

## Cytopathological Studies on Alfalfa Mosaic Virus (AMV) in Potato Plants

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**I**nfectious sap of alfalfa mosaic virus (AMV), inoculated mechanically in different diagnostic plants, reacted positively with each tested plant. Symptoms ranged from local as local lesions in case of *Phaseolus vulgaris* L., to systemic as severe systemic yellow mosaic in case of *Petunia hybrida* L. Infectivity of King Edward potato cultivar was confirmed with Enzyme linked immunosorbent assay (ELISA), Dot blotting immuno-binding assay (DBIA), and Immunosorbent electron microscopy (ISEM). Infected samples reacted positively in comparing with control treatment. Six AMV-infected potato cultivars were positive in ELISA test when examined with AMV antiserum. Gigant was the highly susceptible cultivar, meanwhile Diamant was the lowest susceptible one. Direct electron microscopic examination of crude sap containing AMV showed types of AMV particles ranged from spherical to bacilliform. Some particles were in aggregates. Ultra transverse thin sections showed some changes in cell structures, such as degradation in mitochondria membrane and swelling of chloroplasts, which contained big vacuoles. Electron microscopy studies revealed the presence of virus particles in mitochondria, cytoplasm and nucleus. Ultra transverse thin sections in potato leaves produced from tissue culture technique showed normal cell structures.

**Keywords:** AMV, ELISA, DBIA, ISEM; tissue culture and ultrathin sections.

Potato (*Solanum tuberosum* L.) is one of the world's most popular vegetables. Its importance as a crop is reflected in its large-scale cultivation throughout the world. Potato plants are vulnerable to infect by a large number of pathogens such as fungi, bacteria and viruses (Rahman, 1986; Zaman *et al.*, 2001 and Anonymous, 2007). Many viruses infect potato and cause crop losses. AMV, *Alfamovirus* and *Bromoviridae* used to cause a minor disease in potato but now it occur worldwide and reduce yield and quality of potato (Cordeiro *et al.*, 2003). One of the most important strategies to control plant virus diseases is the tissue culture techniques to produce plants free from viruses. Meristem culture has been used successfully in removal of viruses from potato (Quak, 1977; Mellor and Stace-Smith, 1987; Brown, *et al.*, 1988; Fahmy and Omar, 1988; Anonymous, 2002 and Cordeiro *et al.*, 2003). This study was planned to detect AMV in potato plants and to study the cytopathological changes in infected and in healthy potato plants free from AMV produced by tissue culture technique.

## Materials and Methods

### Source of alfalfa mosaic virus:

Alfalfa mosaic virus was isolated from cv. King Edward which cultivated in EL-Ghorieb farm (Fac. of Agric., Assiut Univ., Assiut, Egypt).

### Virus detection:

#### 1- Diagnostic plants:

Eleven plant Genus (Table 1) were used as indicator plants:

**Table 1. Diagnostic plants for detection of alfalfa mosaic virus**

Latin name	English name
<i>Apium graveolens</i> L.	Celeriac
<i>Gomphrena globosa</i> L.	Globe amaranth
<i>Lycopersicon esculentum</i> L.	Tomato
<i>Ocimum basilicum</i> L.	Basil
<i>Petunia hybrida</i> L.	Garden petunia
<i>Phaseolus vulgaris</i> L.	Kidney & French beans
<i>Salvia splendens</i> L.	Scarlet sage
<i>Cassia angustifolia</i> L.	Indian senna
<i>Vicia faba</i> L.	Bean
<i>Chenopodium amaranticolor</i> L.	Mexican tea
<i>Robinia pseudoacacia</i> L.	False acacia locust tree

The tested plants were grown in sterilized soil in pots (30-cm-diameter) in an insect free greenhouse.

Plants were dusted with carborandum and mechanically inoculated with crude sap obtained from diseased potato plants suffered from bright mosaic. A volume of 0.1 M cold potassium phosphate buffer (pH 7.0) was used for preparing the inoculum. Symptoms were recorded periodically for up to 30 days after plant inoculation.

