

## ASSOCIATION OF PHYTOPLASMAS WITH A YELLOWS DISEASE OF CARROT IN ISRAEL

S. Orenstein, A. Franck, L. Kuznetzova, I. Sela and E. Tanne

Department of Virology, Agricultural Research Organization, the Volcani Center, P.O. Box 6, Bet Dagan 50250, Israel

### SUMMARY

A carrot disease resembling carrot yellows has recently appeared in Israel. Electron microscopy and PCR analyses confirmed that the causative agent is a phytoplasma. This is the first report of carrot yellows in Israel. It was detected in five varieties from different regions of the country. Most of the infected plants carried the AY-type phytoplasma, but a few were found to carry a unique type of phytoplasma, hitherto not reported in carrots, which resembles X-disease agent. Both types of phytoplasma could be transmitted to periwinkle.

### RIASSUNTO

**ASSOCIAZIONE DI FITOPLASMI CON UN GIALLUME DELLA CAROTA.** È apparsa recentemente in Israele una malattia della carota simile al giallume da fitoplasmi, la presenza dei quali è stata accertata nelle piante infette con osservazioni al microscopio elettronico. La malattia, che è nuova per Israele, è stata identificata in cinque diverse regioni del paese. La maggior parte delle piante erano infette da fitoplasmi del gruppo AY (aster yellows) ma alcune contenevano un fitoplasma di tipo diverso, talora in infezione mista con AY, simile all'agente della malattia X (western X disease), non segnalato precedentemente nella carota. Entrambi i fitoplasmi sono stati identificati mediante amplificazione genica (PCR), usando primers gruppo-specifici, seguita da analisi RFLP dei prodotti PCR, e sono stati trasmessi con successo alla pervinca (*Catharantus roseus*).

### INTRODUCTION

Carrot (*Daucus carota*) is an important vegetable, grown in many countries and known for its nutritional value. It is cultivated in Israel in different regions

according to season and the designated market. Carrots are grown for the local fresh market as well as for industry and export, according to growing season and soil. The Israeli carrot industry is affected by various fungal diseases, nematodes and other pests. During the last 3 years, yellows symptoms have been spreading in different growing regions, causing serious damage to fresh and stored carrots. The phenomenon has been recorded in autumn, winter and summer crops, and at various growth stages. The symptoms consist of smaller, feathery leaves, leaf yellowing or reddening, shoot proliferation, and witches' broom growth. Secondary hairy roots are formed in a spiral-like manner, the carrots are abnormally shaped and tapered. Soft rot develops in storage along transparent lines existing on the roots, causing severe losses. In extreme cases, growth stops and the field cannot be cropped.

Yellows diseases, mainly aster yellows (AY), have been recorded in carrots for over 50 years in the USA (Ivanoff and Ewart, 1944), where they have caused various degrees of damage, resulting in up to 70-80% losses (Linn, 1940; Zalom, 1981; Errampalli *et al.*, 1989; Arcelin and Kushalappa, 1991). The disease has been diagnosed through visualization of symptoms and anatomic aberrations, electron microscopy, serology and Western blots (Struckmeyer, 1963; Lin and Chen, 1986; Errampalli *et al.*, 1991).

Phytoplasmas, are the causative agent of yellows diseases in general. These nonculturable Mollicutes are transmitted mainly by leafhoppers. The phytoplasmas can be detected in phloem sieve tubes by electron microscopy (Doi *et al.*, 1967; Hirumi and Maramorosch, 1973), Dienes' stain (Deely *et al.*, 1979) and fluorochromic DNA staining (da Rocha *et al.*, 1986; Hiruki and da Rocha, 1986). Immunological methods have also been developed for some phytoplasmas (Sinha, 1979; Sinha and Benhamou 1983; Lin and Chen, 1986; Errampalli and Fletcher, 1993). However, none of these methods is suitable for the identification of phytoplasmas on a large scale, or their classification. Consequently, molecular methods such as DNA hybridization and polymerase chain reaction (PCR) have been developed for the identification, characterization and classification

Corresponding author: E. Tanne  
Fax: +972.3.9604180  
E-mail: tanne@agri.huji.ac.il

of phytoplasmas (Kirkpatrick *et al.*, 1987; Harrison *et al.*, 1991; Ahrens and Seemüller, 1992; Davis and Lee, 1993; Lee *et al.*, 1993).

