Phytoplasmas as Causal Agents of Celosia Disease in Israel

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Abstract. Recently, yellows diseases have become more common in Israel, and phytoplasmas have been detected in some of these diseased crops. Commercial fields of two celosia species (*Celosia plumosa* L. and *C. cristata* L.) also have exhibited yellows symptoms and total crop failure. Typical mycoplasma-like bodies were observed in infected but not in healthy plants. The same plants were analyzed for the presence of phytoplasma by polymerase chain reaction (PCR), using the universal oligonucleotide pair r16SF2/r16SR2, followed by nested PCR using group-specific primers. Restriction analyses performed with these products indicated that two different types of phytoplasmas are infecting celosia. PCR-RFLP analysis of one type revealed a restriction pattern typical of aster yellows. Similar analysis of the second type indicated possible relatedness, though not identity, to the pattern of phytoplasmas of the Western-X group. This is, to our knowledge, the first report of phytoplasma infection in celosia.

The genus *Celosia* L., a member of the Amaranthaceae, includes many species native to warm regions of Africa and America. They serve as a food source and are used as spices in many African countries. Celosia is grown in Western countries as an ornamental plant, either potted or as a cut flower. The main species are *C. plumosa* L. and *C. cristata* L. Celosia is seed propagated and requires high temperatures to produce the long flower branches demanded by the market (Armitage, 1986). In Israel it is grown mainly in the spring and summer.

Celosia is susceptible to virus infections, and a database search indicated that ~50 different viruses belonging to 21 groups have been found in celosia. Only those belonging to the nepo-viruses are known to be seed-transmissible. Diseases caused by phytoplasmas have not hitherto been reported in celosia.

Phytoplasmas (formerly called mycoplasma-like organisms) are phloem-limited plant pathogens. They are nonculturable prokaryotes lacking a cell wall and are related to the Mollicutes (McCoy et al., 1989).

Phytoplasmas cause diseases in several hundred plant species, with various symptoms, collectively referred to as “yellows diseases” (McCoy et al., 1989). They are transmitted by insect vectors and via vegetative propagules (Carraro et al., 1998; Caudwell et al., 1993; Schvester et al., 1993).

Traditional diagnosis of phytoplasma-associated diseases was based on symptoms and electron microscopy. In the last decade, considerable progress has been made in the detection and identification of phytoplasmas, by means of serological and molecular techniques (Aherns and Seemuller, 1992). The latter are based on the detection of conserved regions of the 16S RNA gene and the 16S-23S spacer region (Boudon-Padieu et al., 1996; Lorenz et al., 1995; Smart et al., 1996) and enable reliable and sensitive detection and characterization of phytoplasmas, as well as classification and identification of some phytoplasma types (Schneider et al., 1993; Seemuller et al., 1998; Vibio et al., 1996).

In Summer 1998, yellows symptoms were observed in celosia grown in central Israel. Plants exhibited leaf yellowing, “little-leaf”, short internodes, flower malformation, or lack of flowering (Fig. 1). Occasionally witches’ broom symptoms were observed. Early seedlings were affected up to 100%, with no marketable flowers; the extent of damage was less in later seedlings. The objectives of the present study were to identify and characterize the causal agent of the yellows disease affecting celosia in Israel.

Plant samples and phytoplasma references. Field samples of symptom-exhibiting and symptom-free celosia plants were used throughout. Celosia plants grown in the greenhouse served as disease-free controls. Reference DNA samples from phytoplasma-infected periwinkles [*Catharanthus roseus* (L.) G. Don] classified as aster yellows (AY) and Western-X (Canadian X type; CX) type, were kindly provided by Dr. R.E. Davis, U.S. Dept. of Agriculture, Beltsville, Md. The Stolbur type of phytoplasma was provided by Dr. M. Maixner, Biologische Bundesanstalt, Bernkastel-Kues, Germany, and was also maintained in periwinkle and used for comparison in RFLP analysis.