#### 2- Electron microscopic examination:

In order to reveal the morphology of AMV, method described by Tzeng *et al.* (1999) was carried out. Drops of crude sap from diseased leaves were placed on electron microscope grids, which previously covered with collodion membrane. Grids were placed on the top of infectious sap for one min., then left to dry. Grids were placed on the drop of specific antiserum of alfalfa mosaic virus for two min then left to dry. Finally the grids were immersed in 2% phosphotungstic acid (pH 7.0) for negative staining. Electron microscope type (JEM 100CX11-EM) in EMU, Assiut University was used for virus examination.

#### 3- Serological techniques:

##### a- Dot blotting immuno-binding assay (DBIA):

Technique of DBIA on nitrocellulose membrane described by Abd El-Salam (1999) was used for detection of AMV. Nitrocellulose membrane 0.2 µm

(Bio-Bind-NC Whatman) was marked with a pencil into squares of 1 x 1 cm. Healthy and AMV infected potato plant samples were grinded in STEP buffer pH 8.3 (1:10 w/v). One micro litre for each of healthy and virus infected samples was applied directly into nitrocellulose membrane for the serological detection of antigen, then blocked for 1 min in polyvinyl alcohol, 17,000 mw, (1mg/ml) and incubated for 2hr with the induced rabbit polyclonal antiserum cross absorbed at dilution 1/1000 in TBST (10 mM Tris-HCl, pH 8.0, 150 Mm NaCl and 0.05% Tween 20) at room temperature. The membrane was then washed three times in TBST for 10 min and incubated with anti-rabbit alkaline phosphatase conjugate (diluted at 1/2000 in TBST) for 2hr, then washed three times, each for 10 min. The blots were then incubated in a substrate solution consisting of fast red TR in 10 ml H<sub>2</sub>O containing 5 Mm MgCl<sub>2</sub> and 4 mg naphthol AS-MX dissolved in 10 ml of 0.2 M Tris-HCl, pH 8.0, as described by Abd El-Salam and El-Sharkawy (1996).

*b- Indirect ELISA:*

Indirect ELISA method was carried out according to Converse and Martin (1990) with some modifications as follows: Microtiter plates were coated with 100 µl/well of leaf extract diluted to 1/10 in coating buffer (pH 9.6) and incubated overnight at 4°C. Wells were washed 3 times with PBST by shaking for 3 min. each time. The microtiter plates were dried after the last wash by pounding the plates on a stack of paper towels by hand then, a blocking agent (5% dried milk powder) dissolved in PBST-PVP was applied for 30 min. at room temperature. The plates were dried without washing. Cross-absorbed antiserum was then added at a dilution of 1/1000 (100 µl/well). Plates were incubated for 3hr. at 37°C or overnight at 4°C, then washed and dried. The antigen was detected with anti-rabbit alkaline phosphatase conjugate (Sigma A-8025) diluted at 1/7000 in conjugate buffer, (pH 7.4), added to the plates using 100 µl/well and incubated for 3hr. at 37°C or overnight at 4°C. The plates were washed and dried. PNPP (1.0 mg/ml) dissolved in the substrate buffer pH 9.8 was added. Absorbance readings at 405 nm were taken after incubation with substrate for 2hr.

*Tissue culture technique:*

To obtain plants free from AMV, medium developed by Murashige and Skoog (1962) was used. Sprouts from different potato cultivars were chosen as starting materials. They were rinsed in water, then sterilized with 0.5% mercuric chloride for 5min., then washed thoroughly with sterile water. Leaves surrounding the meristem of their apical buds were removed. The shoot tip about 0.5-8 mm containing the meristem dome and 2-3 leaves primordial were dissected under a stereoscopic microscope and then transferred to tubes containing 10ml solid medium. Produced plants were grown in a green house and examined under electron microscope.

*Ultra-thin sectioning technique:*

The following method was carried out according to DE Bokx and Waterreus (1971). A small piece of infected potato leaves of King Edward cv. showing calico pattern (yellow blotching symptoms) and infected with AMV were cut into small pieces (1x1mm), and then fixed in 2.5 glutaraldehyde and Epon 812.

Ultra-thin sections of the aforesaid materials with thick 50-80 nm were transversely cut with a glass knife and picked up on 200 mesh grids. The section-mounted grids were positively stained with saturated aqueous uranyl acetate 2% for 25 min and followed by lead citrate for 5min. Sections were observed and photographed under electron microscope. The same technique was done with healthy plants formed by tissue culture.

## Results

### 1- Symptomatology:

Results of AMV infection on diagnostic plants are shown in Table (2).