AY is one of the most important phytoplasma groups, affecting over 300 plant species and causing disease mainly in vegetable and flower crops. Carrot AY is transmitted by the aster leafhopper, *Macrostelus quadrilineatus* Forbes (Kunkel, 1926; Chiykowski and Chapman, 1965; Granados and Chapman, 1968; Mahr *et al.*, 1993). Canadian-X disease (CX), belonging to the Western-X group (Seemüller *et al.*, 1998), is found in woody and herbaceous plants, but its economic impact is felt mainly in stone fruits in North America (Gilmer *et al.*, 1954; Chiykowski and Sinha, 1982; Kirkpatrick *et al.*, 1990).

We here report on the first investigation of carrot yellows in Israel, aimed at studying the etiology and spread of the disease and characterizing the associated phytoplasma by PCR-based techniques.

*Key words:* phytoplasma, carrot, PCR, yellows.

## MATERIALS AND METHODS

**Sources of healthy and diseased plants.** Samples of symptom-expressing carrots and healthy controls were collected in three different growing regions in Israel: the Bet She'an Valley (North), the Sharon (Center), and Sa'ad (South). Samples of the varieties 'Tino', 'Newton', 'Presto', 'Senior' and 'Nairobi' were collected from commercial plots in autumn, winter and spring.

**Electron microscopy.** Pieces of petioles and roots (about 1 mm<sup>2</sup>) from healthy and symptomatic carrots were fixed in 2% glutaraldehyde in 20 mM phosphate buffer (pH 6.8) for 1 h. Post fixation was done with 2% osmium tetroxide in 20 mM phosphate buffer (pH 6.8), followed by dehydration in acetone and embedding in Agar-100, as described by Orion and Franck (1990). Sections were cut with an ultramicrotome (Ultratom III, LKB), stained with 2% uranyl acetate and lead citrate, and examined in an electron microscope (Jeol JEM 100 CXII).

**DNA extraction.** DNA extracts were prepared from leaves, hairy roots and main roots as described by Maixner *et al.* (1995). Fresh or frozen tissue (0.1 g) was ground in 1.5 ml microcentrifuge tubes in 1 ml of extraction buffer (100 mM Tris-HCl pH 8.0, 2% cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 20 mM EDTA, 0.2%  $\beta$ -mercaptoethanol). The slurry

was incubated for 20 min at 60°C, and centrifuged (10 min, 3200 g). The supernatant fluid was collected and extracted with an equal volume of chloroform: isoamyl alcohol (24:1 v/v), followed by centrifugation and precipitation with 1 volume of isopropanol. Following 20 min at -20°C the preparation was centrifuged at 14,900 g for 20 min. The pellet was washed twice with 70% ethanol, dried and resuspended in 50  $\mu$ l distilled water.

**Polymerase chain reaction.** A universal primer pair, r16SF2/r16SR2, was synthesized according to the sequences published by Lee *et al.* (1993), amplifying a 1.2 kbp fragment of the conserved region of the 16S rDNA of all known phytoplasmas. This primer pair was used in PCR to detect the presence of phytoplasma in the tested carrot DNA extracts. Group-specific primers for AY and WX (Canadian isolate; CX) were also synthesized and used for nested PCR (Lee *et al.*, 1993; Lee *et al.*, 1994); 20 ng of DNA was used in all PCR assays. The DNA was heated to 94°C for 5 min, after which *Taq* polymerase was added, and the mixtures were subjected to 35 PCR cycles (92°C, 30 s; 55°C, 30 s; 72°C, 50 s). PCR was terminated with a 7 min chain elongation step. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel, followed by staining with ethidium bromide and visualization of DNA bands using a UV transilluminator.

DNA extracted from asymptomatic plants served as a negative control in PCR. DNA extracted from periwinkle carrying either AY-type or WX-type phytoplasma served as the positive control for the PCR assays.

**RFLP analysis of PCR products.** PCR products were analyzed by restriction digestion, using *Mse*I, *Alu*I and *Kpn*I endonucleases. The DNA was digested in the specific buffers appropriate to the enzymes at 37°C for 2 h. Fragments were separated by electrophoresis on 5% polyacrylamide gels, followed by staining with ethidium bromide as described above.

