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OCCURRENCE OF MULBERRY MOSAIC VIRUS IN EGYPT

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Abstract- Mosaic, yellowing and malformation symptoms were detected on leaves of mulberry plants (*Morus spp.* L.), grown in the Experimental Farm of Faculty of Agriculture, Ain Shams University, Cairo, Egypt, during the spring of 2012. Viral assay experiment which carried out on sensitive host (*Phaseolus vulgaris*), resulted in appearance of virus-like symptom. The detected virus was found to have a narrow host range. Electron microscopy studies of ultrathin sections of healthy and infected mulberry leaves revealed that the detected virus affected the plant cell structure of the diseased plants. The viral particles were icosahedral in symmetry and double capsid of 57 nm in diameter and the diameter of the inner core was found to be 42 nm. The isolated mulberry mosaic virus is a tentative member of the Genus *Begomovirus* in the Family *Geminiviridae*. Isozyme polymorphism in the healthy and infected plants was studied. Differences were detected in the ideogram of peroxidase, α-esterase and β-esterase isozymes of the infected plants relative to that of the healthy plants. According to the available literature, this may be the first report of mulberry mosaic virus in Egypt.

Keywords- Mulberry mosaic virus, Transmission electron microscope, Cytopathic effect, *Geminiviridae*, Isozyme polymorphism

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Introduction

Mulberry is distributed in a tropical, subtropical, temperate and subarctic zones [1], and it is widespread in Egypt [2]. At the beginning of spring, mulberry eatable fruits, with a variety of colors: white, red, pink and even black, ripen and silkworm's larvae feed on its leaves. It has been reported that mulberry plants are susceptible for different viral diseases, such as mulberry ring spot *Nepovirus* [3]; mulberry latent *Carla virus* [4]; yellow streak virus [5]; mosaic dwarf viroid-like disease [6,7], hop stunt viroid [8], mulberry badna virus 1 [9].

Analysis of isozyme is a powerful biochemical technique with numerous applications in plant pathology and studying of population genetics of fish, insects, nematodes, mammals, and higher plants [10-12]. Isozymes are multiple molecular forms of a single enzyme. These forms usually have similar enzymatic activities, but different isoelectric points due to the presence of different amino acid composition as a result of the differences in nucleotide sequence of the DNA that code for their protein. Often the only difference among isozymes is the substitution of one to several amino acids [10].

Isozymes have been reported to be useful in defining systematic phylogentic relationships and to assess the genetic diversity between taxa [13,14]. Enzyme polymorphism has been successfully used to assess genetic variability in many plant species.

This investigation was carried out to identify a virus isolated from naturally infected mulberry trees showing mosaic, yellowing and malformation symptoms. Also, host range, particle size and morphology; thermal inactivation point, dilution end point and longevity *in vitro* of the isolated virus as well as cytopathic effects and isozyme polymorphism were studied.

Materials and Methods

Seeds and Growth of Seedlings

Seeds of cucumber (*Cucumis sativus*), tomato (*Lycopersicon esculentum*), zucchini (*Cucurbita pepo*), white and pinto beans (*Phaseolus vulgaris*), *Datura stramonium*, *D. metel*, *Nicotiana tabacum*, *N. glutinosa* and *Chenopodium amaranticolor* were obtained from Microbiology Dep.- Faculty of Agriculture, Ain Shams University and sown in plastic pots with diameter of 25 cm and containing 500 gm of sterile clay soil. Five seeds were sown in each pot, then pots were placed on a bench in the green house at 25 to 28ºC and watered as required.

Sample Collection

Fifty symptomatic leaf samples were collected during the spring of 2012 from mulberry trees grown at the Experimental Farm of Faculty of Agriculture, Ain Shams University, Cairo, Egypt and kept in sterile plastic bag then maintained at -20ºC for further analysis.

Plant Inoculation

Seedlings of the different plants were mechanically inoculated through leaves as follows: frozen diseased leaves were ground in extraction buffer (0.2 M phosphate buffer pH 7 containing of 0.4% mercaptoethanol) in sterilized mortar and pestle. The extracts were filtered through two layers of sterilized cheese cloth. Five replica of tested seedlings were dusted with 600-mesh carborundum and inoculated with cotton swabs which had been dipped in the inoculums. About 5 min. post-inoculation, leaves were rinsed with distilled water. Controls of corresponding seedlings were inoculated with the extraction buffer alone [15].