**Table 2. Symptoms developed on diagnostic plants mechanically inoculated with AMV**

Diagnostic plant	Time needed for syndrome appearance	Symptoms appeared on differential host leaves
<i>Apium graveolens</i> L.	20-30 days	M or M
<i>Gomphrena globosa</i> L.	10-15 days	W
<i>Lycopersicon esculentum</i> L.	20-25 days	Y, BL
<i>Ocimum basilicum</i> L.	4-10 days	YB
<i>Petunia hybrida</i> L.	25-30 days	SYM
<i>Phaseolus vulgaris</i> L.	10-15 days	Y, NLL
<i>Salvia splendens</i> L.	12-17 days	S
<i>Cassia angustifolia</i> L.	8-10 days	Y, VN, W
<i>Vicia faba</i> L.	11-15 days	VC, Y, SS
<i>Chenopodium amaranticolor</i> L.	6-15 days	MCS
<i>Robinia pseudoacacia</i> L.	10-25 days	CL

S = Pink spots.

YB = Yellowing with blisters.

Y, BL = Yellowing on inoculated leaves later blotches with central necrosis.

W = Inoculated leaves became pale and later withered.

VC, Y, SS = Vein clearing, yellowing and small spots.

MCS = Minute chlorotic spots on inoculated leaves.

Y, VN, W = Yellowing, Vein necrosis and withering of inoculated leaves.

Y, NLL = Yellowing, necrotic local lesions.

M or M = Mosaic or mottling on the new leaves

CL = Chlorotic lesions later became necrotic

SYM = Severe systemic yellow mosaic on the new leaves.

Reaction and symptoms developed on the tested plants differed from one plant species to another. Symptoms ranged from local to systemic. *Apium graveolens* L. and *Petunia hybrida* L. produced mosaic. However, *Salvia splendens* L. produced pink spots only. *Chenopodium amaranticolor* L. reacted with minute chlorotic spots on inoculated leaves. Only *Robinia pseudoacacia* L. produced chlorotic lesions and later turned to necrotic. In case of *Cassia angustifolia* L. symptoms varied between yellowing, veinal necrosis and withering.

## 2- Serology:

## A) DBIA:

DBIA test confirmed the infectivity of King Edward potato with AMV as shown in Fig. (1).

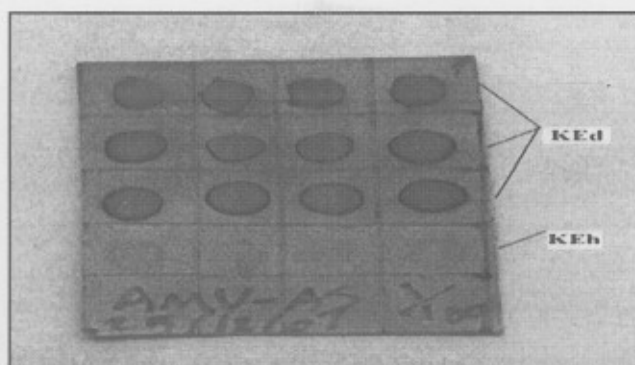


Fig. 1. Dot blotting immuno-binding assay (DBIA) of extracted sap obtained from potato diseased King Edward cultivar, which infected with alfalfa mosaic virus (AMV). This reaction is compared with control (Healthy plants obtained through tissue culture technique).

## B) ELISA test:

Figure (2) show the results of six potato cultivars infected with AMV and tested after six weeks. These results showed that cv. Gigant was the highest in virus concentration value, followed by Burren, then Mirakel, King Edward, Spunta and the least one was Diamant cv., in comparing with the control.