The positive controls were DNA extracts from periwinkles infected with type AY or WX (CX-type) phytoplasma, kindly provided by Dr. R.E. Davis, USDA, Beltsville, MD, USA.

**Graft transmission.** Young leaves attached to a piece of carrot root were grafted onto healthy periwinkle plants. The graft site was wrapped with Parafilm, and the plant was kept in a moist atmosphere for several days. Grafted plants were then transferred to a greenhouse for several months, observed for symptoms, and analyzed by PCR to detect graft-transmitted phytoplasma.

## RESULTS

A typical diseased carrot with crown shoot proliferation, witches'-broom symptoms, secondary hairy roots and a deformed main root, is shown in Fig. 1. Pleomorphic, membrane-bound phytoplasmas were observed by electron microscopy at high concentration in phloem sieve tubes of infected plants (Fig. 2). No phytoplasmas were observed in sections from healthy control plants.

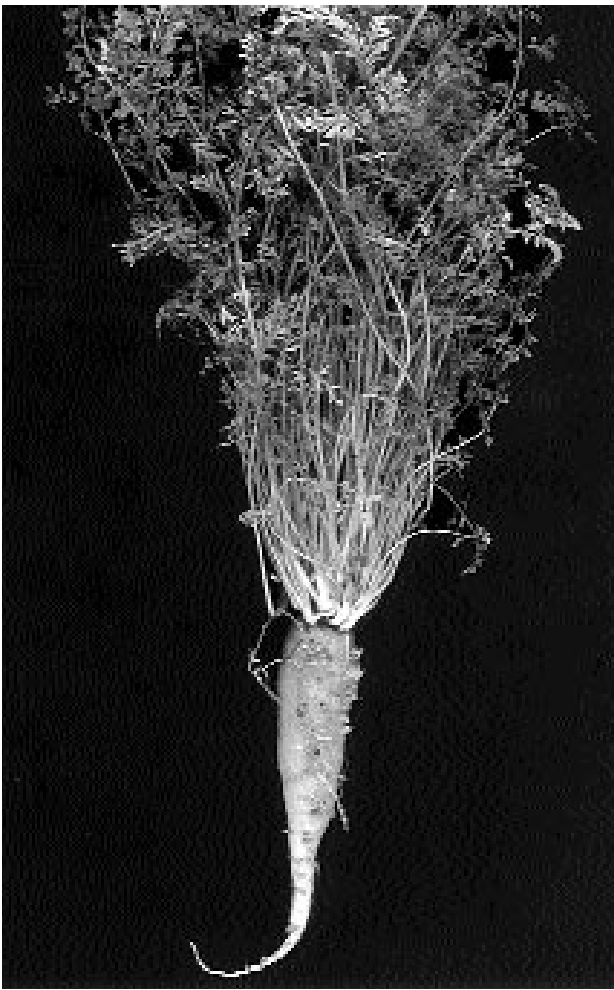


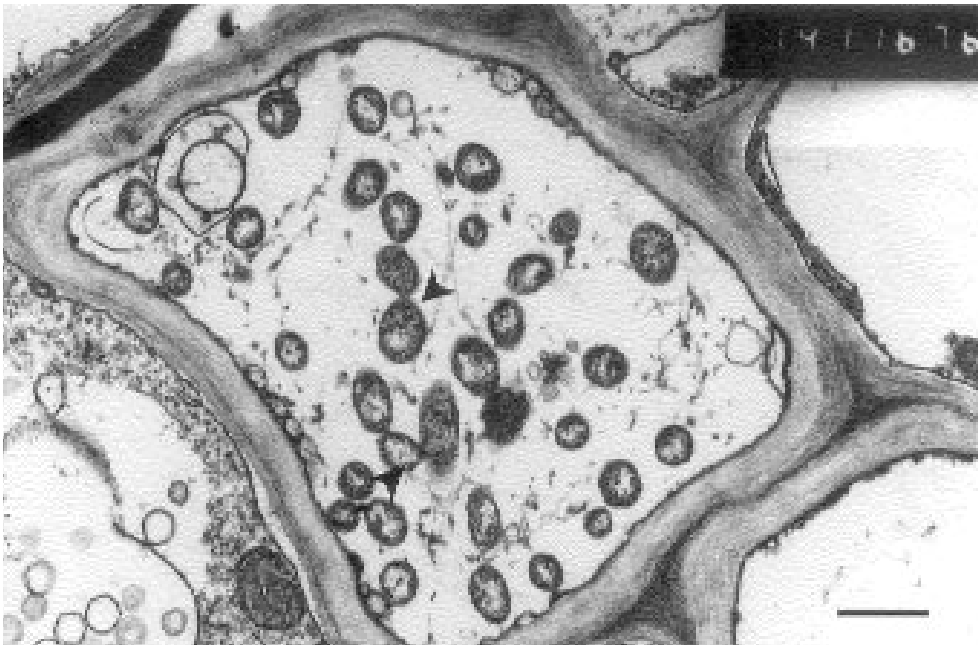
Fig. 1. Yellows symptoms on carrot.

