rDNA of all known phytoplasmas, and was used to detect the presence of phytoplasma in plant and leafhopper samples. Polymerase chain reaction (PCR) amplification of DNA was performed using a T gradient thermocycler (Tamar Laboratory Supplies, Israel). The reaction was carried out according to Orenstein *et al.* (2001): amplification was carried out in a total of 30 µl; 0.3 µl *Taq* polymerase (Promega, imported by Ornat Rehovot, Israel), 3 µl amplification buffer without $MgCl_2$, 2 µl 25 mM $MgCl_2$, 2 µl 2 mM dNTPs, 0.5 µl of 50 ng/µl each primer and 5–10 ng DNA. The reaction mixture was incubated for heat shock at 94°C (5 min), 50°C (2 min) and 72°C (2 min); 35 cycles of 92°C (45 s), 50°C (45 s) and 72°C (90 s). The last cycle (72°C) was extended for 7 min as an elongation step. The amplified PCR products were analysed by electrophoresis of 12 µl of reaction mixture in 1.5% agarose gel (40 mM Tris–HCl pH 7.5, 20 mM acetic acid, 1 mM EDTA), stained with ethidium bromide and visualized with a UV transilluminator. The size standard 100 bp DNA ladder used in gels was obtained from MBI Fermentas (imported by Eisenberg Brothers Ltd, Israel). DNA extracted from asymptomatic plants served as a negative control, and DNA extracted from periwinkle identified as carrying AY phytoplasma served as the positive control for the PCR assays.

Restriction fragment length polymorphism analysis (RFLP)

In order to classify the phytoplasma, each positive PCR product was analysed separately by RFLP reaction in the presence of restriction enzymes *Tru9* I and *Alu* I (Promega, Ornat Rehovot, Israel). From the PCR product, 15 µl were digested for 2 h at 37°C. The restriction products were then separated by electrophoresis through a 5% polyacrylamide gel and stained with ethidium bromide, and visualized with a UV transilluminator.

Results

Kibbutz Sa'ad, 1999 and 2000

Table 1 shows the total number of leafhopper and planthopper species that are known to be vectors of phytoplasmas, spiroplasmas or viruses that were trapped from the commercial carrot fields at Sa'ad in 1999 and 2000. At no time during the monitoring did the density of any one species of leafhopper exceed 2 individuals/trap/week. Additionally, the vacuum sampling yielded very few *Empoasca* spp. in both the years. The percentage of carrot plants showing signs and symptoms of phytoplasma disease at the time of harvest was $<3\%$ in 1999; and $<1\%$ in 2000.

Gilat, 2000

Table 2 shows the total number of leafhopper and planthopper species that are known to be vectors of phytoplasmas, spiroplasmas or viruses that were

Table 1. Total number of known leafhopper vectors caught on yellow sticky traps at Kibbutz Sa'ad in 1999 (12 traps) and 2000 (10 traps)

		Year	
Subfamily	Species	1999	2000
Agalliinae	*Anaceratagallia laevis*	0	1
	Austroagallia sinuata	0	5
Iassinae	*Batracomorphus glaber*	23	3
Deltocephalinae	*Cicadulina bipunctella*	43	16
	Circulifer haematoceps	111	52
	Circulifer/Neoaliturus sp.	31	23
	Exitianus capicola	7	3
	Macrosteles quadripunctulatus	10	31
	Neoaliturus fenestratus	46	0
	Orosius orientalis	9	10
	Psammotettix sp.	0	11

Table 2. Total number of known phytoplasma or spiroplasma vectors caught in two carrot fields at Gilat Research Center in 2000 (8 traps per field, dates of monitoring indicated)

Subfamily	Species	Early-planted field 4 April–25 July	Late-planted field 19 April–8 August
Agalliinae	*Anaceratagallia laevis*	16	16
	Austroagallia sinuata	57	490
Iassinae	*Batracomorphus glaber*	105	337
Deltocephalinae	*Cicadulina bipunctella*	14	16
	Circulifer haematoceps	293	994
	Exitianus capicola	12	3
	Neoaliturus fenestratus	254	39
	Orosius orientalis	33	71
	Psammotettix sp.	41	14
Cixiidae	*Hyalesthes obsoletus*	0	5

trapped from the commercial carrot fields at Gilat in 2000. The dynamics of those leafhoppers species in which more than 2 individuals were caught per trap per week are shown in Fig. 1. Although the planting dates were only 21 days apart and the fields 30 m apart, large differences were observed in the density of leafhopper populations. Populations of *N. fenestratus* were about six times higher in the early-planted field (planted on 9 March). Populations of *C. haematoceps* and *B. glaber* were about three times as high and those of *A. sinuata* were about eight times as high in the later-planted field (planted on 30 March). Vacuum samples reflected the sticky trap catches, but the number of individuals caught was very low; after four weeks, vacuum sampling was discontinued.