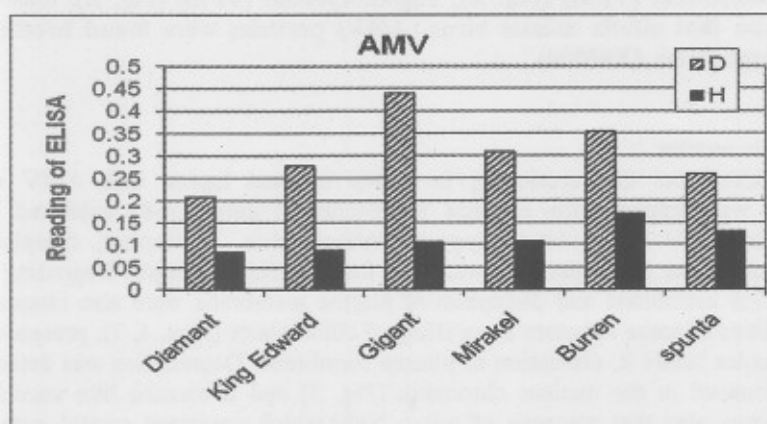
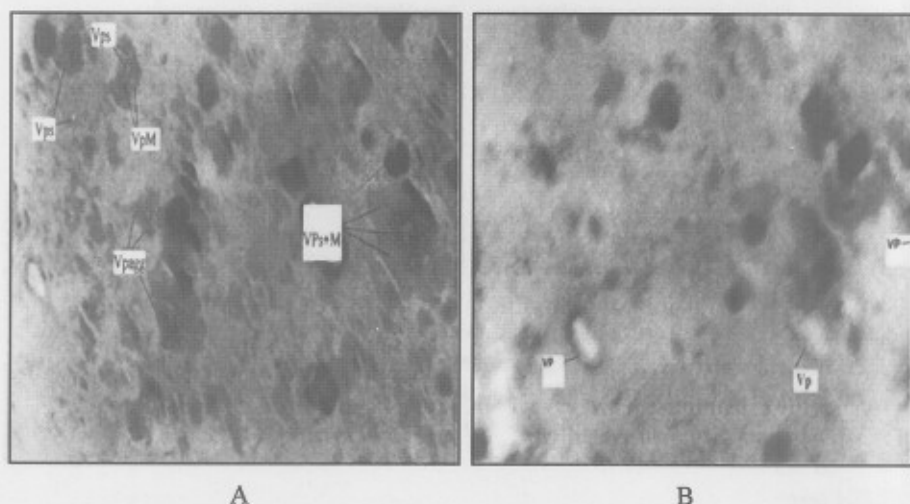


Fig. 2. ELISA value of 6 potato cultivars infected with alfalfa mosaic virus.

### 3- Electron microscopic examinations:

#### a) Immunosorbent electron microscopy:

The crude sap of AMV infected potato leaves was examined with electron microscope to detect virus particles. Fig. (3) show that virus particles had different types of shapes ranging from spherical to bacilliform. The particles were also found in aggregates.



**Fig. 3.** Alfalfa mosaic virus particles under electron microscopy in infectious potato sap. Virus particles negatively stained with 4% of sodium phosphotungstate. Note the virus particles of alfalfa mosaic virus (AMV) have different types (Spherical particles (VPs), Middle - component (VPM) (Fig. A), Top-component (VPtb) (Fig. B). Observe also that alfalfa mosaic virus (AMV) particles were found herein as aggregates. (X40000).

#### b) Ultrathin section:

Ultra transverse thin sectioning in potato infected leaves with AMV was examined with transmission electron microscope. Figures (4-8) exhibited the presence of AMV in dumbbell shape of mitochondria, chloroplast, cytoplasm, vacuoles and grana. AMV particles were also found in chloroplastids. Degrading the mitochondria membrane and disruption of plasma membrane were also observed. Abnormalities of some structure as swelling of chloroplasts (Figs. 4, 7), presence of a big vacuoles inside it, disruption of plasma membrane. Degradation was detected also pronounced in the nucleus chromatin (Fig. 5) and it became like vacuoles. Fig. (8) show also that presence of micro body which contained crystal with an irregular shape, a lot of peroxisomes in diseased tissues.