Over 500 samples of symptomatic carrots from fields in three regions of Israel were PCR-assayed over a 3 year period. Asymptomatic carrots were included in every experiment. PCR with universal primers amplified a band of *ca* 1.2 kbp from all symptomatic carrot samples, but not from asymptomatic carrots. Phytoplasmas were found in all five varieties tested. Fifty positive samples were subjected to typing experiments using nested PCR

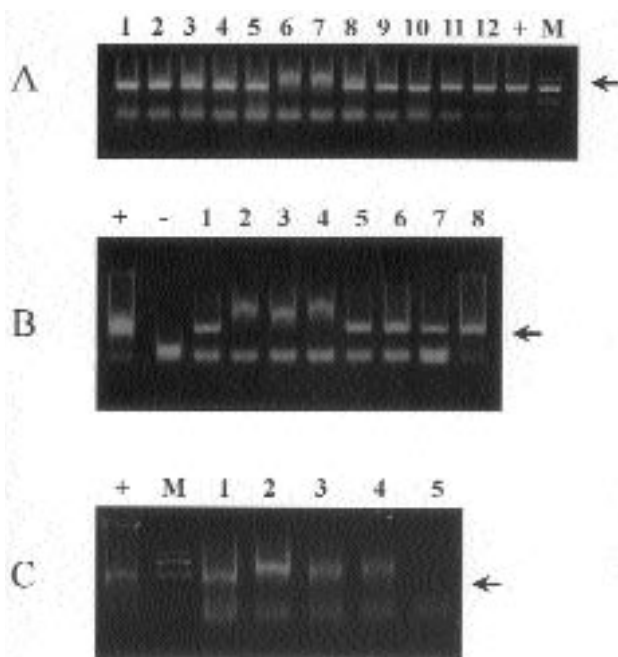
with group-specific primers for AY (resulting in a 1.1 kbp fragment) and CX (resulting in a 0.8 kbp fragment). AY-type phytoplasma was found in 44 of these plants, and 6 plants were found to carry another type of phytoplasma which was amplified by the CX-specific primers. Four of these plants carried both types of phytoplasma. An example for the AY-specific amplification is shown in Fig. 3. Most of the samples reacted with the AY-specific primers, but several did not (*e.g.* lanes 6 and 7 in panel A). The CX-specific amplification is shown in Fig. 4, and only a few samples were found to react with the CX-specific primers (*e.g.* lanes 2 and 3 in panel A). AY-type phytoplasma was detected in all varieties tested from all parts of Israel. WX-type phytoplasma, which previously has not been recorded in carrot, was detected in only about 12% of the phytoplasma-positive samples in the varieties 'Tino', 'Newton' and 'Nairobi' in the north and south, but not in the center of Israel. Double infections were also detected (compare Figs 3 and 4). The validity of AY-typing by nested PCR was corroborated by RFLP assays with three different enzymes. The RFLP pattern resembled that of other AY-type phytoplasmas reported (Lee *et al.*, 1994; Berges *et al.*, 1997) and that of the Maryland AY serving as an internal control. An example of such a RFLP analysis with six samples positive for AY by nested PCR is given in Fig. 5. WX was detected infrequently, and an example of its RFLP typing is shown in Figs 6 and 7. The *AluI* and *KpnI* restriction patterns of the WX-specific nested band were very different from those of the AY-specific nested band. The restriction patterns of this WX-specific nested band, were quite similar, but not identical to the CX-type reference obtained from Beltsville (Lee *et al.*, 1994).