Virus Isolation

Five grams of diseased mulberry leaves were homogenized in 0.2 M phosphate buffer (pH 7) containing 0.4% (v/v) β-mercaptoethanol. Two weeks old *P. vulgaris* seedlings, the most sensitive indicator plant to mulberry viruses [4,16] were used to test the presence of the virus. Seedlings were mechanically inoculated with 500 µl sap extract of diseased leaves as previously described and kept at moderate temperature (25-28°C) in an insect proof greenhouse. Plants were daily inspected for symptoms appearance.

Host Range

Seedlings of 10 plant species belong to 4 families (*Chenopodiaceae*, *Cucurbitaceae*, *Leguminosae* and *Solanaceae*) were used as hosts. These plants are cucumber (*Cucumis sativus*), tomato (*Lycopersicon esculentum*), zucchini (*Cucurbita pepo*), white and pinto beans (*Phaseolus vulgaris*), *Datura stramonium*, *D. metel*, *Nicotiana tabacum*, *N. glutinosa* and *Chenopodium amaranticolor.* Five identical seedlings of each plant were mechanically inoculated through leaves with the extract of diseased mulberry leaves as previously described. Seedlings inoculated with the extraction buffer were considered as a control. All inoculated seedlings were maintained in an insect proof greenhouse until symptoms appeared.

Thermal Inactivation Point (TIP)

Eighteen Eppendorf tubes, each containing 1 ml of crude extract of diseased mulberry leaves were divided into 9 sets, each consisted of 2 tubes. One set was incubated in a water bath for 10 min at one of the following temperature degrees: 50, 55, 60, 65, 70, 75, 80, 85 and 90ºC. Then tubes were cooled under tap water and pathogenicity test was carried out by inoculating the treated extract into *P. vulgaris* seedlings as previously described [15].

Longevity *in Vitro* (LIV)

Eppendorf tubes each containing 1ml of crude extract of diseased mulberry leaves were kept at room temperature and the infectivity of the virus was tested every 12 h. for up to 7 days [15].

The Dilution End Point (DEP)

Serial ten-fold dilutions of infectious crude extract were prepared (*i.e.* from 10^{-1} to 10^{-6}). The infectivity of each dilution was assayed [15].

Study of Cytopathic Effects on Mulberry Plants by Ultrathin Section

Leaf blade segments (1 mm) were cut from healthy and infected leaves. The selected tissues samples were fixed and examined in Jeol-Jem 1010 transmission electron microscope using ultra-thin section technique as described by Luft [17].

Partial Purification and Morphological Characteristics

Five hundred grams of mulberry leaves which exhibited the mosaic symptoms were ground in 0.2 M phosphate buffer (pH 7) containing 0.4% (v/v) β-mercaptoethanol. The homogenates were strained through two layers of cheesecloth and clarified by mixing with two volumes of cold mixture of n-butanol and chloroform (1:1) followed by centrifugation at 4ºC and 6000 rpm for 15 min. Virus particles were precipitated by addition of 8% polyethanol glycol (MW 6000) and NaCl followed by centrifugation at 30.000 rpm for 90 minutes. Purification of virus partials was carried out according to Steere [18] with minor modification. The partially purified virus particles were stained with 2% (W/V) phosphotungstic acid for 15 seconds. The grids were air dried and examined in Jeol-Jem 1010 transmission electron microscope.

Isoenzyme Electrophoresis

One gram of each healthy and infected leaf tissues was homogenized with 0.9 M Tris-HCl buffer pH 7.9 containing 14 mM β-mecrcaptoethanol according to the method described by Tuzun, et al [19]. Each sample was vortexed for 15 sec. and the homogenates were centrifuged at 10000 rpm and 4°C for 20 min, then the supernatants were used as enzyme sources for one isozyme namely esterase (Est) using two different substrates, α and β-naphthyl acetate. The supernatants were then separated in 8% polyacrylamide gel electrophoresis (PAGE) according to Stegemann, et al [20]. After electrophoresis, the gels were individually stained according to gels staining protocols of Heldt [21] for peroxidase (Px) and Scandalios [22] for α and β-Est.; Gels were scanned using the Gel Doc-2000 Bio-Rad system.