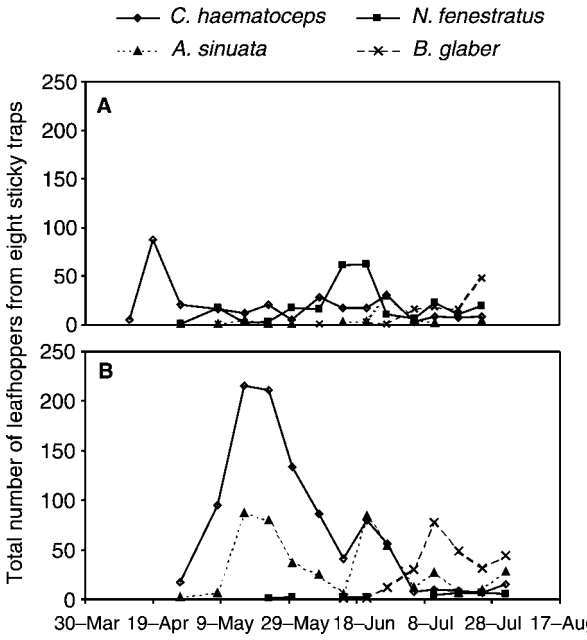

Fig. 1. Total number of leafhoppers caught per week on 8 yellow sticky traps at the Gilat Research Center fields planted on 9 March 2000 (A) and 30 March 2000 (B)

Results of the field evaluation for symptoms of phytoplasma infection are shown in Table 3. In the field planted on 9 March and harvested on 25 July, 21% of the carrots showed symptoms of phytoplasma infection. In that field, an average asymptomatic carrot weighed 44 g, whereas an average carrot with phytoplasma symptoms weighed 101 g. In the field planted on 30 March and harvested on 14 August, 12% of the carrots showed symptoms of phytoplasma infection. In this field, an average asymptomatic carrot weighed 64 g and an average carrot with phytoplasma symptoms weighed about 78 g.

For PCR and RFLP analysis, leafhoppers of the same species, removed from sticky traps from the two fields, were pooled. For PCR analysis, DNA was extracted from groups of five leafhoppers; a minimum of half of the leafhoppers caught on each date was analysed. The results of the PCR analysis for the presence of phytoplasma DNA in leafhoppers is shown in Fig. 2 and Table 4. Of the known vector species, only four, namely *C. haematoceps* complex, *N. fenestratus*, *A. sinuata* and *B. glaber*, tested positive for phytoplasma DNA. Phytoplasma DNA was first detected in *N. fenestratus* on 16 May, in *A. sinuata* on 30 May, in *B. glaber* on 27 June and finally in *C. haematoceps* complex on 11 July. Analysis by RFLP showed that all individuals carried DNA for a phytoplasma close to Elm Yellows (Fig. 2). Fifteen carrots showing all symptoms of the diseases were analysed for the presence of phytoplasma. Of these, 8 were completely negative and 7 were weakly positive. Those that were positive matched the profile for a phytoplasma close to Elm Yellows (Fig. 3).