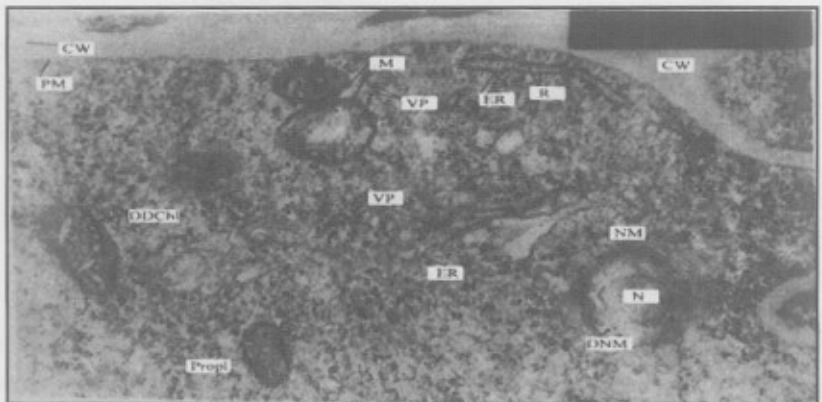


Fig. 4. Ultra transverse thin section in potato leaf (*Solanum tuberosum* L.) infected with alfalfa mosaic virus and showing mesophyll cells under electron microscopy. Note the large vacuoles in chloroplast due to the damage induced by alfalfa mosaic virus. Also, observe that the degradation of diseased mitochondria. Virus particles (vp), chloroplast (Chl), mitochondria (m), nuclear membrane (NM) nucleolus (N), endoplasmic reticulum, (ER) Proplastid, (Propl), Destructive degradative of chloroplast (DDChl), disrupted of nuclear membrane (DNM). Note that virus particles are detected in mitochondria, and in the cytoplasm of mesophyll cells (X 11000).

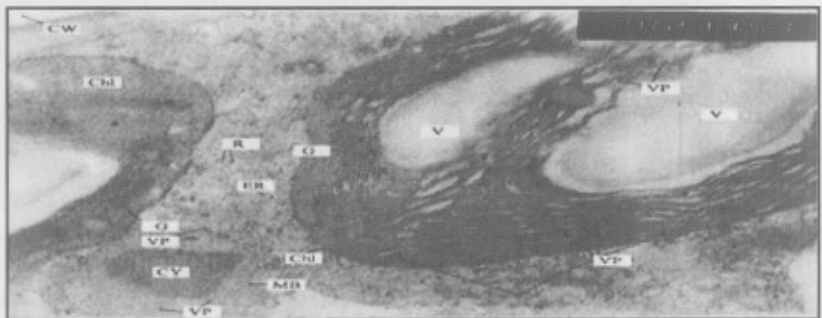


Fig. 5. Ultra transverse thin section in potato leaf (*Solanum tuberosum* L.) infected with alfalfa mosaic virus and showing mesophyll cells under electron microscopy. Note that the swelling of the chloroplasts and presence of vacuoles in its matrix was induced by the damage due to viral infection with alfalfa mosaic virus. Also, note that (VP) virus particles were found in the cytoplasm, Chloroplasts and micro body (MB), which also contained a crystal -containing an irregular shape. Chloroplast (Chl), vacuole (V), grana (G), mitochondria (M), ribosome's (R), cell wall (CW) (X 20000).

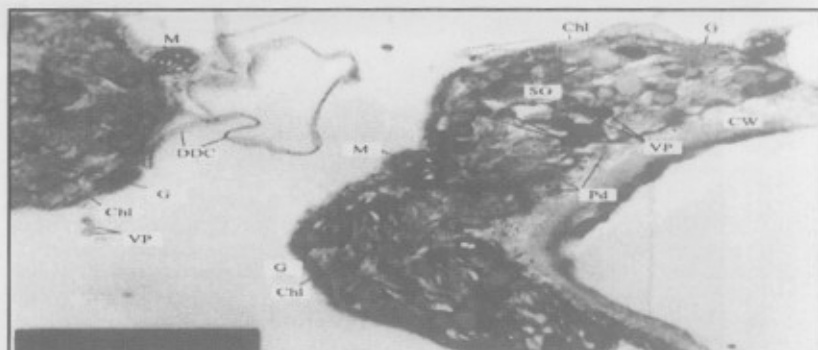


Fig. 6. Ultra transverse thin section in potato leaf (*Solanum tuberosum* L.) infected with alfalfa mosaic virus and showing mesophyll cells under electron microscopy. Note that particles (VP) of alfalfa mosaic virus were found in the chloroplast and in the cytoplasm. Chloroplast (Chl), vacuole (V), grana (G), cell wall (CW), mitochondria (M), starch grain (SG), plasmodesmata (Pd). Note also that viral infection caused destructive, degrading of cytoplasm (DDC) (X11000).

