

COMPARATIVE ULTRASTRUCTURAL CYTOLOGY OF BROAD-BEAN INFECTED TISSUES WITH ALFALFA MOSAIC ALFAMOVIRUS, BROAD BEAN MOTTLE BROMOVIRUS AND BEAN YELLOW MOSAIC POTYVIRUS.

Mervat M. Fath-Allah

Plant Pathology Institute, Agric – Res. Center, GISA

ABSTRACT

The effect of three different viruses, i.e., alfalfa mosaic alfamovirus (AMV), broad bean mosaic bromovirus (BBMV) and bean yellow mosaic potyvirus (BYMV) on the cytopathic changes in cv. Giza Blanka (*Vicia faba* L.) as well as their pathogenicity and effect on cells of affected host were studied. The viruses varied in their aggressiveness and effect on host cells, giving rise to various deformations and deteriorations of host cell organelles and formed various types of cytoplasmic inclusions, in addition to virions accumulations.

The aggressiveness of virus isolates seemed to be correlated to their cytopathic effect on host cell organelles and was time dependant. AMV, with the greatest damaging effect on organelles, forming abundant virion accumulations but poor development of inclusions (tubes and scrolls), was the most aggressive. BBMV, having less cytopathic effect, with abundant accumulation of virions but no formation of specific inclusions, was relatively, less aggressive. BYMV showed a slight effect on host organelles with poor virions accumulations but formed numerous types of inclusions, was the lowest aggressive.

Viruses, with spherical virions tended to form no inclusions, with filamentous virions formed inclusions abundantly, of different types and that of multiple particles was intermediate in this respect.

Keyword: alfalfa mosaic virus, broad bean mottle virus, bean yellow mosaic virus, inclusion bodies, ultrastructural cytology.

INTRODUCTION

The cultivated areas of faba bean (*Vicia faba* L.) in Egypt and many Arab countries are affected by several diseases caused by seed-borne viruses. Of these, alfalfa mosaic alfamovirus (AMV), broad bean mottle bromovirus (BBMV), broad bean stain comovirus (BBSV), broad bean true mosaic comovirus (BBTMV), bean yellow mosaic potyvirus (BYMV) and pea seed borne mosaic potyvirus (PsbMV) were most prevalent (EL- Afifi and EL-Dougdoug 1997, EL- Hammady *et al* 2000, Fegla *et al* 2003, Fortass and Eos 1991, Makkouk *et al* 1994 and Najar *et al* 2000). The incidence of mosaic disease symptoms ranged from 11 to 64 % Fegla *et al* (2003).

Recently, virus like particles were detected in the nuclei of young vascular cells of *Phaseolus vulgaris* L., infected with bean golden mosaic (BGMV) Kim (1978), Honda *et al* (1973) reported in tobacco plants, infected with cucumber mosaic virus, the virus particles to be scattered or aggregated in small amounts in the cytoplasm, but not in nuclei or other organelles. Moreover, no virus particles were found in chloroplast, mitochondria or vacuoles of *Chenopodium amaranticolor* infected with tobacco necrosis virus

(TNV) Gana *et al* (1982), in cucumber, affected by cucumber soil borne virus (CsBV) (Koenig *et al* 1983) or in tobacco infected with peanut stunt virus Sanger *et al* (1998).

Tomato infected with tomato yellow mosaic virus (mosaic Amarillo del tomate) (MAT) showed striking changes in nuclear morphology. 6 days after inoculation, mainly, hypertrophy of the nucleus. The virus-like particles were observed as large masses in the nuclei of infected phloem cells Lastra and Gil (1981). Two types of crystalline inclusions were observed in the epidermis covering the abaxial surface of the leaf veins of *Capsicum annuum* inoculated with cucumber mosaic virus (Moorman, 1982; and Moorman and Woodbridge 1983).

Ultrathin sections of leaves from plants infected with potyviruses revealed the cylindrical or nucleolar inclusion in cytoplasm. Cylindrical inclusions appeared as scroll-type, pin wheels, tubes, bundling, short, and laminated aggregates (DeBokx and Watteraus 1971, Lisa *et al* 1981, Hunst and Tolin, 1982, Edwards and Christie 1983, Giorda *et al* 1986, Wiles *et al* 1987, Thomas *et al* 1997, Younes 2003 and Liang *et al* 2004).

The present work aimed to investigate two major criteria, i.e., inclusion bodies and deterioration of host cell organelles due to viral infection. Moreover, attention was paid to detect whatever these changes were associated with virus type and/or symptoms severity.

MATERIALS AND METHODS

Source and Maintenance of Viral Isolates:

Alfalfa Mosaic Virus:

The virus was isolated from alfalfa plantation at Desert Development Center Farm. The virus was isolated from its original host with typical disease symptoms, identified on the basis of diagnostic hosts and serological reactions. AMV antiserum used in this study was locally prepared during the course of her study for Ph.D. The virus was maintained on *Nicotiana glauca* plants, grown in insect proof greenhouse, until needed.

