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RESEARCH

Identification, characterization and ultrastructure aspects of *Alfalfa mosaic virus* infecting potato (*Solanum tuberosum* L.) in Egypt

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ABSTRACT

Background: *Alfalfa mosaic virus* (AMV) is only virus in the genus Alfamovirus and has very wide ho range among weed and crop plants which produces a variety of symptoms. It can cause problems in potat in some regions where vectors easily move into potato fields from reservoir host, particularly if a tube necrosis-causing strain is involved.

Objective: : The purpose of this study is to characterize biologically and serologically AMV infectin potato (*Solanum tuberosum* L.) in Egypt. Moreover, the study described the histological and cytologic effects of AMV infection in potato leaf cells.

Methods: Leaf samples were collected on the basis of visual symptoms from potato plants with yellow blotching symptoms, called "Calico" and leaf distortion. A sap-transmitted virus isolated from potato was biologically purified after three successive single local lesion passages onto *Chenopodium ammaranticolor* which reproduced prominent local lesions. The virus isolate was then propagated in potato Ditta cv. plants, The virus was identified on the bases of host range, symptomatology, transmission and serological diagnosis, in addition to the ultrastructural changes produced in potato leaf cells infected with AMV .

Results: Reaction of thirteen plant species and cultivars belonging to four families (*Amaranthaceae, Solanaceae, Fabaceae and Laminaceae*) to AMV infection was demonstrated**.** The presence or absence of the virus was verified by back inoculation onto healthy indicator host plant and/or ELISA test**.** AMV was readily transmitted by mechanical means and by *Myzus persicae* with percentage of 60 %. In addition to visible symptoms, infection with AMV also causes ultrastructural changes in potato leaf cells. Examination of epidermal strips of *N. tabacum* cv. White Burley using light microscope showed amorphous cytoplasmic inclusion bodies seemed to be attached to the nucleus from one or two sites, while those inclusions have never been observed in the epidermal stripes of healthy leaves. Electron microscopy, revealed cytological and histological changes induced by *Alfalfa mosaic virus* infection in potato leaves

Conclusion: In this work, *Alfalfa mosaic virus* (AMV) characterized and ultrastructure aspected of infecting potato (*Solanum tuberosum* L.) in Egypt illustrating important effect of AMV on potato plant. Finally our recommendions to use a ground zeolite alone as a potential and highly economic adjuvant in FMD vaccine for cattle.

Key words : Potato (*Solanum tuberosum*), *Alfalfa mosaic virus*, Host range, Transmission, ELISA, Electronmicroscope and Ultrastructural changes.

BACKGROUND

Potato (Solanum tuberosum L.) is the world's most important vegetable crop. Moreover, potato is, a staple food of the world's population, In 2104, four million and eight hundred thousand (4800,000) tons of tubers were harvested from 439855.8 feddans of potato grown in Egypt according to the FAO (2014), plants are infected by many viruses under field conditions (Al-Shahwan et al., 2002). Potato is a semi-perishable crop susceptible to many diseases and insect pests. About 30 viruses and virus like agents infect potato. These being systemic pathogens, are perpetuated through seed tubers and pose a major threat to potato production (Naik and Karihaloo, 2007). Virus diseases are numerous and almost contribute to cause great

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economic losses and are considered to be the major limiting factors of potato production. Among such viral diseases Alfalfa mosaic virus (AMV) was found to be widely distributed on potato plants. AMV is the type member of the genus Alfamovirus in the Bromoviridae family of plant viruses. AMV is a world-wide distributed virus (Jasper and Bos, 1980) with a very wide host range. In Egypt, AMV is one of the most important and widely distributed virus, appeared on naturally infected potato plants in several locations in Egypt, causing severe loses. Several authors isolated AMV from potato (El-Helaly et al., 2012). In this study, Alfalfa mosaic virus was isolated from naturally infected potato plants (Solanum tuberosum L.) grown in Badr Center in Behera Governorat, Egypt with symptoms as yellow blotching and bright mottling of potato leaves (calico). However AMV was considered as economically less important when compared to potato viruses, but was considered as one of the most prevalent viruses. Anatomic studies have abundant proof that the type of symptoms induced by viruses frequently reflects histological and cytological effects of virus induced in plants. Light microscopy is still important in study of histological abnormalities induced by viral infection.

