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Occurrence of fig mosaic virus in Egypt

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Different patterns of chlorotic to yellowish mottling, mosaic spots and deformation were observed in leaves of field grown figs. The causal virus was identified as Fig mosaic virus (FMV). Electron microscope observations of thin-sectioned tissues from symptomatic leaves or from purified preparations showed double membrane bodies (DMBs) characteristic for FMV particles, which belong to family of Bunyaviridae. FMV infection also caused increases in H_2O_2 and malondialdehyde (MDA) contents. These results suggest that FMV infection causes oxidative stress in fig leaves leading to the development of epidemiological symptoms.

Keywords: Bunyaviridae, FMV, FMV

Introduction

The first critical study of fig mosaic disease (FMD) was conducted by Condit and Horne (1933). The disease has been widespread in several fig growing countries (Alhudaib, 2012). Although it was not sap- or seed-transmissible (Martelli *et al*., 1993; Elbeaino *et al.*, 2006), successful transmission of the disease by an eriophyid mite, *Aceria ficus* Cotte, has been reported by Flock and Wallace (1955). The aetiology of the disease is still uncertain. The disease was thought to be of viral origin until ultrastructural observations revealed the occurrence of intracytoplasmic enveloped spherical bodies in infected fig cells (Bradfute *et al*., 1970; Plavsic and Milicic, 1980; Appiano *et al*., 1990). In the following years, the agents of the disease were called "disease associated bodies" (DABs), which differed in shape and size (Martelli et al., 1993 and Elbeshehy, 2009). Putative potyviruses were reported from Croatia (Grbelja, 1983), and then the pathogen was assumed as a member

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of the *Potyviridae* family by Brunt *et al*. (1996). Some double membrane-bound bodies (DMBs) (Elbeaino *et al*., 2009a) and rod shaped virus particles (720 nm in size) showing a tail of 230 (Serrano *et al*., 2004), which might responsible for the disease, were described as possible agents of the disease. Complete nucleotide sequence of four RNA segments of fig mosaic virus (Elbeaino *et al*., 2009b). Double stranded RNAs (dsRNA) with a size ranging from 0.6 to 6.6 kb were obtained from infected trees in Turkey (Acıkgoz and Doken, 2003).

Recently Elbeaino *et al*. (2006) showed that figs host a putative closterovirus species, for which the name of *Fig leaf mottle-associated virus* (FLMaV) is proposed. Then the same agent was reported from Tunisia (Nahdi *et al*., 2006) and Egypt (Fayes and Mahmoud, 2011). A second member of the *Closteroviridae* from the mosaic-diseased fig was identified and named *Fig leaf mottle-associated virus 2* (FLMaV-2) (Elbeaino *et al*., 2007). Objectives of the present study were identification the putative agent of fig mosaic disease, also, effect of viral infection on physiological parameters of fig leaves.

Materials and methods

Plant samples

Symptomatic leaves showing different patterns of chlorotic to yellowish mottling and deformation were collected from fig-grown at Baltim region, Kafr El-Sheikh Governorate, Egypt.

Transmission electron microscopy (TEM)

Fresh leaf samples were fixed in 3% glutaraldehyde prepared in 0.05 M phosphate buffer (pH 7), at field sites for 3 h. Samples were rinsed several times in 0.05 M phosphate buffer and then were post fixed with 1% OsO4 in 0.05 M phosphate buffer for 2 h. Samples were rinsed several times with 0.05 phosphate buffer and taken to the laboratory. Samples were then dehydrated in a gradient acetone series, and embedded in Spurr's (1969) medium. Ultrathin sections (60–70 nm thick) were stained with uranyl acetate and lead citrate. Specimens were viewed with a Jeol-1010 transmission electron microscope at 100 kV (Unit of Electron Microscopy at Sohag University, Egypt).

Purification

Fig leaves exhibited the characteristic symptoms were collected and used as a source for virus purification. One hundred gm of leaf tissues were