Discussion

Transmission of pathogens by insects is dependent on the abundance of vector(s) and their interplant movement (Irwin and Ruesink, 1986; Power, 1987,

Table 3. Percent phytoplasma infection from carrots harvested from fields planted 9 and 30 March 2000 at the Gilat Research Center

Location	No. asymptomatic	Wt (kg)	No. symptomatic	Wt (kg)	% Infection
Planted 9 March, harvested 25 July					
West	215	13.1	30	2.9	14.0
Centre	319	11.4	62	6.1	19.4
East	308	12.5	88	9.2	28.6
Planted 30 March, harvested 14 August					
West	206	14.4	14	1.0	6.8
Centre	210	14.4	36	3.3	17.1
East	200	10.5	24	1.5	12.0

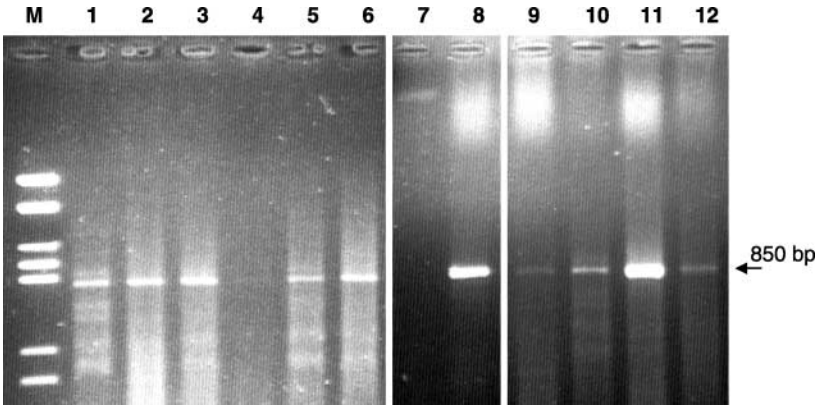

Fig. 2. Photograph of 1.5% agarose gel stained with ethidium bromide, showing amplified products from universal primers rU3 and fU5 for carrots and leafhoppers. Lane M, molecular markers; 1–3, symptomatic carrots; 4, asymptomatic carrot; 5–6, symptomatic carrots; 7–8, *Neoaliturus fenestratus*; 9, *Austroagallia sinuate*; 10–11, *Batracomorphus glaber*; 12, *Circulifer haematoceps*

1992); interplant movement is affected by the vectors' sex in some cases (Hunt *et al.*, 1993; Beanland *et al.*, 1999), but also by temperature, precipitation, age of the host plant (Chiykowski, 1981) and host plant density (Power, 1987, 1992). In the mid-western USA and Ontario, Canada, an AYI (Aster Yellows Index) has been established for head and leaf lettuce, celery and carrots based on leafhopper density and the percentage of infective individuals. On the basis of this index, recommendations are made whether or not to treat crops for leafhoppers. The situation in the mid-western USA and Canada is relatively clear-cut; there is one species, *Macrosteles quadrilineatus* (= *fascifrons*) Forbes, vectoring this phytoplasma (Hoy *et al.*, 1992). On the other hand, in California, the situation is not as simple, since there are many vectors (Severin, 1934; Severin and Frazier, 1945). In the early 1930s in California, yellows diseases were severe enough as to warrant ploughing under whole fields (Severin and Frazier, 1945). Since at least the mid-1940s yellows diseases in carrots, celery and lettuce have persisted but have not been of economic importance.

In our trials, we monitored leafhopper populations in an effort to determine which species vectored phytoplasma to carrots, and which specific phytoplasma occurred. It is well known that non-vector leafhoppers can acquire phytoplasmas (Purcell *et al.*, 1981), and test positive for the presence of phytoplasma DNA (Vega *et al.*, 1993). In spite of this, and because of the low levels of carrot infection, we analysed known vectors for the presence of phytoplasma DNA in an attempt to narrow the list of potential vectors.

Monitoring leafhoppers in carrot fields at Kibbutz Sa'ad yielded very low densities on sticky traps and virtually no leafhoppers by vacuum sampling. This was likely as a result of insecticide treatments in all fields surrounding and adjacent to the one monitored.

In Gilat, there were clear differences in the vector populations between the two adjacent fields; since there was no barrier between the fields, it is possible that the vector populations were responding to the maturation of the carrot plants. Even though *Circulifer* spp. appeared in the fields first, they were not found positive for phytoplasma DNA

Table 4. PCR analysis of leafhoppers caught on sticky traps from two fields in 2000 at the Gilat Research Center

Species	Date	No. groups analysed[+]	No. groups positive
C. haematoceps	11 July	3	3
	18 July	3	1
N. fenestratus	16 May	4	1
	30 May	4	2
	7 June	4	1
	14 June	7	2
	11 July	4	3
	18 July	2	2
	25 July	3	3
	1 August	1	1
A. sinuata	30 May	4	3
	7 June	3	1
	14 June	1	1
	21 June	7	4
	27 June	9	4
	5 July	2	1
	18 July	1	1
	1 August	2	1
B. glaber	27 June	2	2
	5 July	6	3
	25 July	9	2

+ DNA was extracted from groups of 5 individuals then analysed by PCR.

