Phytopath. medit., 1996, *35*, 19-23

Detection of maize yellow stripe tenui-like virus by ELISA and dot-blot tests in host plants and leafhopper vector in Egypt

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Summary. Antisera to the nucleoprotein of maize yellow stripe tenui-like virus (MYSV) were produced and used for detection of this virus in several host plants and vector leafhoppers in Egypt. Dot-blot and direct antigen coating (DAC) ELISA were used to detect MYSV in naturally or experimentally infected maize, wheat, barley, oats, and the graminaceous weeds *Bromus wildenowii, Cenchrus biflorus, Dichanthium annulatum, Digitaria sanguinalis, Echinochloa colonum, Setaria verticillata* and *S. viridis*. In maize leaves, differences in virus titer appeared to be correlated with leaf age and with MYSV symptom-types. Dot-blot and DAC-ELISA were used also to detect MYSV in naturally or experimentally infective leafhoppers (*Cicadulina chinai*).

Introduction

The nucleoprotein and non-capsid protein of the leafhopper-borne maize yellow stripe virus (MYSV), a tentative member of the tenuivirus group, have been recently purified and partially characterized (Ammar *et al.*, 1990a; Thouvenel *et al.*, 1995). MYSV symptom-types include fine stripe, coarse stripe, and chlorotic stunt; these symptom-types usually appear on different leaves of the same plant (Ammar *et al.*, 1990a). MYSV was detected, through symptomatology and insect transmission, naturally infecting maize, sorghum, wheat, barley (Ammar *et al.*, 1989, 1990a), and some graminaceous weeds in Egypt (Sewify, 1994). It is thought that wheat, barley and graminaceous weeds may play a role in the over wintering of both MYSV and its vector leafhopper, *Cicadulina chinai*. However, due to the lack of MYSV antisera, epidemiological studies on this disease have so far been very difficult and time consuming, depending mainly on the use of symptom observation and insect transmission for diagnosis. Thus, in the present work, we produced specific antiserum to the nucleoprotein of MYSV; and we used it, through ELISA and dot-blot methods, for detecting MYSV in several host plants and weeds as well as in the vector leafhopper *C. chinai*. Additionally, we report differences in the virus concentrations that are correlated with leaf age and with MYSV symptom-types.

Materials and methods

Sources and maintenance of virus and vector. The MYSV isolates and the cultures of the leafhopper vector *C. chinai* were the same as those used by Ammar *et al.* (1989, 1990a). MYSV was maintained in maize seedlings cv. SC 10 or DC 204 in a greenhouse by serial transmission with *C. chinai*. Non-infective leafhoppers were maintained on caged healthy maize plants that were checked regularly for virus symptoms.

Purification of virus nucleoprotein. The method used for purification of the MYSV nucleoprotein was as follows:

infected maize or sorghum tissue (100-200 g of leaves or stems) were blended in grinding buffer 0.2 M Tris/boric acid, Polyvinyl Pyrrolidone 2%, mercapto-ethanol 0.5% pH 7.0 (1 g/5 ml). After grinding for 3-5 min, the extract was filtered through cheesecloth and treated with 25% (v/v) chloroform. After shaking for 10 min, the emulsion was centrifuged at 7,000 rpm for 10 min. The clarified extract was centrifuged at 40,000 rpm for 2.5 h at 5°C (Type 55.2 rotor). The pellets were resuspended in pellet buffer (0.01M KH_2PO_4, 0.01M Ethylene diamine tetraacetic acid (EDTA), pH 7.0) and kept overnight at 4°C or directly dissolved, stirred and then layered onto 6 ml of 20% sucrose (w/v) in pellet buffer and centrifuged at 40,000 rpm for 3 h at 5°C. Pellets were resuspended in the pellet buffer and kept overnight at 4°C. The suspension was clarified and layered on 10-40% sucrose density gradients in phosphate buffer (0.01M KH_2PO_4, pH 7.0) and centrifuged at 25,000 rpm for 4 h at 5°C (Beckman SW 28 rotor). One zone was observed by scanning at 260 nm wavelength, diluted in distilled water and pelleted at 40,000 rpm for 2 h at 5°C then resuspended in phosphate buffer.