MATERIALS AND METHODS

Isolation and identification

Source of the virus isolate:

Leaf samples were collected on the basis of visual symptoms from potato (*Solanum tuberosum* L.) plants cultivated in Bader Center, Behera Governorate, Egypt during the growing season of 2011-2012. The observed symptoms, yellow blotching, called "Calico" and leaf distortion.

Virus isolation and propagation:

Leaf samples showing symptoms suspected to be due virus infection were prepared for mechanical inoculation by homogenizing leaf tissues in 0.1M potassium phosphate buffer, pH 7.0-7.2 (1:3 wt/vol). The diagnostic study of the pathogen was done under greenhouse at 25-30c^{o} using standard methods for mechanically transmitted viruses (Noordam, 1973). Seedlings of healthy tested plants of *Solanum tuberosum*, *Nicotiana tabacu*m cv. White Burley, *Vicia faba* cv. Giza 2 and *Vigna unguiculata* cv. Baladi were mechanically inoculated. The virus under study was biologically purified after three successive single local lesion (Kuhan, 1964) passages formed onto *Ch. amaranticolor* which reproduced prominent local lesions. The virus isolate was then propagated in potato Ditta cv. plants. It was identified on the bases of host range, symptomatology, transmission, serological diagnosis, and histological and cytological studies as described below.

Virus identification:

Host range and symptomatology:

Ten seedlings from each of fifteen plant species and cultivars belonging to five families were mechanically inoculated by the virus isolate, additional 10 seedlings were left without inoculation as control treatment. Inoculated plants were kept under observation in insect proof greenhouse for 30 days and were periodically sprayed with insecticides to prevent virus contamination. Four weeks later, symptomless plants were assessed for latent infection on the local lesion host plant and/or ELISA test.

Modes of transmission:

a. Mechanical transmission:

Inoculum and mechanical inoculation were undertaken as mentioned above (virus isolation). All inoculated and healthy plants were kept in greenhouse conditions, observed and recorded the development of symptoms daily for 25 days and assayed by back inoculation and/or ELISA.

b. Insect transmission

Colonies of non-viruliferous aphids, *Myzus persicae* Sulz, the most common aphid species frequently observed on potato plants in the field were obtained from Insect Dep., Agri. Fac., Cairo Universty. The aphids were maintained on healthy faba bean plants in insect prof cages. The aphid culture was routinely checked by allowing a group of them to feed for one hour on healthy indicator plants. These plants remain healthy, thus proving that the tested aphids were virus-free during the course of study.

Non-viruliferous aphids were starved for 2h, given an acquisition feeding period of 2-4 min. on infected potato plants Ditta cv. and then transferred to healthy potato plants for an inoculation feeding period of 20 min. after which they were killed by spraying with insecticide (Tafaban 0.15%). Aphids used for control treatment received the same care except that they were feed on healthy plants. Ten aphids were used for each plant and ten seedlings were used for this experiment. Symptoms and percentage of transmission were recorded for a period of 30 days.

Serological detection:

a- The identity of the virus was confirmed by indirect-ELISA (Hampton *et al.*, 1990) using IgG specific for *Alfalfa mosaic virus* by Bioreba AG. The reaction was assessed by measurement at 405 nm in Vniskan ELISA reader. Reading greater than twice the value of healthy controls was considered positive.