Results

Symptomatology and Virus Isolation

During the spring of 2012, mosaic, yellowing and malformation symptoms were observed on the leaves of mulberry trees grown in the Experimental Farm of Faculty of Agriculture, Ain Shams University, Cairo, Egypt [Fig-1]. *Phaseolus vulgaris* was used as sensitive indicator host for mulberry viruses.

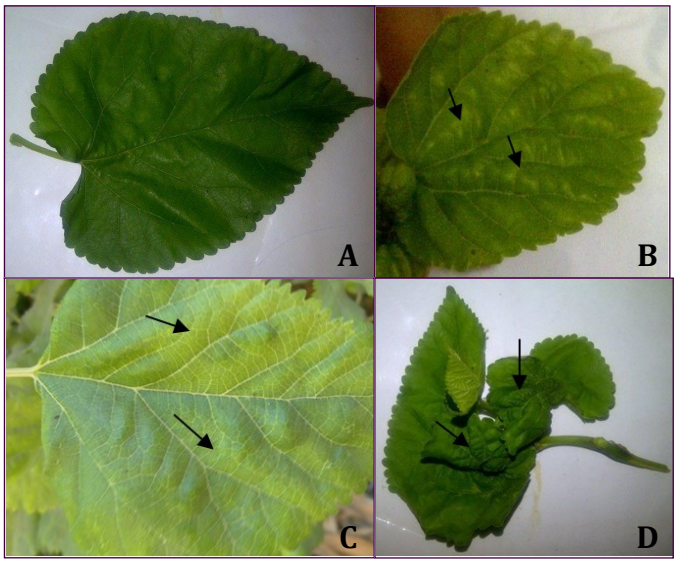

Fig. 1- Mulberry leaves (**A**) Healthy leaf (**B, C & D**) Naturally infected leaves showing mosaic, yellowing and malformation symptoms.

Two weeks old *P. vulgaris* seedlings were inoculated with extract of diseased mulberry leaves. After 21 days, local necrotic lesions, leaf crinkle and malformation symptoms were detected on the infected leaves [Fig-2].

Host Range

Ten plant species belong to four different families were used to determine the host range of the isolated virus using mechanical inoculation. The obtained results indicated that, among all the inoculated seedlings, the symptoms were observed only on white *P. vulgaris* after 21 days [Fig-2]. No symptoms were detected on the other tested plants.

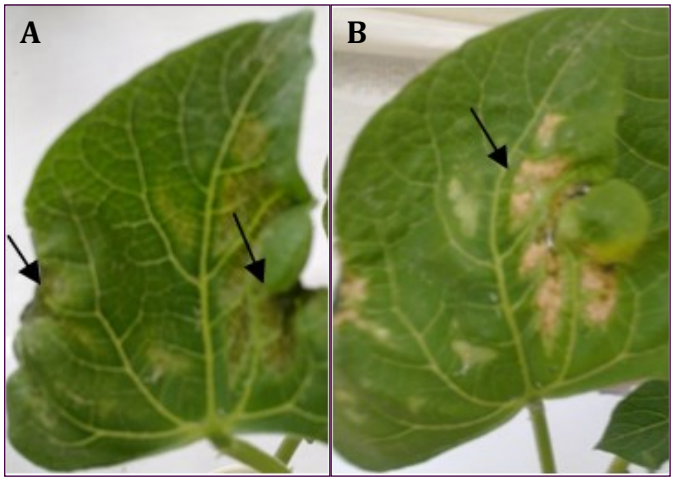

Fig. 2- Viral symptoms on sensitive host (*Phaseolus vulgaris*) showing local lesions, leaf crinkle and malformation

Properties of the Isolated Virus

Infectious crude sap that extracted from infected mulberry plant was used to determine the properties of the isolated virus. The dilution end point, longevity *In vitro* and thermal inactivation point of the isolated virus were found to be 10^{-4}, 4 days and 60ºC, respectively.