Periwinkles exhibited yellows symptoms 3-4 months after grafting. PCR tests of grafted symptomatic periwinkle and healthy controls indicated that the phytoplasma had indeed been transmitted. As found previously for grapevine phytoplasma (Tanne and Orenstein, 1997), the phytoplasma in the recipient plants was identical to that of the carrot source plants as determined by nested PCR and restriction analysis of periwinkle (data not shown). Thus a method of maintaining the carrot phytoplasma for further studies in periwinkle was provided.

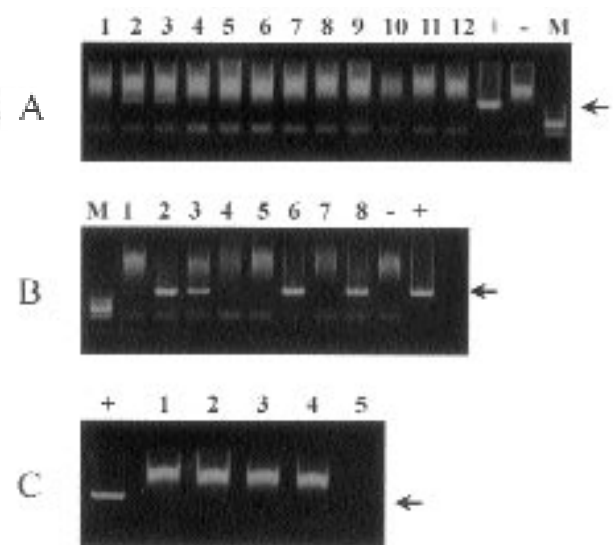
A high correlation was found between the presence of phytoplasmas in the tissue, as detected by electron microscopy, and the positive results of the molecular tests.



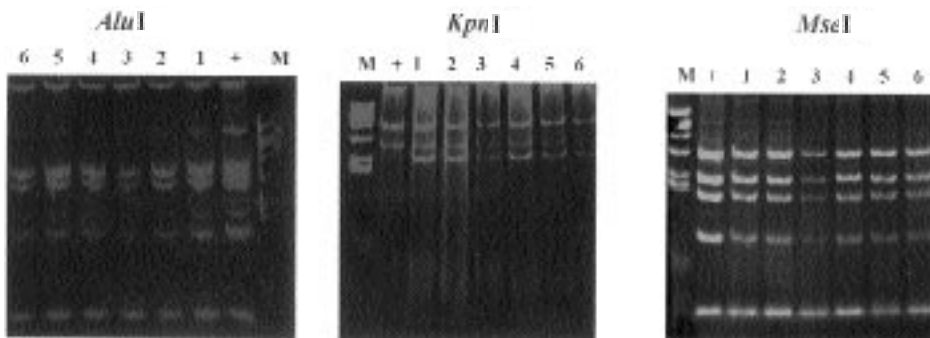
**Fig. 2.** Electron micrograph of a section of a companion cell of a petiole of infected carrot.



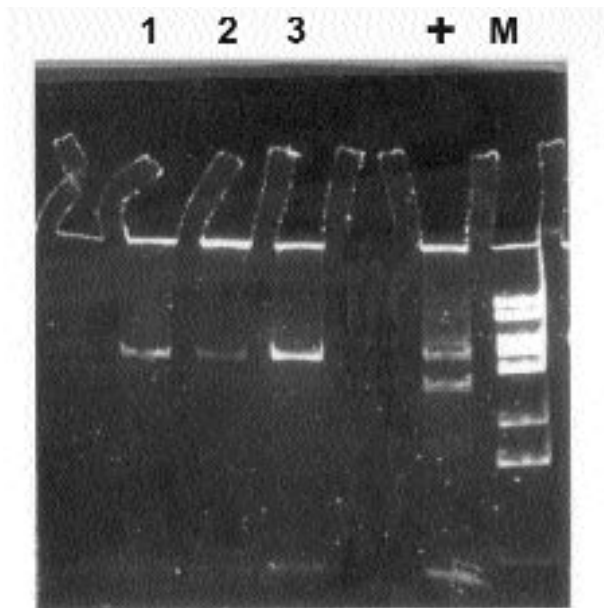
**Fig. 3.** Nested PCR analyses of carrots exhibiting yellows symptoms. PCR was primed with AY-specific primers. **Frames A-C:** samples from the North, South and Center of Israel, respectively. M: Size markers; (+): positive control; (-): negative control. Bands were not amplified from asymptomatic carrots. Lane numbers indicate different plants. Arrows indicate products of the expected size.