Histological and Cytological studies

a. Light microscopy:

Inclusion bodies produced by *Alfalfa mosaic virus* in *Nicotiana tabacum* L. cv. White Burley after mechanically inoculation were visualized by light microscope. Epidermal stripes of both healthy and infected leaves were removed with forceps from under-side of leaves. The strips were dipped for 5 minutes in 5% Trediton X-100 solution. After that, strips were immersed in a stain mercuric bromo phenol blue stain containing $100mgl^{-1}$ bromo phenol and $10gl^{-1}$ mecuric chloride in 100ml distilled water for 15min. The treated strips were placed in 0.5% acetic acid for 15min., then washed in tap water for 15min. (Mazia *et al.*, 1953). Finally, strips were examined by light microscope (OptiKa B-353, Italy) with a camera (ATPEK) attached.

b. Semi thin and ultra-thin sections were prepared for presented the histological and cytological changes induced by *Alfalfa mosaic virus* infection in potato leaves. In regarding to histological study, the midrib was examined and small parts from the leaf blade of potato leaves of healthy and infected ones. Semithin sections (1 µm thick) were cut using Leica Ultracut UCT Ultramicrotome and stained with toluidine blue for 90 sec. then examined by previous light microscope. Specimens were examined by TEM according to the procedure described by John *et al.* (1966). Ultra-thin sections (50-80 nm thick) in Copper hexagonal mesh, 2.05-mm grids staining by double stains (Uranyl acetate 2% for 15 min. followed by Lead citrate for 15 min), Speciment were examind with a JEOL 1010 Transmission Electron Microscope at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University.

RESULTS

Isolation and identification:

Virus isolation and propagation:

The virus under study was isolated from naturally infected potato plants collected from Bader center, Behera Governorate, Egypt, showing the characteristic symptoms of AMV which included yellow blotching and bright mottling (calico) (Fig. 1b). It was biologically purified by single local lesions developed on *Ch. amaranticolor* leaves and then propagated in potato cv. Ditta. Identification was undertaken based on host range, modes of transmission, serological detections, in addition of histological and cytological studies.

Fig. 1: Naturally infected potato plants showed yellow blotching and bright mottling (b) and healthy (a)

Virus identification:

Host range and symptomatology:

Reaction of thirteen plant species and cultivars belonging to four families (*Amaranthaceae, Solanaceae, Fabaceae and Laminaceae*) to AMV infection is presented in Figs. (2).

The presence or absence of the virus was verified by back inoculation onto healthy indicator host plant and/or ELISA test. It is obvious that, plants tested for virus infection could be summarized as follows:

a- Plants reacted with systemic symptoms only: *Nicotiana tabucum* L. cv. White Burley; *Capsicum annuum* L. cv. Gedeon F1; *Pisum sativum* L.; *Medicago sativa* L.; *Glycine max* L. cv. Giza 111; *Solanum tuberosum* L. cv. Ditta.

b- Plants reacted with local and systemic symptoms: *Vicia fabae* L. cv. Balady and *Phaseolus vulgaris* L. cv. Giza 4, *Ch. amaranticolor* and *Ch. Qui*noa

c- Plants could not be infected with the isolated virus: *Vigna unguiculata* L. and *Ocimum basillicum* L.

Modes of transmission:

a- Mechanical transmission: AMV was readily transmitted by sap extracted from infected potato leaves. Calico symptoms were appeared on potato plants, whereas chlorotic local lesions and systemic chlorotic spots were developed on *ch. amaranticolor*.

b- Insect transmission: *Myzus persicae* was checked for ability to transmit the AMV isolate from infected potato seedling to healthy ones after acquisition feeding period of 2-4 min., the percentage of transmission was 60 %.