Cytopathic Effect of Mosaic Virus on Mulberry Plant Cells

In the electron micrograph, [Fig-3](A&B) the cell wall thickness, the nucleus and the photosynthetic lamella of the healthy mulberry leaf cells appeared normal. Whereas, the cells of infected mulberry leaves [Fig-4] showed highly thickening of cell wall [Fig-4](A), the cytoplasm appeared more aggregated, and tubular shape inclusions were observed [Fig-4](B). Virus particles were clearly shown [Fig-4](D) with formation of empty spaces [Fig-4](C,E&F) in the infected cells due to degeneration of internal cell components [Fig-4](F). The viral infection leads to whole decomposition of internal plant cell components and formation of inclusion bodies.

Particle Size and Morphology

Partially purified virus particles isolated from infected mulberry leaves were negatively stained with 2% (W/V) phosphotungstic acid and examined in electron microscope.

As shown in [Fig-5] the virus particle was found to be icosahedral of double capsid with diameter of 57 nm and the diameter of inner core was found to be 42 nm.

Isozyme Polymorphism

Two isozymes including esterase (Est) using two different substrates, α and β-naphthyl acetate and peroxidase (Px) were used to study the different gene/genes expression for healthy and virus-infected plants.

The resolved isozymes included 2 to 4 bands depending on the sample. [Fig-6](A) illustrated the results of peroxidase isozyme and its ideogram and the relative front value of each band was calculated and presented in [Table-1]. The results showed two bands at healthy sample as well as at the virus infected sample with relative front (Rf) 0.3 and 0.5, respectively. While the band with Rf 0.5 was demonstrated more density in the virus infected sample than in the healthy one.

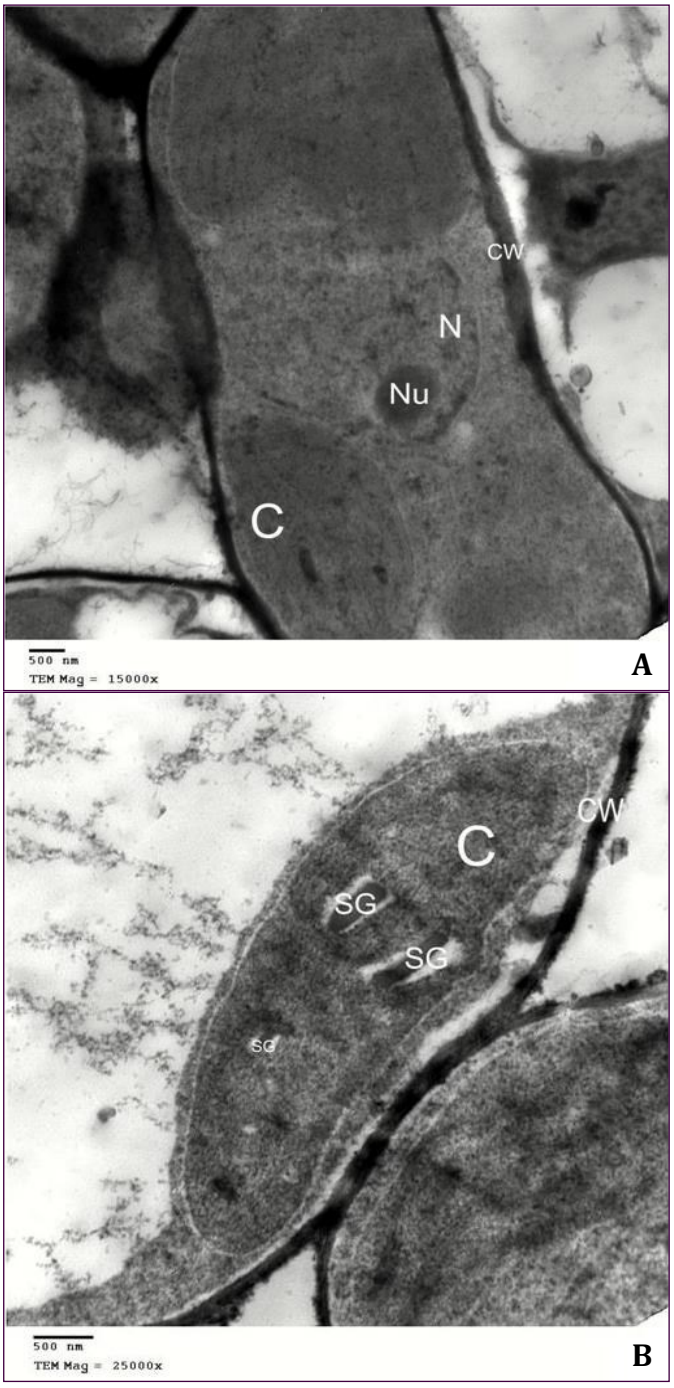

Fig. 3- Ultrathin section of healthy mulberry leaf tissue showing normal cell wall thickness & nucleus (A) and normal chloroplast (B).