Production and titration of antiserum. Two New Zealand white rabbits were immunised by three weekly intramuscular injections of purified MYSV nucleoprotein emulsified with an equal volume of Freund's complete adjuvant for the first and second injections, and with incomplete adjuvant for the third injection. Blood was collected on the 10th day after the third injection.

The titre of the antiserum produced to the virus nucleoprotein was estimated by agar gel double diffusion tests (titre 1/64). Infected maize tissue was used as antigen. It was ground in 0.9% NaCl (1g/10 ml), and squeezed through two layers of cheesecloth. The antiserum was first cross-absorbed by mixing with healthy maize leaves extract, and then centrifuged at 7,000 rpm for 10 min. The pellet was discarded and the supernatant used as antibodies fraction.

Enzyme-linked immunosorbent assay (ELISA) test. A direct antigen coating (DAC) ELISA was used as described by Converse and Martin (1990) with a few modifications. Test samples were ground with a homogenizer in coating buffer pH 9.6 (1 g/10 ml) applied to the microtiter plate and incubated overnight at 4°C. Wells were washed with PBS-Tween buffer 3 times, then a blocking agent (5% non fat dry milk powder) was applied at room temperature for 30 min. Antiserum was then added at the dilution of 1/1,000 in PBS-Tween buffer pH 7.4 containing 2% polyvinyl-pyrrolidone-40 (PVP-40) and incubated for 3 h at 37°C, or overnight at 4°C. The plate was washed, dried and the conjugate to anti-rabbit alkaline phosphatase, diluted at 1/2,000 in conjugate buffer containing ovalbumin 0.2 g/l in PVP-PBS-Tween pH 7.4 was added and incubated 3 h at 37°C or overnight at 4°C. The plate was then washed and dried. The p-nitrophenyl phosphate (PNPP) substrate was dissolved in substrate buffer pH 9.8 (1 mg/ml). Absorbance values were measured at 405 nm wavelength. Absorbance values more than twice those of healthy controls were considered positive.

Dot-blot immunoassays of plants and leafhoppers. The method used was that of Lin *et al.* (1990) with a few modifications. For some experiments nylon membrane (0.45 µm pore-Amersham), or preferably nitro-cellulose membrane (0.20 µm-Bio Bind-NC-Whatman) was directly used. Tissue extract (0.1 g infected maize plant/1 ml buffer or 1 insect/10 µl buffer) were applied on the membrane after grinding in PBS buffer, pH 7.3 followed by clarification. The membrane was dried for at least 5 min, immersed in PBS-Tween containing 5% non fat dry milk, incubated for 45 min at 37°C, shaken for 15 min. It was then washed with PBS-Tween, for 3 times by shaking it (2-5 min each), incubated with antiserum (cross-absorbed) at a dilution of 1/1,000 in PBS-Tween for 2 h at 37°C, or overnight at 4°C, shaken for 15 min, then washed as above. The membrane was later incubated with conjugated anti-rabbit alkaline phosphatase diluted at 1/2,000 in PBS-Tween for 2 h at 37°C, washed as above and then incubated with substrate containing 30 µl BCIP, 30 µl NBT/15 ml substrate buffer, pH 9.8. The membrane was washed by distilled water to stop the reaction. All incubations were done in the dark.

Host plant studies. The above mentioned serological methods were used to detect MYSV in naturally or experimentally infected winter host plants and graminaceous weeds. During october 1990, june 1991 and april 1993, diseased weeds showing symptoms similar

to those of MYSV were collected from the farm of the Faculty of Agriculture, Cairo University, at Giza, Egypt. These weeds were: *Digitaria sanguinalis* and *Setaria viridis.*

Additionally, the following plants were experimentally inoculated, by feeding on them MYSV-infective leafhoppers for 1-7 days (5 insects/plant): maize (*Z. mays* L.), wheat (*T. sativum* L.), barley (*Hordeum vulgare* L.), oats (*Avena fatua* L.), and the graminaceous weeds *Bromus wildenowii, Cenchrus biflorus, Dichanthium annulatum, Echinochloa colonum* and *Setaria verticillata.* These plants were checked serologically for virus presence 8 weeks after the inoculation.

The virus titer was assessed by DAC-ELISA in different parts of infected maize plants (leaves, stems and roots) as well as in leaves of different ages showing different symptom-types.