Fig. (2a-k) Symptoms of AMV on susceptible host range as follow:

a) Chlorotic local lesions and systemic chlorotic flecking on *Ch.amaranticolar*
b) Leaf distortion or crinkling on *Ch.quinoa*
c) *Solanum tuberosum* L. cv. Ditta is beginning of calico symptoms produced by AMV.
d) *Nicotiana tabacum* L. cv. White Burley showed systemic symptoms such as (mild mottle), bright chlorotic and vein-banding
e) *Lycopersicum esculantum* L. cv. Casel Rok and Hienz H8704 showed that bright yellow blotches with some mottle. Leaves eventually develop a bronze discoloration
f) *Datura metel* L. showed systemic and Necrotic spots which enlarged and spreading necrotic areas along the leaves, vein clearing and puckering symptoms
g) *Capsicum annuum* L. cv. Gedeon F1with mosaic folloed by deformation
h) *Vicia fabae* L. cv. Balady with black necrotic local lesions followed by stem necrosis
i) *Pisum sativum* L. showed mosaic, yellowing of leaves and death of the plant
j) *Phaseolus vulgaris* L. with chlorotic local lesions and systemic mild mosaic
k) *Medicago sativa* L. Hegazy reacted with yellow striks and mosaic

Serological detection:

Positive reaction was obtained between sap of infected potato leaves and AMV specific antiserum using indirect-ELISA technique. This result indicated that the virus under study is AMV

Histological and cytological studies

a- Inclusion bodies: Examination of epidermal strips of *N. tabacum* cv. White Burley using light microscope (Fig. 4) showed amorphous cytoplasmic inclusion bodies seemed to be attached to the nucleus from one or two sites (Fig. 4a), while these inclusions have never been observed in the epidermal stripes of healthy leaves (Fig. 4b)

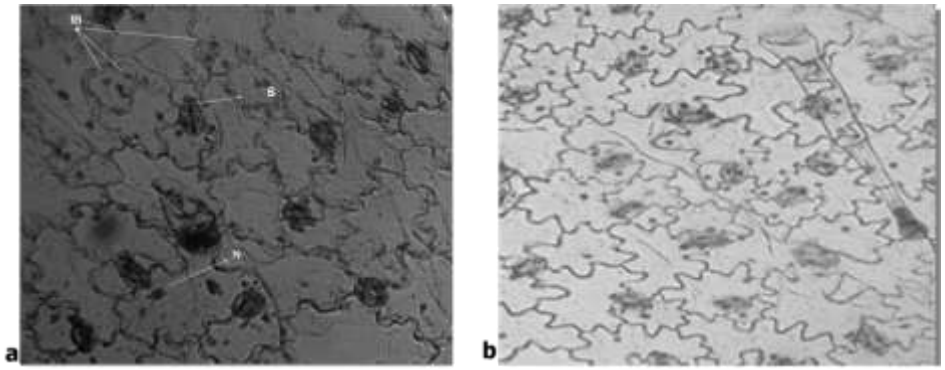

Fig. 4: Light microscopy of epidermal cells from infected White Burley tobacco leaf with AMV stained with mercuric bromophenol blue, showed inclusion bodies (a) and healthy (b). Magnifiction 400X

b- Histological study: Leaf sections of AMV infected healthy potato leaves were stained with toluidine blue and examined via light microscope. The obtained results in (Fig. 5a-d) showed histological abnormalities the AMV infected-potato leaf cells. Large differences were observed between infected and healthy tissues:

- Upper epidermal cell layers were reduced and became irregular also the thickness in lower surface were reduced (Fig. 5a,c) as compared to the healthy plant cells (Fig. 5b,d)
- Palisad mesophyll cells were separated, increased shorter than normal, irregular and contain large intercellular space in infected plants compared with healthy ones (Fig. 5b,d)
- Unorganized and compact large size of spongy mesophyll without air space between cells (Fig. 5a, c).
- Reduction of vascular bundle cells of midrib which tended to be incomplete circular shape which lost its arrangement and normal shape (Fig. 5a, c) comparing with healthy cells (Fig. 5b, d).