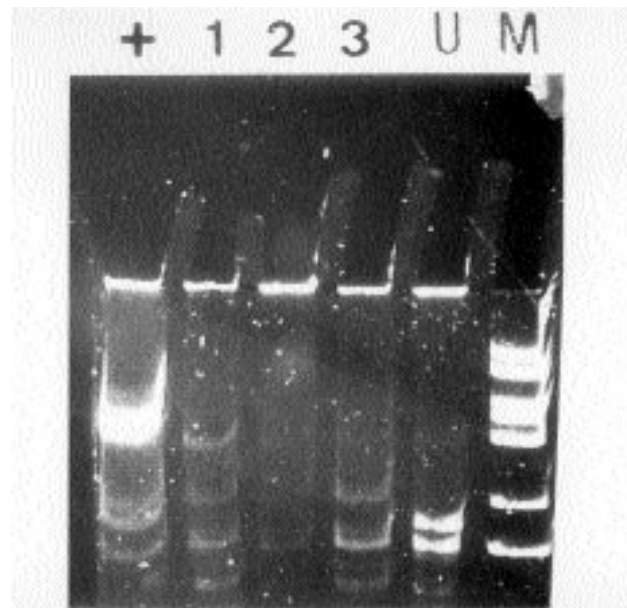
**Fig. 4.** Nested PCR analyses of the same carrots as in Fig. 3. PCR was primed with WX-specific primers. Frame designation and symbols are as in Fig. 3.



**Fig. 5.** RFLP analyses of nested, AY-specific, PCR products following cleavage with the restriction endonucleases *AluI*, *KpnI* and *MseI*.



**Fig. 6.** RFLP analysis of nested, WX-specific, PCR products following cleavage with *AluI*. Lanes 1-3: WX-specific nested PCR of carrot samples; (+): Positive control (analysis performed with DNA from CX-infected periwinkle); M: Size markers.



**Fig. 7.** RFLP analysis of nested, WX-specific, PCR products following cleavage with *KpnI*. Symbols as in Fig. 6. U: a pattern of an unrelated sample (phytoplasma of another plant).

## DISCUSSION

Yellows disease has been reported in carrots in North America for many years (Kunkel, 1926; Ivanoff and Ewart, 1944). The disease, caused by phytoplasmas and transmitted by *M. quadrilineatus*, inflicted various degrees of damage. The aim of this study was to detect and characterize the putative causal agent of carrot yellows in Israel. Phytoplasmas were PCR-detected in carrots exhibiting either one or all described symptoms in five varieties grown in different regions, and could not be detected in symptomless carrots. In previous reports, carrot yellows was attributed to an AY-type phy-

toplasma (Errampalli *et al.*, 1991). In Israel, AY was also found to be the predominant type of carrot phytoplasma. However, in a few samples, a different type of phytoplasma was detected (sometimes as a mixed infection with AY). Its restriction pattern resembled that of WX, and therefore it is tentatively suggested to be a member of the X-disease group. PCR typing was carried out with group-specific primers as well as by RFLP analysis of the nested PCR products. PCR and RFLP analyses performed with the grafted periwinkle (data not shown) indicated the phytoplasma type in carrot and the one transmitted to periwinkle were the same, proving the fidelity of transmission by grafting. Hence,

phytoplasmas of this annual crop can be preserved for long-term studies in periwinkle. The correlation between the presence of pleomorphic MLOs in phloem of carrots exhibiting symptoms – but not in symptomless ones – and the PCR results indicates that the carrot yellows disease in Israel is caused by phytoplasmas.

This paper is the first to describe yellows diseases in carrots in Israel, and the occurrence of a non-AY-type (possibly WX) phytoplasma in this plant.

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