Attempts to separate MYSV symptom-types. Three types of MYSV symptoms that occur on naturally infected plants, i.e. fine stripe, coarse stripe and chlorotic stunt, were previously recorded (Ammar *et al.,* 1989, 1990). In a trial to separate these symptom types, healthy *C. chinai* leafhoppers were starved for 1-2 h before virus acquisition feeding for 15, 20, 30 or 35 min on pieces of maize leaves showing only one type of symptoms. Insects were then transferred to healthy maize seedlings for 1 week to allow for virus incubation in the vector. Leafhoppers were then placed on maize seedlings (10 insects per plant) for 1-2 wks inoculation tests. The symptom-type was recorded on each test plant 2-3 weeks later.

Results

Serological tests on plants. MYSV was detected by either DAC-ELISA or dot-blot in naturally infected maize and graminaceous weeds *Digitaria sanguinalis* and *Setaria viridis.* It was also detected serologically in experimentally inoculated maize, wheat, barley, oats, and in the graminaceous weeds *B. wildenowii, C. biflorus, D. annulatum, E. colonum* and *S. verticillata.*

In DAC-ELISA, MYSV antiserum had a titer of 10^{-5} with leaf extracts or purified virus (O.D. 1.39) diluted to 10^{-3} (Fig. 1). In dot blot tests, the titer for MYSV antiserum was 10^{-4}.

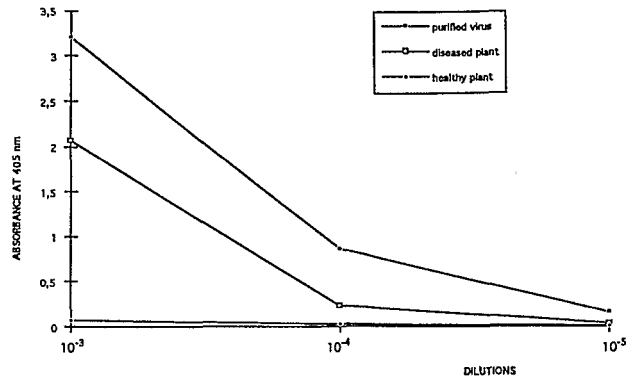

Fig. 1 - Detection of MYSV in maize plants using Direct Antigen Coating - ELISA method. Antigen was diluted (extract and purified virus - 0,4 O.D. -) in coating buffer. Absorbance values were recorded after 30 min.

Detection in the leafhopper vector. Using DAC-ELISA and the MYSV antiserum, the virus was detected in clarified extracts of infective *C. chinai* leafhoppers at a dilution of 10^{-3} (Fig. 2). Using the dot-blot method, MYSV was detected in clarified extracts of single infective insects at the dilution of 1/4 (single insects).

Correlation of MYSV titer with leaf age and symptom-type. The three types of MYSV symptoms, i.e. fine stripe, coarse stripe and chlorotic stunt usually appear at this order on different leaves of MYSV-infected maize plants. However, the chlorotic stunt type usually appears only on naturally-infected field plants. Attempts to separate these

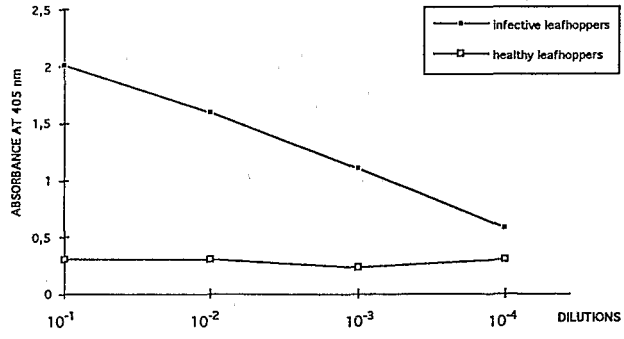

Fig. 2 - Detection of MYSV in the leafhopper vector using Direct Antigen Coating - ELISA method. Antigen was diluted (extract) in coating buffer. Absorbance values were recorded after 30 min.

symptom-types through short acquisition access period (AAP) of 15, 20 and 30 min of the leafhopper vector *C. chinai* on leaf pieces showing only one symptom-type were unsuccessful. No transmission occurred following the 15 min AAP, whereas both fine and coarse stripe resulted from longer AAP on leaves showing fine stripe, coarse stripe or chlorotic stunt.