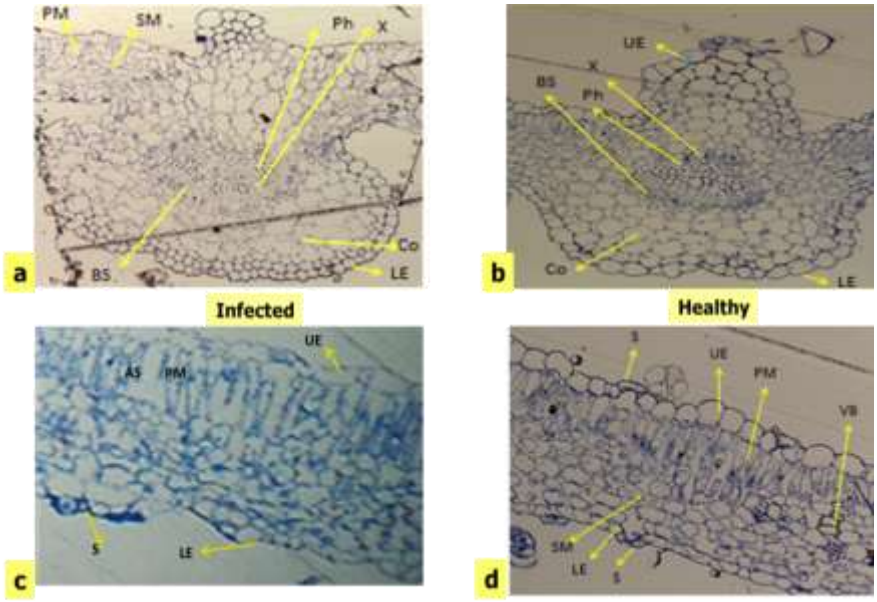

Fig. 5: Light microscopy of semi thin section of AMV infected tissue (a and c) and healthy tissue (b and d) UE: upper epidermis, LE, Lower epidermis, PM: Palisade mesophyll, SM: Spongy mesophyll, AS: Air space, VB: Vascular bandle (X: Xylem, Ph:Phloam), Co: Collenchema, BS: Bbundle sheath cells, S:Stomata - magnification: 400 X

C- Cytological studies

Electron Microscopic examination of *Alfalfa mosaic virus* infected potato leaf cells revealed significant ultrastructural changes (Fig. 6).