CW: Cell Wall C: Cytoplasm SG: Storage Granule Nu: Nucleolus N: Nucleus

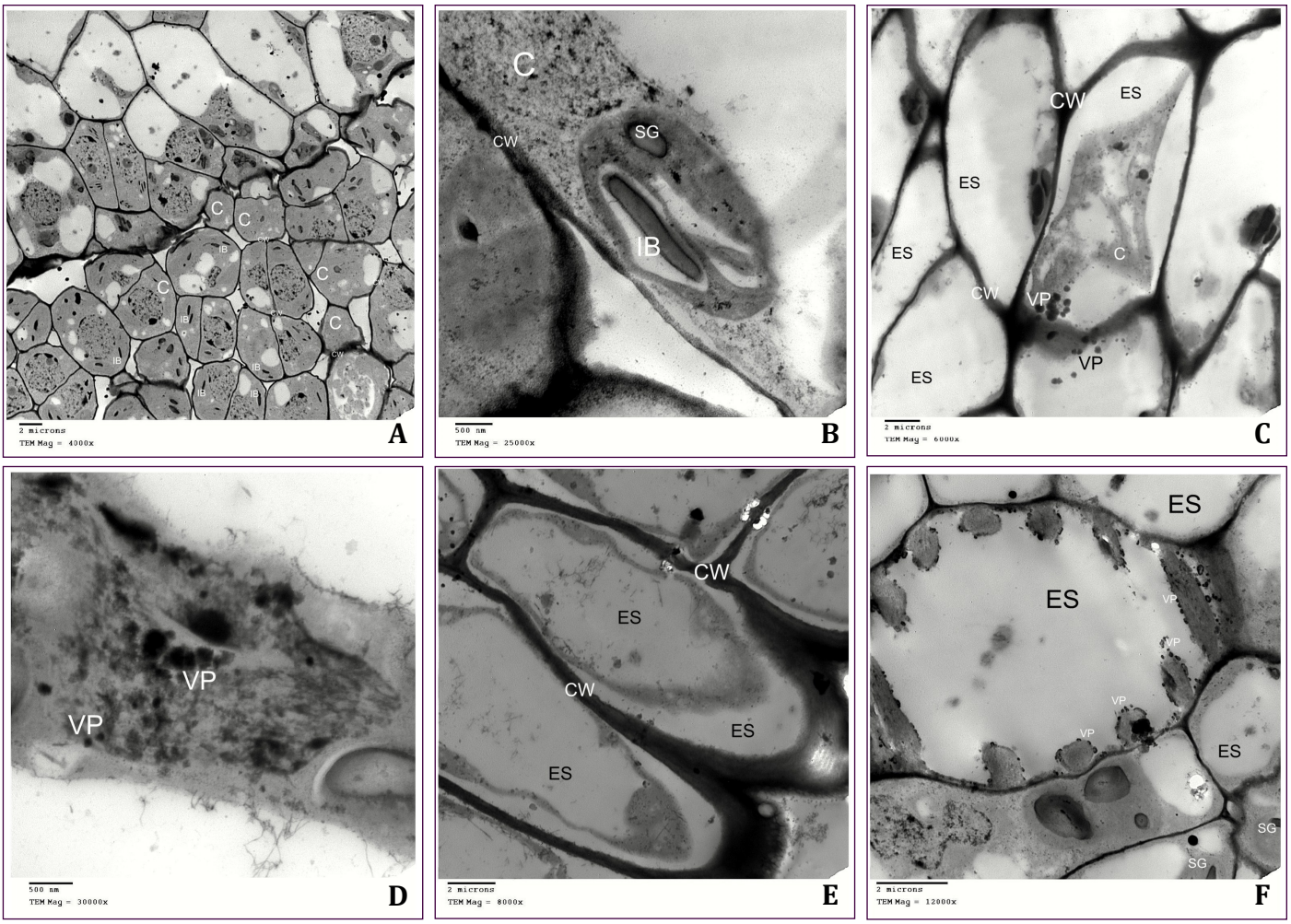

Fig. 4- Ultrathin section of infected mulberry leaf tissues showing increase in cell wall thickness (A & E), tubular viral inclusion bodies (B), cytoplasm aggregation (C), virus particles (D)and empty spaces (E & F).