homogenized in 0.1 M phosphate buffer pH 7.0 containing 0.5 % 2-mercaptoethanol, 200 µg bentonite per millilter and 0.01 M EDTA (3 ml of buffer for each gm of plant) according to (Ahemaidan, 2000). The juice was stirred overnight with gentle agitation, squeezed through two layers of cheesecloth, then clarified with 1/3 volume cold chloroform and centrifuged for 10 minutes at 8000 in a Beckman J-21C centrifuge using JA-20 rotor. The virus suspension was stirred for a minimum 1 hour, then centrifuged for 10 minutes at 1000 rpm in a Beckman J-21C centrifuge using JA-20 rotor. The supernatant was resuspended in 2 ml pad of 20% sucrose (w/v) followed by ultracentrifugation for 3 hr at 23,000 rpm in a Beckman Ti25 rotor. Pellet was resuspended in 0.01M phosphate buffer pH 7.0 containing 1% Triton X-100 (v/v) and stirred on ice for 30 minutes, followed by a low speed centrifugation at 8000 rpm for 10 minutes. One ml of suspension was layered over the sucrose gradients (10-40%) and centrifuged at 40,000 rpm for 3.5 hr in a Beckman SW 60TI. Virus band was collected from the sucrose gradient using a syringe, diluted in 0.01 M potassium phosphate buffer, pH 7, centrifuged at 45,000 rpm for 90 min, then pellet resuspended in 0.01 M potassium phosphate buffer then measured spectrophotometrically and examined by electron microscopy (EM).

Negative staining and TEM

Microscope grids coated with baralodion films were floated for 1 min on droplets of purified suspensions, then, excess fluid was removed with filter paper. The grids were floated again for 1 min on droplets of 2% phosphotungestic acid-stain pH, 7 as described by Noordam (1973). After air drying, grids were viewed with electron microscopy a Jeol-1010 transmission electron microscope (Unit of Electron Microscopy at Sohag University, Egypt).

H_2O_2, MDA contents and antioxidant activity

H_2O_2 content was determined colorimetrically as described by Jana and Choudhuri (1981). H_2O_2 was extracted by homogenizing 0.5 g leaf tissue with 3ml phosphate buffer (50 mM, pH 6.5). The homogenate was centrifuged at 6000 rpm for 25 min. To determine H_2O_2 level, 3 ml of extracted solution was mixed with 1ml of 0.1% titanium sulfate in 20% H_2SO_4. The mixture was then centrifuged at 6000 g for 15 min. The intensity of the yellow color of the supernatant at 410nm was measured. H_2O_2 level was calculated using the extinction coefficient (E = 0.28 µmol cm^{-1}). Lipid peroxidation was expressed as malondialdehyde (MDA) content and was determined as 2thiobarbituric acid (TBA) reactive metabolites according to Zhang (1992). Lipid peroxidation was expressed as nmol (g fresh weight)$^{-1}$ by using an extinction coefficient (E =

155mM cm^{-1}). Antioxidant activity (AOA) was calculated according to Arabshahi et al. (2007) and expressed as percentage inhibition of lipid peroxidation relative to the control using absorbance at 532nm obtained from MDA analysis according to the following equaion:

$$\text{AOA activity (\%)} = \frac{absorbance\ of\ control - absorbance\ of\ sample}{absorbance\ of\ control} x100$$

Results

Field observations and TEM

The most common symptoms observed on leaves were yellow mosaic spots, contrasting with normal green colour of the foliage. The margins of the yellow spots blended gradually from a light yellow colour into the dark green of healthy tissue. Later in the season, death of epidermal or sub-epidermal cells was appeared. Deformed leaves sometimes occurred on the same twig with normal leaves. Mosaic and necrotic spots on the fruit were very similar to those observed on leaves (Figure 1).

Fig. 1. Typical mosaic (left) and necrotic spots (right) on fig leaves.

FMV-infected cells (Fig. 2 left) contained structures like double membrane bodies (DMBs). DMBs has size (50 to 250 nm) and observed in leaves showing only yellow mosaic. Also, deformation on cell wall was

noticed. On the other hand, abnormal channels across cell wall seem to be modified plasmodesmata are shown (Fig. 2 right) in FMV-infected plants only.

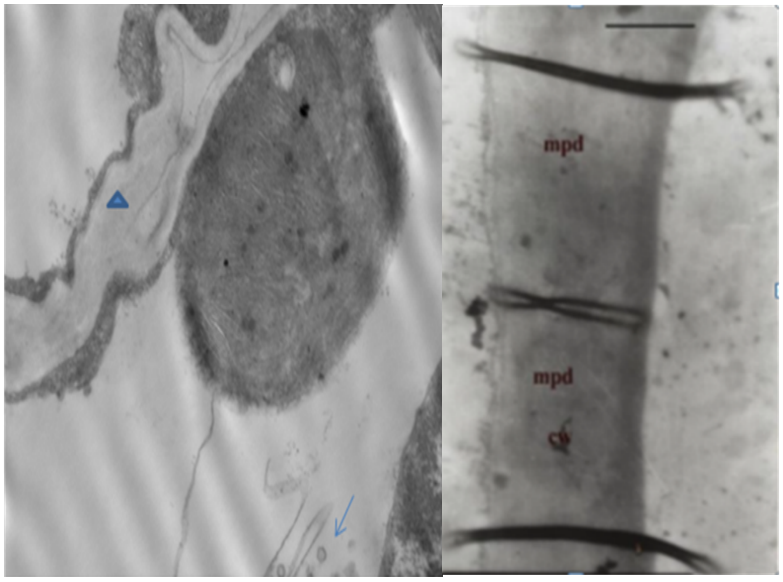

Fig. 2. Electron micrographs of portions of mesophyll cell. (Left) Double membrane bodies (DMBs) associated with fig mosaic symptoms (see arrow), with evident signs of plasmolysis and cell wall malformation (arrow head). (Right) Abnormal channels across cell wall seem to be modified plasmodesmata (mpd) are shown in FMV infected cells.