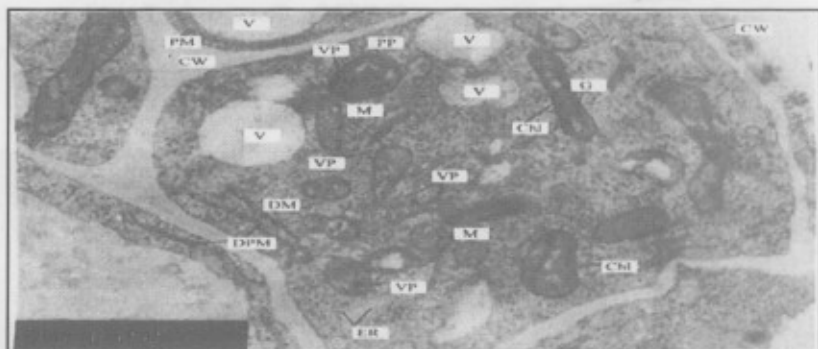


Fig. 7. Ultra transverse thin section in potato leaf (*Solanum tuberosum* L.) infected with alfalfa mosaic virus and showing mesophyll cells under electron microscopy. Note that particles (VP) of alfalfa mosaic virus were found in the proplastid (PP), chloroplast and in the cytoplasm. Chloroplast (Chl), vacuole (V), grana (G), cell wall (CW), mitochondria (M), endoplasmic (ER) reticulum, plasma membrane (PM), disrupted plasma membrane (DPM) due to the viral infection with alfalfa mosaic virus. Viral infection caused destructive, degrading of mitochondria (DDM) and its dumbbell shape clearly appeared (X 18000).



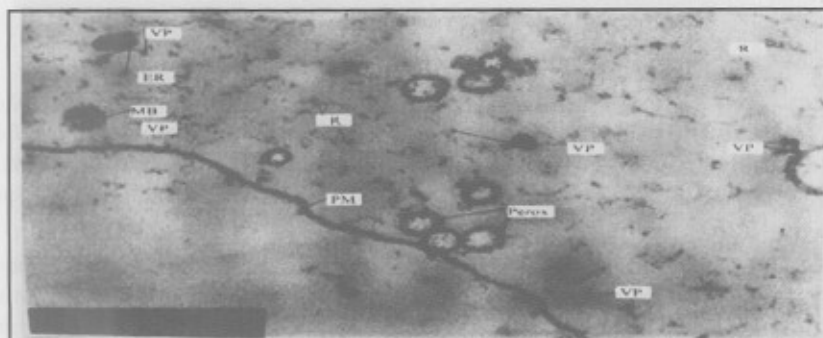


Fig. 8. Ultra transverse thin section in potato leaf (*Solanum tuberosum* L.) infected with alfalfa mosaic virus and showing mesophyll cells under electron microscopy. Note that a lot of peroxisomes (Perox) were found in diseased potato tissues. Note that (VP) virus particles were found in the cytoplasm separately or in aggregates. (PM) plasma membrane, (R) ribosome, (MB) micro bodies, (ER) endoplasmic reticulum (X 18000).

Ultra transverse thin section in mesophyll cell of healthy potato leaf obtained from AMV free plants produced through tissue culture technique showed normal mitochondria and chloroplast. Mitochondria free from vacuoles with dense matrix, grana clearly appeared, also all of the components such as ribosomes, nucleus, nucleus nuclear membrane, nuclear envelop, endoplasmic reticulum, nuclear envelop pore, Golgi stacks and cell wall (Fig. 9).

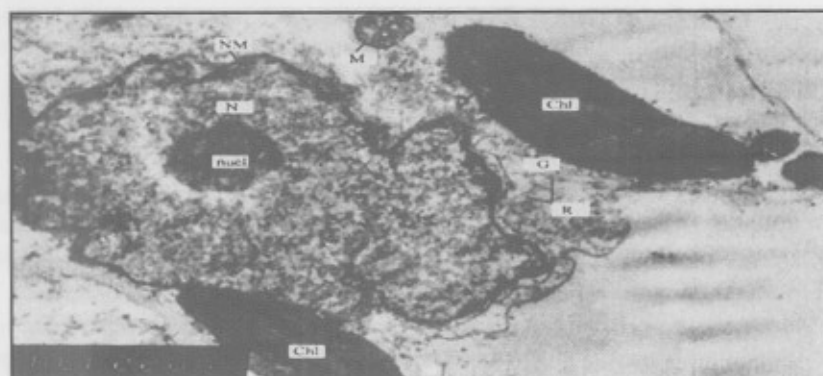


Fig. 9. Ultra transverse thin section in the mesophyll cell of healthy potato leaf obtained from virus-free plants produced through tissue culture technique under electron microscopy showing mitochondria and chloroplast. Note the normal mitochondria free from vacuoles with dense matrix, which grana clearly appeared, mitochondria (M), chloroplast (Chl), ribosomes (R), nucleus (N), nucleolus (nucl), nuclear membrane (NM) (X 88.000).