Mechanical inoculation. Leaves exhibiting disease symptoms were ground in 0.01 M phosphate buffer, pH 7, and the slurry was rubbed onto carborundum-dusted leaves of the following test plants: *Gomphrena globosa* L.; *Nicotiana glutinosa* L.; *N. benthamiana* Domin.; *N. rustica* L.; *N. tabacum* L., cvs. Samsun and Xanthi-nc.; *N. sylvestris* Speg. & Comes; *N. clevelandii* Gray; *Datura stramonium* L.; *Chenopodium amaranticolor* Coste & Reynier.; *C. quinoa* Willd.; *Cucumis sativus* L., cv. Bet Alpha; *Lycopersicon esculentum* Mill, cv. Marmand; *Capsicum annuum* L.; and *Petunia hybrida* Hort. Plants were rinsed with water and kept in an insect-proof greenhouse at 22 °C for observation of symptoms.

Electron microscopy. Crude plant extracts in phosphate buffer were negatively stained with 1% uranyl acetate and examined for virus in a Jeol 100CX II (Jeol, Tokyo) or a Zeiss EM 906 (Carl Zeiss, Oberkochen,

Fig. 1. Celosia plants exhibiting yellows symptoms.

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Germany) electron microscope. Pieces of petioles and roots ($\approx 1 \text{ mm}^2$) from healthy and symptomatic celosia plants were fixed in 2% glutaraldehyde in 20 mM phosphate buffer (pH 6.8) for 1 h. Postfixation 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 in an ultramicrotome (Ultratom III; LKB-Produkter AB, Bromma, Sweden), stained with 2% uranyl acetate and lead citrate, and examined in an electron microscope (Jeol 100 CX II; Jeol, Tokyo).

DNA extraction. Extracts were prepared from leaves, hairy roots and main roots as described by Maixner, et al. (1995). Fresh or frozen tissue (0.5–1.0 g) was ground in 5–7 mL 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 (10 min, 3200 $\times g$). The supernatant fluid was collected and extracted with an equal volume of chloroform : isoamyl alcohol (24:1 v/v) and precipitated with 1 volume of isopropanol. Following 20 min at –20 °C, the preparation was centrifuged at 14,900 $\times g$ for 20 min. The pellet was washed twice with 70% ethanol, dried and resuspended in 50 μL distilled water.

Polymerase chain reaction (PCR). The universal phytoplasma primer pair P1/P7 (Schneider et al., 1993) amplifies a 1800 bp-long fragment that extends from the 5' end of the 16S RNA gene to the 5' region of the 23S DNA. This primer pair was used for phytoplasma detection in DNA extracts of the tested plant tissues. The P1/P7 PCR product was further amplified by nested PCR with the U5/U3 primer pair (Lorenz et al., 1995). Another universal primer pair, r16SF2/r16SR2 was synthesized according to published sequences (Lee et al., 1993), and used to amplify a 1.2-kbp fragment of the conserved region of the 16S rDNA of all known phytoplasmas. In this case, the PCR was made directly with the r16SF2/r16SR2 primer pair, followed by a nested PCR with group-specific primers for AY and CX that were also synthesized according to Lee et al. (1994) and Lee and Davis (1998). The primer pair STOL11 f2/f1, amplifying a stolbur-specific 1.7 kbp-long fragment, was synthesized according to Daire et al. (1997) and used for PCR. The DNA template in all PCR assays was 20 ng in a 30- μL assay. 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). The PCR program ended with a 7-min elongation step at 72 °C, and the products were analyzed by electrophoresis on a 1.5% agarose gel, followed by staining with ethidium bromide and visualization of DNA bands by means of a UV transilluminator.

The DNA extracted from symptom-free plants and that extracted from greenhouse-grown plants served as negative controls in

PCR. DNA extracted from periwinkles identified as carrying AY-type, CX-type and Stolbur-type phytoplasma, served as positive controls.

RFLP analysis of PCR products. The PCR products were analyzed by restriction endonuclease digestion, using *MseI*, *AluI*, and *KpnI*. The DNA was digested (37 °C; 20 h) in the specific buffers corresponding to the enzymes. 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 the aforementioned phytoplasma-infected periwinkle plants.