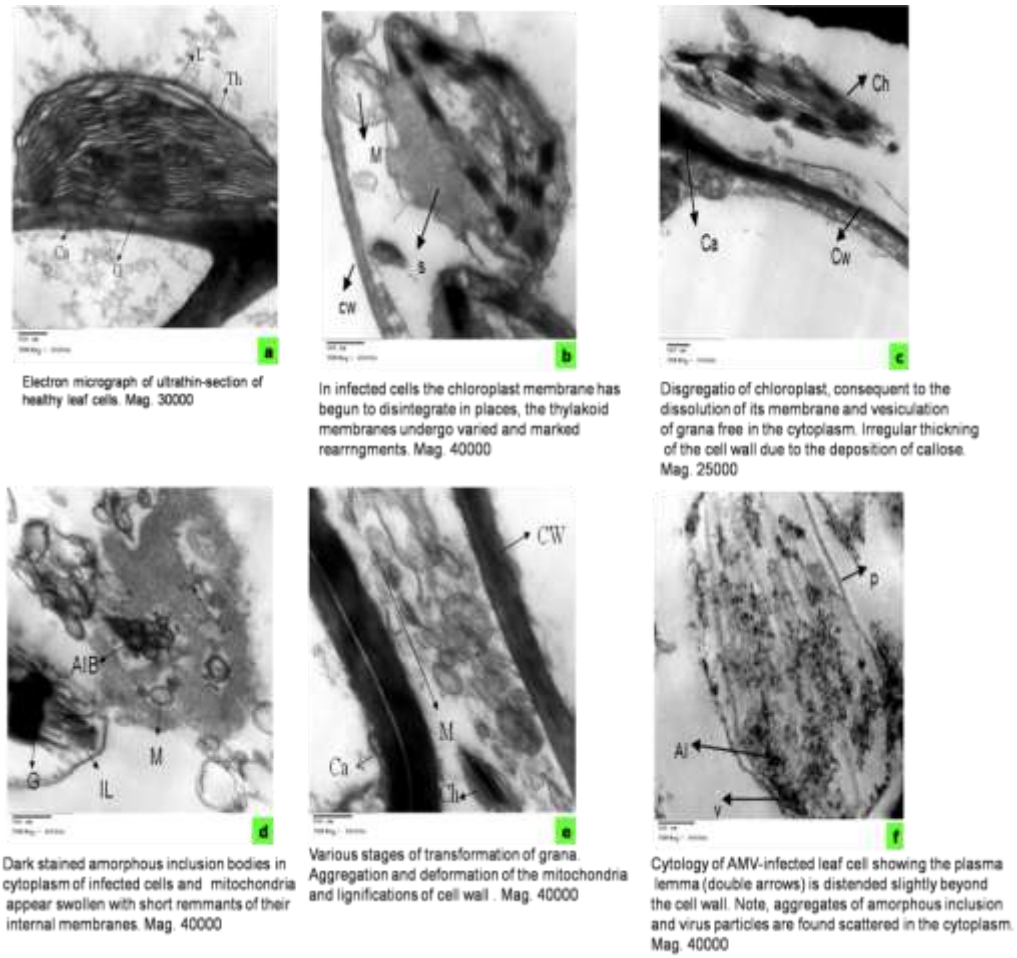

Fig. 6: Effect of AMV on ultrastructural changes in potato leaf cells infected with AMV**,** ch- chloroplast, G- grana, IL- intergranal lamella, S-stroma, CW- cell wall, M- mitochondria, N- nucleolus, Nu- nucleolus, AI- amorphous inclusions, P- plasmalema,V- virus particles – magnification of (b,d,e,f) 40000, (a) 30000 and (c) 25000

These could be summarized as follows:

Chloroplasts

- In the less damaged cells, the chloroplasts slightly damaged, showed a reduced number of discs in their grana (Fig. 6b) comparing with healthy control (Fig. 6a)
- In severely damaged cell, the chloroplast membrane begins to dissolve in places, the thylakoid membranes undergo varied and marked rearrangements, the number of stromatic lamella is also diminished, they appear reduced to short fragments which trend to keep the same alignments as the lamellae from which they originate (Fig. 6c)
- Disaggregation of chloroplast, consequent to the dissolution of its membrane and veseculation of grana free in the cytoplasm (Fig. 6c).
- The chloroplast membranes dissolved until it almost disappears and the whole chloroplast content is poured into cytoplasm (Figs. 6d)

Mitochondria

Particular of a dissolved cell, mitochondria appear swollen with short remnants of their internal membranes and sometimes they were aggregated or deformed (Fig. 6de)

- Abnormal irregular thickening, due to the 3- Cell walls:

Three kinds of abnormality have been observed in or near the walls:

deposition of callose (Figs. 6c, e)

- Protrusions of cell wall into cytoplasm (Fig. 6e).
- Deposition of electron-dense material or dark bodies between the cell wall and plasma membrane (Fig. 6f)
- Dark stained amorphous inclusions in the cytoplasm of infected cell and mitochondria either appear swollen with short remnants of their internal membranes (Figs. 6d).

DISCUSSION

Alfalfa mosaic virus (AMV), also known as *Lucerne mosaic virus* or *Potato calico virus*, is a worldwide distributed phytopathogen that can lead to necrosis and yellow mosaic on a large variety of plant species, including commercially important crops. In 1931 Weimer was the first to report AMV in alfalfa (*Medicago sativa*) (Jaspers and Bos, 1980).

In this investigation, AMV was isolated from naturally infected potato plants collected from Bader Center, Behera Governorate, Egypt, showing the characteristic symptoms of AMV including yellow blotching and bright mottling. In Egypt, several investigators also isolated this virus from potato El-Helaly *et al*., 2012).

A pure isolate of the virus under study, established through single-lesion isolation was identified as such based on host range, symptomatology, modes of transmission, serological detection, in addition of histological and cytological studies. Correct diagnosis of the virus causing a particular disease is essential if effective control measures are to be developed.