CW: Cell Wall C: Cytoplasm SG: Storage Granule IB: Inclusion Body ES: Empty Space VP: Virus Particle

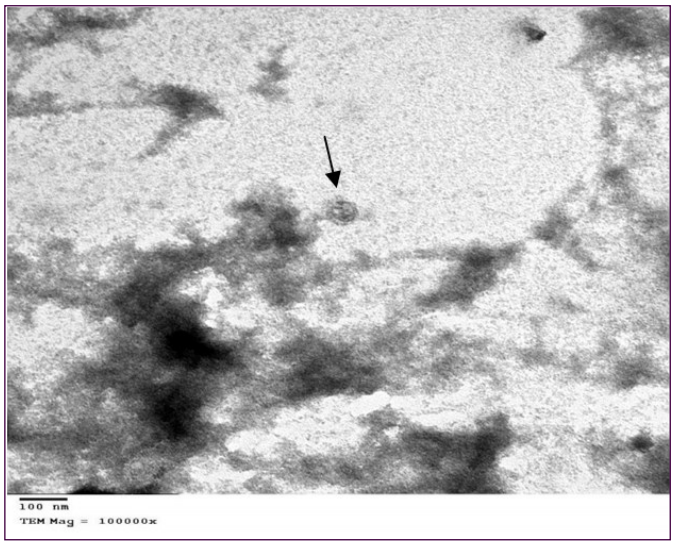

Fig. 5- Electron micrograph of mulberry mosaic virus. The viral particle is indicated by arrow

The electrophoretic patterns of α-esterase and β-esterase isozymes and their ideograms are presented in [Fig-6](B&C) and the corresponding Relative front (Rf) represented in [Table-1]. The results of α-esterase showed differences between that of the infected plants and the healthy ones. Three bands were with corresponding Rf (0.2, 0.6 and 0.8), were observed. Meanwhile, the three bands in the sample of the healthy plants showed lower density than in the sample of the infected ones. Although, the results of β-esterase showed differences between infected and healthy plant samples.

Table 1- Corresponding relative front of bands presented by peroxidase, esterases α-naphthyl acetate and β-naphthyl acetate

Isozymes Groups	Relative Front	Samples	
		Healthy (1)	Infected (2)
Peroxidase Groups	0.3	++	++
	0.5	-	+
α Esterase Groups	0.2	+	++
	0.6	-	++
	0.8	+	++
β Esterase Groups	0.2	+	+
	0.6	+	+
	0.7	-	+
	0.9	+	-

Four bands were demonstrated in both healthy and infected samples with corresponding Rf 0.2, 0.6, 0.7 and 0.9. Moreover, the band of virus-infected sample with Rf value 0.7 showed more density than healthy one. While, lower density in the virus infected plant sample than in the healthy one was observed in band with corresponding Rf value 0.9.