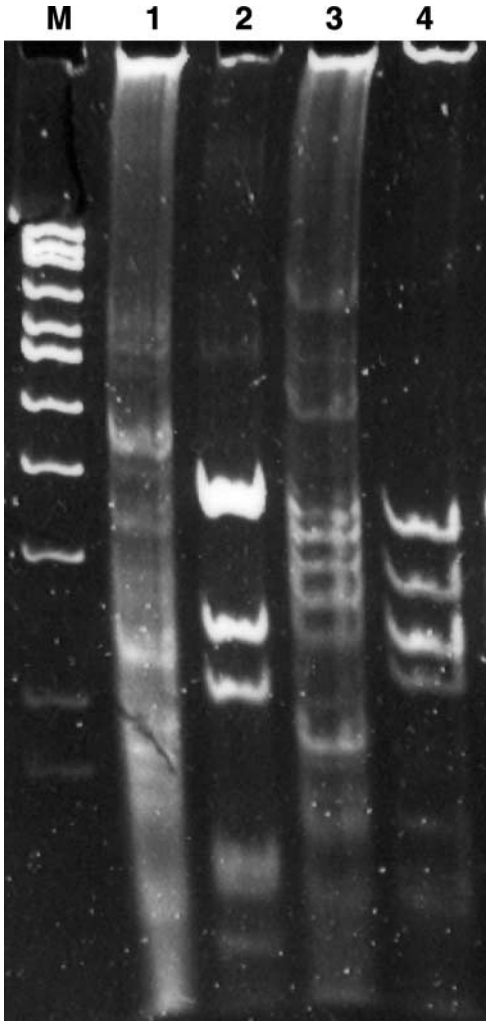

Fig. 3. Photograph of 5% polyacrylamide gel, stained with ethidium bromide, showing the restriction pattern profile from four different phytoplasma DNAs treated with restriction enzymes *Tru9* I and *Alu* I. Lane M, molecular markers; 1, Aster Yellows; 2, Western-X; 3, Elm Yellows; 4, Stolbur

until more than a month after they first appeared, and therefore, could not possibly have vectored it into the field. It is argued that *Circulifer* spp. may serve to increase the rate of infection within an already infected field, but they are not the primary vector initiating the infection cycle. Similar to *Circulifer* spp., *A. sinuata* were found early in the second field but tested positive for phytoplasma only on 30 May, when their numbers were declining.

Neoaliturus fenestratus was caught early in the season and found to have phytoplasma DNA. About twice the number of plants were infected with phytoplasma in the field planted at the beginning of March as compared to the field planted three weeks later. *Neoaliturus fenestratus* populations were caught in the early-planted field at levels six times greater than in the later-planted field. Furthermore, phytoplasma DNA was detected in *N. fenestratus* two weeks before it was detected in any other leafhopper species.

Batracomorphus glaber is a summer species, and while it was found to be infected shortly after it was first caught, it appears too late in the season to initiate the cycle of infections. It may, however, help to maintain or increase infections within an already infected field.

Although all 15 carrot samples analysed by PCR for the presence of phytoplasma DNA showed full symptoms of infections, only half of them were [weakly] positive. We detected a phytoplasma similar to Elm Yellows, the first time this has been recorded in carrots. Plants showing typical phytoplasma symptoms, but having negative PCR results, are not unique to Israel; similar results have been reported from Italy (Marzachi *et al.*, 1999) and Australia (Gibb *et al.*, 2001). In the Australian study, symptomatic carrots were negative for phytoplasma in both single and nested PCR analyses; further analysis revealed that the symptomatic plants were infected with Proteobacteria. Symptoms similar to phytoplasmas have been shown to be caused by other prokaryotes such as rickettsia or rickettsia-like-organisms (Giannotti

et al., 1974; Fránová *et al.*, 2000) or by insect toxins (Bedendo *et al.*, 1999).

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