Using DAC-ELISA with MYSV nucleoprotein antiserum, MYSV titer was assessed in different parts of infected maize plants including stems, roots, and leaves of different ages showing different symptom types. As indicated in Fig. 3, MYSV titer was higher in both leaves and stems then in the roots of infected maize plants. Also, virus titer was higher in younger leaves showing coarse stripe, than in older ones showing fine stripe.

Discussion

Previous studies on MYSV had to rely mainly on symptomatology and the laborious and time consuming insect-transmission tests for identification of this virus in different host plants in Egypt (Aboul Ata and Ammar, 1985; Ammar *et al.*, 1984, 1987, 1989; Sewify, 1994). Production and use of specific antiserum to the nucleoprotein of MYSV in the present work will facilitate further epidemiological studies on this virus and its vector in Egypt.

Furthermore, it will help to identify this virus or other viruses serologically related to it in other maize-producing countries particularly in Africa or in the Middle East. In our study, both DAC-ELISA and dot-blot methods were successful in detecting low concentrations of MYSV in host plants or in leafhopper vectors. DAC-ELISA is more economic and less complicated than other ELISA methods and is thus more useful in field surveys. However, the dot-blot method may be more sensitive for detecting MYSV in single leafhoppers. Either method can be used for future studies to determine if MYSV replicates in its leafhopper vector, which is a crucial characteristic for the classification of MYSV as a tenuivirus (Gingery, 1988; Ammar *et al.*, 1990a).

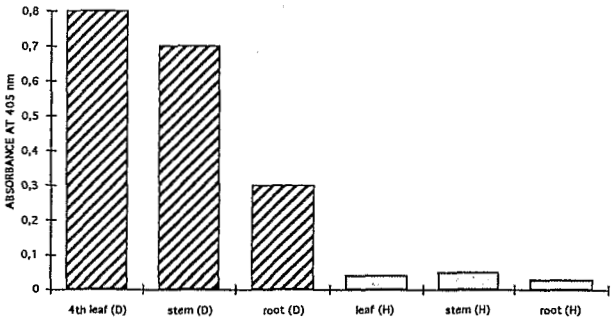

Fig. 3 - Detection of MYSV in different parts of the same plant by Direct Antigen Coating-ELISA method. Absorbance values were recorded after 30 min.

In the present study, using either DAC-ELISA or dot-blot serological methods, we confirmed and added to the previously recorded host range of MYSV. In Egypt, MYSV has been found naturally infecting maize, sorghum and wheat plants (Ammar *et al.*, 1990) as well as some graminaceous weeds (Sewify, 1994). Furthermore, MYSV can experimentally infect barley (Ammar *et al.*, 1989), oats and several other graminaceous weeds. It has been suggested that wheat, barley, and some graminaceous weeds may favour the overwintering of MYSV and its leafhopper vector in Egypt (Ammar *et al.*, 1989; Sewify, 1994), but further work is still required.

The three symptom-types associated with MYSV, i.e. fine stripe, coarse stripe and chlorotic stunt, usually appear in this order on successive leaves of the same maize plant (Ammar *et al.*, 1989; 1990a). These symptom-types could not be separated using short acquisition feeding periods of the vector (Ammar *et al.*, 1989; and present study). Electron microscopy of thin sections of maize leaves revealed no differences in the cytopathology and ultra structure of leaves showing any of these symptom-types (Ammar *et al.*, 1990a). Here in DAC-ELISA tests, MYSV titer was higher in younger maize leaves showing coarse stripe, than in older leaves showing fine stripe. These results suggests that MYSV symptom-types may be correlated with both leaf age and virus concentration in maize leaves. It is possible that the first leaves to be infected may get lower concentration of MYSV and thus develop fine stripe, whereas younger leaves may get higher concentration of virus and thus develop coarse stripe or chlorotic stunt. However, more serological studies, that take in consideration the plant and leaf ages during infection under field as well as greenhouse conditions, are needed to substantiate and expand this hypothesis further.

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Accepted for publication: October 18, 1995

Volume XXXV - No. 1 April 1996

PHYTOPATHOLOGIA MEDITERRANEA

FOUNDED BY

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PRINTED WITH A FINANCIAL SUPPORT OF THE "CONSIGLIO NAZIONALE DELLE RICERCHE" ROMA, ITALY

ISSN = 0031-9465

MEDITERRANEAN PHYTOPATHOLOGICAL UNION