### Discussion

The results of DBIA of extracted potato leaves infected with AMV showed the presence of AMV in each tested replicate. ELISA value of six potato leaf cultivars, showed that the AMV was found in all tested cultivars with the highest concentration in Gigant, followed by Burren, Mirakel, King Edward, Spunta and finally Diamant.

Electron microscopic examination of crude sap of infected leaf showed that presence of virus particles with types ranging from spherical to bacilliform shapes. It also showed that aggregation of the virus particles. Our obtained results are in agreement with Hull (2001).

Virus infection frequently alters many components and shape of cell structure, Ultra thin transverse sections showed degradation in mitochondria membrane, which may be due to presence of AMV in mitochondria, also, swelling of chloroplasts, presence of AMV particles in cytoplasm induced destructive degrading of cytoplasm. These results are in harmony with Zechmann *et al.* (2003).

The results also revealed a degradation of chromatin and nuclear membrane. The appearance of perioxomers in noticeable numbers in diseased potato leaf tissues with AMV may be play an important role in viral replication, these finding are in agree with Navro *et al.* (2006) who reported cytopathological effects due to virus infection .On the other hand, the ultrathin sections of plants produced from tissue culture technique showed normal cell structure and absence of the virus particles.

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## دراسات سيتوباثولوجية على ألفا ألفا موزايك فيروس

### Alfalfa mosaic virus (AMV) في البطاطس

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اثبتت هذه الدراسة أن عدوى بعض العوائل المفردة بصير من نباتات البطاطس المصابة بفيروس AMV قد أدت إلى ظهور أعراض مرضية متباينة واختلفت تلك الأعراض تبعاً لنوع السلل المفرق، وتراوحت تلك الأعراض ما بين بقعاً موضعية قد ظهرت على نباتات الفاصوليا بعد إجراء تلقيح لورقها ميكانيكياً بصير من نباتات بطاطس مصابة بـ ألفا ألفا موزايك فيروس ، في حين ظهرت أعراض جهازية شديدة على نباتات البتونيا.

وقد تم إجراء بعض الاختبارات الميولوجية مثل الـ Enzyme linked immunosorbent assay (ELISA) واختبار الـ Dot blotting immuno-binding assay (DBIA) واختبار الـ Immunosorbent electron microscope (ISEM) والتي أعطت نتائجاً موجبة مع عصور النباتات المصابة بالفيروس مقارنةً بالمتحكم.

وباستخدام اختبار الـ ELISA على ستة أصناف من البطاطس اتضح أن الصنف جيجانت Gigant كان أكثر الأصناف قابلية للإصابة بالفيروس ، بينما كان الصنف ديمنت Diamant أقلهم قابلية للإصابة بالفيروس.

وعند فحص عصور نباتي تم الحصول عليه من نباتات بطاطس مصابة بفيروس AMV بواسطة الميكروسكوب الإلكتروني مع استخدام الصغ الصلب الـ Spherical إلى Bacilliform لجزيئات الفيروس، كما شوهدت أيضاً جزيئات الفيروس في هيئة كتل.

وقد تم أيضاً عمل مقاطع عرضية دقيقة وتم فحصها بالميكروسكوب الإلكتروني في أوراق نباتات بطاطس مصابة بالفيروس وكذلك في أوراق نباتات بطاطس سليمة خالية من الفيروس نتيجة من زراعة الأنسجة الميرستيمية، ووضحت المقاطع وجود الفيروس في السيتوبلازم والميتوكوندريا والنواة في خلايا النسيج المصاب ، وشوهدت للكلوروبلاستيدات في خلايا أوراق البطاطس المصابة بالفيروس متضخمة مع كبر حجم الفجوات بها، كما أحدث الفيروس تلقاً لغشاء النواة ، وقد استحدث هذا الفيروس إنتاج عدد كبير من الـ microbodies وكانت في هيئتها تشبه الكريستال.