In this work, symptoms produced on the artificially inoculated plants by the virus isolate (thirteen plant species and cultivars belonging to four families, *Amaranthaceae, Solanaceae, Fabaceae and Laminaceae*) varied in severity in the selective host plants studied from wilting, malformation like dwarfing, ring spots, mottle, mosaic to necrosis.

Inoculated *C. amaranticolor*, *C. quinoa* plants produced symptoms typical of AMV, chlorotic local lesions ccompanied chlorotic flecking and crinkling respectively. Whereas, infected *N. tabacum* cv. White Burley exhibited bright yellow mosaic. Similar results were obtained by Hajimorad and Franki (1988), Parrelia *et al*. (2010) and Wintermantel and Natwick (2012). On the other hand, symptoms produced by some other isolates varied in plants previously recommended as diagnostic plant indicator for AMV including *C. amaranticolor*, *Pisumsativum*, *Phaseolus vulgaris*, *V. faba* and *Vigna unguiculata* (Jaspars and Bos, 1980) indicating that it would be difficult to unequivocally identify the isolates as AMV by the reaction to indicator plants.

Virus will usually depend for survival on being able to spread from one susceptible individual to another fairly frequently. Knowledge of the ways in which viruses are transmitted from plant to plant is important for recognize a particular viral disease (Matthews, 1991). For experimental point of view, the virus under study was easily transmitted mechanically to different hosts and by *Myzus persicae* by 60 %. Twenty-nine aphid species were reported to be efficient vectors to transmit AMV in a non-Persistent manner (Edwarson and Christle, 1997).

Clark and Adams (1977) showed that, the microplate method of ELISA could be very effectively applied to the detection and assay of plant viruses. Since that time the method has become to be more and more widely used. Positive reactions were (A 405 nm values more than two times greater than those of the negative control-non-infected plants) obtained with AMV

specific antiserum by indirect-ELISA technique provided further evidence that the virus under study is AMV.

Light microscopy has demonstrated that inclusions are neither uniformly distributed nor necessarily correlated with symptom expression (Edwardson and Christie, 1997). In this respect examination of the epidermal strips of AMV-infected White Burley leaf cells revealed amorphous cytoplasmic inclusion bodies. These inclusions seemed to be attached to the nucleus from one or two sites and have never been observed in epidermal strips of healthy ones. These results are consistant with those described by several investigators (Jaspars and Bos, 1980 and Shafie *et al.*, 1997).

In this investigated point, cleared histopathological variations were observed between AMV-infected and healthy plants. These variations are mainely restricted in the reduction of the thickness of the epidermis either upper or lower epidermal layers. The results exhibited reduction of midrib zone, palisade and spongy tissues, leaf blade thickness, length and width of both protoxylem and metaxlem vessles these results are in harmony with Hamed (2011) on *Onion yellow dwarf virus*, Abd El-Baset (2014) on *Apple mosaic virus*, Badr *et al*. (2014) on *Bean common mosaic virus* and Badr *et al*. (2015) working on *Potato virus Y*.

Cytological effects of virus infection have been well illustrated by Franki *et al.* (1985). They stated that external symptoms are reflections of disturbed cell metabolism leading to modifications in tissues, cells and cell organelles. Eloctron micrographs of potato plants infected with AMV revealed various cytological changes in chloroplasts, mitochondria, cell membrane and cytoplasm. The chloroplast become rounded, clumped together and fragmented. The thylakoid membranes undergo varied and marked rearrangements. The number of stromatic lamella is also diminished. The mitochondria appear swollen and sometimes were aggregated or deformed. It has, been found that uneven thickeneing in the cell walls, due to the deposition of callos. Dark salined amorphous inclusions were also observed in the cytoplasm of infected cells. Similar results were obtained by different investigators (Zielinska *et al.*, 2012 and Badr *et al.*, 2014, 2015)

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