Purification and electron microscopy

Low virus concentrations were obtained in final preparations, this was clear when preparation were measured spectrophotometrically. Although, final preparations contained a substantial amount of plant contaminates, DMBs, round to ovoid (50 to 200 nm in diameter) (Fig. 3) were showed in purified preparation extracted from tissues having yellow mosaic and malformation.

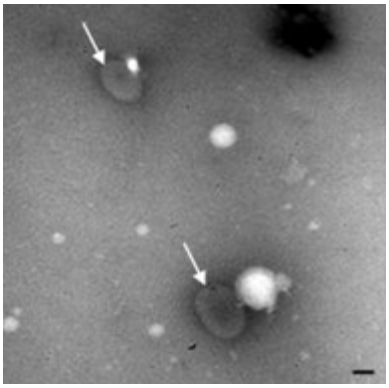

Fig. 3. Purified preparations of FMV, which showed as double membrane bodies (DMBs). Scale bars = 50 nm

H_2O_2 and MDA contents

Due to FMV infection, MDA and H_2O_2 concentrations increased significantly in fig leaves. The AOA of leaf extracts were calculated on the basis of relative amounts of MDA, which indicated lipid peroxidation, with respect to control MDA concentration. In response to virus infection, an elevated level of lipid peroxidation was observed in infected leaves. This increase is 38.8% as compared to that of healthy leaves (Table 1).

Table 1. Effect of FMV infection on H_2O_2 concentration (mmol g^{-1} FW) and MDA content (nmol g^{-1}FW) of fig leaves

Treatments	H_2O_2 (Nmol g^{-1} FW)	MDA (µmol g^{-1} FW)	AOA
Healthy	9.36	7.96	
Infected	16.28	11.05	- 38.8

Statistical significance of differences compared to healthy: **, significant at < 0.01.

Discussion

Different patterns of chlorotic to yellowish mottling and deformation were observed in leaves of fig grown at Kafr El-Sheikh Governorate. These results confirm results obtained earlier by several investigators (Harry and Burnett, 1960, Kitajima *et al*., 2003, Elbeaino *et al*., 2006, Elbeaino *et al.*, 2007 and Castellano *et al*., 2007). FMD is widespread in the Mediterranean basin, and different types of symptoms can be commonly seen in infested fig plantations. The cytopathological patterns observed in many studies revealed that disease was caused by double membrane-bound bodies (DMBs) (Martelli *et al*., 1993; Çağlayan *et al*., 2009). The similar structure was also observed in

this study. DMBs were surrounded by a fibrillar matrix in most of the infected cells, similar to some animal viruses (Appiano *et al*., 1990). These bodies were only observed in chlorotic leaves. It was previously reported that FMD can be transmitted by vegetative propagation material and an eriophyd mite, *A. ficus* (Flock and Wallace, 1955), but not by seeds (Martelli *et al*., 1993). In this case, FMD must have been transmitted into these seedlings by eriophyd mites.

Induction of H_2O_2 was recorded in our experiments under FMV infection. Formation of H_2O_2 in the cells during disease development showed previously by (Clarke *et al.*, 2002). Oxidative stress can be demonstrated by MDA concentration which is considered a general indicator of lipid peroxidation (Chaoui *et al*., 1997). Generally, high MDA contents were detected in FMV-infected leaves. Increased lipid peroxidation can be observed both in plants with mosaic symptoms and in plants showing leaf-yellowing and malformations. In other words, lipid peroxy-radicals are known to oxidize several pigment molecules leading to bleaching of these pigments. This co-oxidation of pigment molecules might be responsible for yellowing of virus invaded tissues. Kluge *et al*., 1995, reported that yellowing of virus infected leaves of *Beta vulgaris* has been attributed to this co-oxidation. The present results indicated presence FMV and suggested further experiments via PCR to confirm the virus definition. Also, application of exogenous materials (such as salicylic acid), which could induce plant defense mechanisms against FMV infection through antioxidant system.

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