Results and Discussion

A typical infected celosia is illustrated in Fig. 1. Test plants, mechanically inoculated with sap from infected celosia, did not exhibit any symptoms related to viral infection, indicating that no mechanically transmissible virus was associated with the disease. Electron microscope observations of crude plant extracts revealed no virus or virus-like particles (not shown).

The observation of typical phytoplasma bodies in thin sections of phloem sieve tubes from infected celosia plants (Fig. 2), and their absence from the corresponding symptom-free plants, indicated that a phytoplasma could have been the causative agent of the disease.

No viral particles could be detected in these thin sections, which supports the aforementioned mechanical inoculation tests in suggesting that no viral infection was involved.

Use of the universal primers P1/P7 indicated that all celosia plants showing symptoms were infected with phytoplasma, while all symptom-free plants were phytoplasma-free (data not shown). Group-specific PCR assays, using the universal 16SF2/16SR2 primer pair and followed by nested PCR with group-specific primers, indicated the involvement of AY in celosia infections in most of the infected plants (Fig. 3A). This was corroborated by restriction analysis both of the PCR product of the nested AY-specific bands (Fig. 3 B and C) and of the P1/P7 bands (data not shown). However, a few infected plants produced amplification products with the CX-specific primers. The resultant products were somewhat longer than that of the CX-positive control (Fig. 4A), and the restriction analysis indicated a pattern similar but not identical to that of the control CX band (Fig. 4B). Negative results were obtained with the Stolbur primers (data not shown). We propose a new type of phytoplasma resembling CX and the AY type of phytoplasma as the causative agents of celosia yellows disease in Israel. Double infection, in which a plant carried both phytoplasma types, was not observed.

In recent years, several types of phytoplasma have been identified in various crops,

Fig. 2. Electron micrograph of a thin section through sieve cells of yellows-infected celosia containing phytoplasma-like bodies.

Fig. 3. Detection of AY-type phytoplasma in celosia. (A) Amplification with universal primers followed by nested PCR with AY-specific primers. Lane 1: positive AY control; lanes 2–4: samples of yellows-diseased celosia plants. Fragment size: ≈ 800 bp. (B) *AluI* restriction analysis of nested AY fragments. Lane 1: positive AY control; lanes 2–4: samples from yellows-diseased celosia plants. Size-range of fragments: 150–350 bp. (C) *KpnI* restriction analysis of nested fragments. Lane 1: positive AY control; lanes 2–4: samples from yellows-diseased celosia plants. Size range of fragments: 300–1200 bp.

such as grape (*Vitis vinifera* L., Tanne and Orenstein, 1997) and carrot (*Daucus carota* L., Orenstein et al., 1999) in Israel. The aster yellows type along with the Western-X and Stolbur types is the most prevalent, and mixed infections sometimes occur. Phytoplasma has been associated with disease symptoms in a few ornamental plants, such as *Anemone coronaria* L., lisianthus (*Eustoma russellianum* L.), and *Lilium longiflorum* L. (Cohen et al., 1999), but no severe damage has been recorded

Summary

The objective of this study was to determine the causative agent of a yellows-type disease That causes severe damage to celosia. Molecular analysis and electron microscopy demonstrated that the disease was caused by phytoplasmas. Two different types of phytoplasma could be detected in the diseased plants (but not in healthy ones), one belonging to the AY group and the other, closely related, but not identical, to the CX group. The same CX-resembling type has also been detected in carrots in Israel (Orenstein et al., 1999). The characterization of this phytoplasma and the search for the possible vector(s) are currently in progress.

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Fig. 4. Detection of CX-resembling phytoplasma in celosia. (A) Nested PCR of celosia DNA. PCR was conducted first with the 16SF2/16SR2 universal primer pair, followed by nested PCR with CX-specific primers. Lane 1: positive CX control; lanes 2–4: samples of yellows-diseased celosia plants. Fragment sizes: control fragment $\approx$ 800 bp; amplified celosia DNA $\approx$ 950 bp. (B) *Mse*I restriction analysis of the nested CX fragments. Lane 1: positive control; lanes 2, 3, and 5: samples of yellows-diseased celosia plants (lane 4: empty). Size range of fragments: 70–300 bp.

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