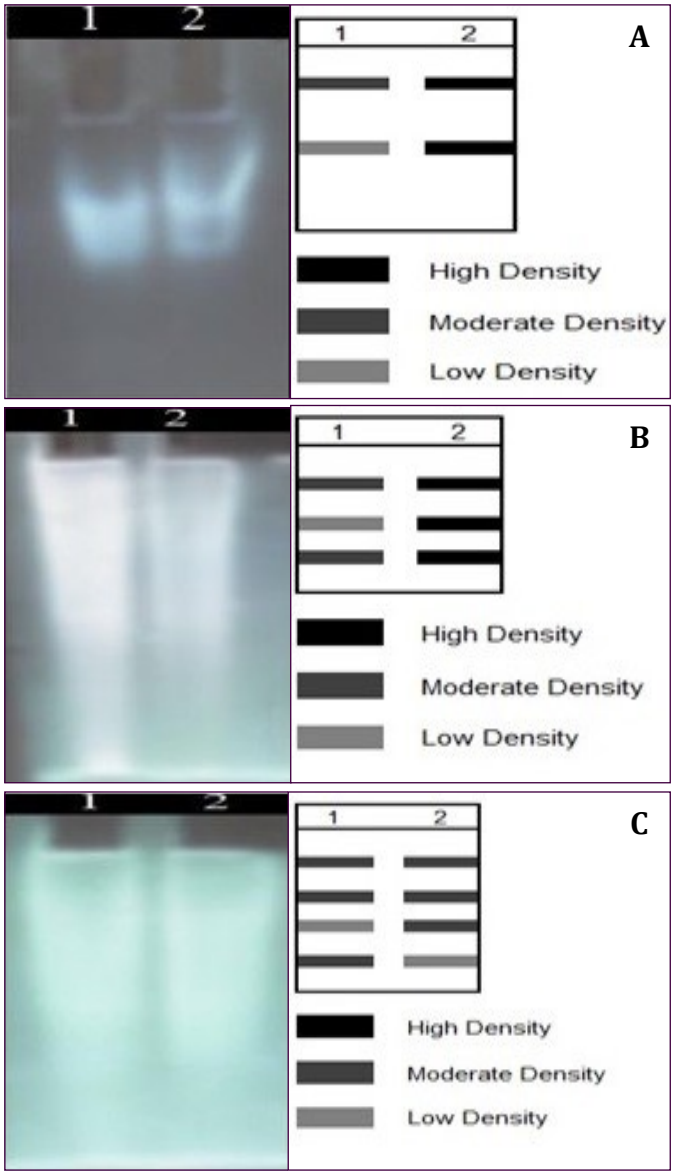

Fig. 6- Zymogram and Ideogram profiles of (**A**) peroxidase, (**B**) esterase α-naphthyl acetate and (**C**) esterase-β-naphthyl acetate among the healthy (1) and the virus infected (2) samples.

Discussion

Various virus-like symptoms including mosaic, yellowing and malformation were observed on mulberry trees grown in Egypt. The isolated mulberry mosaic virus was found to be of a limited host range as found in other mulberry ring spot virus [3]. The isolated virus proved to be transmitted mechanically. Only one plant species (*P. vulgaris*) has developed symptoms, among five plants tested, belong to different families. This could be due to the effect of the coat protein on host range and symptoms [23]. Changes in physiological and biochemical parameters in mulberry leaves due to the infection of mulberry mosaic symptom virus were evaluated. The thermal inactivation point, dilution end point and longevity *in vitro* were studied for the isolated virus and were found to be 60ºC, 10^{-4} and 4 days, respectively. These results are relatively in agreement with those obtained for other mulberry viruses; Mulberry ring spot *nepovirus* [3] and Mulberry latent *carlavirus* [4].

Electron microscopy examination of the infected mulberry sections demonstrated some changes in cell wall thickness, storage granules and cytoplasm as compared to those of the healthy ones. Tubular inclusion bodies were also found in the infected tissues. These results are in agreement with those obtained by Walkey & Webb [24].

The mulberry mosaic virus is icosahedral in morphology and double capsid with diameter of 57 nm and the diameter of the inner core was found to be 42 nm. Mulberry mosaic virus isolate is a tentative member of the genus *Begomovirus* in the family *Geminiviridae*. These results were relatively in agreement with those obtained by Sherry, et al [25].

Isozymes merely represent different structural configurations of the same polypeptide chain of an enzyme [26]. Ideogram pattern of electrophoresis for peroxidase isoforms of virus infected mulberry exhibited more density of peroxidase isozyme at Rf 0.5 than healthy plant. Which prove the peroxidase isozymes role in a variety of catalyzing reactions IAA catabolism, subrization of cell wall, lignification and H_2O_2 detoxification system in plant cell when the plant infected with pathogen [27,28]. While ideogram patterns of electrophoresis for α- esterase isozyme present three bands more density at virus infected plant than healthy one with corresponding Rf values 0.2, 0.6 and 0.8. Additionally, ideogram patterns for β–esterase figure out a variation between infected and healthy plants at band with corresponding Rf values 0.7 and 0.9. Which agreed with the result reported by Van Loon [29] and Baaziz [30].

Conflicts of Interest: Author declares no conflicts of interest.

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