Broad Bean Mottle and Bean Yellow Mosaic Virus:

The viruses were isolated from broad bean plant samples collected from plantations grown at the Experimental Farm of Faculty of Agriculture, Alexandria University, at Abeer location as well as from new reclaimed lands at Behera Governorate, which had typical symptoms of either virus. The isolates were transferred to new healthy faba bean plants and identified on the basis of indicator plants and serological reactions.

The antisera for BBMV and BYMV were kindly provided by Antiserum-Bank, Institute of seed pathology for Developing Countries, Denmark. The obtained isolates were identified as broad bean mottle virus and bean yellow mosaic virus by using serological method as indirect ELISA.

The two viral isolates were maintained on *Vicia faba* by mechanical transmission and kept under greenhouse insect free condition.

Host Inoculation:

The inocula were prepared by grinding 1g leaf tissues of artificially infected plant in 10 ml buffer. In case of AMV, SB buffer (0.07 M Sorensen's Phosphate buffer pH 7.5) was used, where in case of BBMV and ByMV Phosphate buffer, pH 7.5 was used. Broad bean (*Vicia faba* L.) var. Giza Blancka seedlins, grown from seed, at 4-5 leaf stage, i.e., 15 days after seed germination were mechanically inoculated. Inoculated plants were kept under careful observation in a green house. Two weeks after symptoms development, leaflets of infected broad bean were collected for further investigations.

Disease Severity:

Inoculated plants were red due to number of diseased plants and type of symptoms developed. Three replicates, 15 plants each, were inoculated by each viral isolate. The developed symptoms ranged from necrotic local lesion (NLL), black necrotic local lesion (BNLL), mild mosaic (MM), sever mosaic (SM).

Electron Microscopy:

Leaf tissues (1x3 mm) were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.05 M cacodylate buffer pH 7.0, for 2 hr at room temp. Under vacuum, rinsed with the same buffer, then postfixed in 1% osmium tetroxide (O₅O₄) for 2 hr. Sample were prestained in 0.5% uranyl acetate at 4°C, overnight. Tissue dehydration was achieved by running through an ethanol series, then imbedded in Spurr's epoxy-resin. Ultra thin sections of 10 nm thickness were cut by glass knife on a LKB ultramicrotome. Sections were double stained with 2% uranyl acetate for 10 min then lead citrate for 5 min and examined in a Jeol JEM-10 ex electron microscope.

The studied samples were taken at two intervals, i.e., 4 and 15 days after host inoculation.

RESULTS

Disease Incidence and Severity:

Three different viruses, one of each viral group, i.e. AMV representing virions bacilliform, mostly 30, 35, 43 and 56 nm length and 18 nm wide, BBMV, representing virions isometric, 26 nm in diameter and ByMV representing virions filamentous, 75 nm length and 12-15 nm wide, were used during the course of this study. The infection percentage as well as disease severity (symptom type) on the host, *faba bean* cv. Giza Blancka were determined 15 days and one month after inoculation. The results were represented in Fig 1 and Table 1.

The most aggressive isolate was that of AMV which gave the highest infection rate of 75% and the severest symptoms. Symptoms started as brown necrotic spots 10 days after inoculation, then death of whole plant by the end of the month.

BBMV gave moderate infection of 40% , but more severe symptoms, developed as necrotic local lesion in the first inoculated leaves, 5-7 days after inoculation, then vein clearing, accompanied by the more distinct symptoms

of mottling mosaic on the apical leaves ended by death of whole plant 60 –70 days after inoculation .

BYMV caused 65% infection with less aggressive symptoms expressed as chlorosis of the veins and bright green or yellow mosaic spots on the leaves followed by severe mosaic.

Electron Microscopy:

The careful examination of ultrathin sections of infected broad bean leaflet tissues, by means of transelectron microscope, (two fields each of 10 sections / infected tissue with each viral isolate), revealed occurrence of lot of cytological diversities, in comparison with healthy control. Such differences, i.e., size, shape, number and structure of most of cell organelles as well as, specific inclusions could be observed concerning each infection status as follow.

AMV Infected Broad Bean Leaflet Tissues:

Nucleus showed different stages of deterioration, it appeared some how spherical, almost with normal size but with indistinct envelope and marginal distribution of hetero (high density) chromatin DNA and RNA. Moreover, turned into pycnotic (lycid) nucleus. Fig. 2 (A & B).

Mitochondria undergo morbid structure. They appeared with ellipsoid to spherical shape, with indistinct membranes and cristae in structure. Some other mitochondria were distorted (Fig. 2B).

Chloroplast had poor undifferentiated lamellar structure. Cell cytoplasm was occasionally occupied with multivacuolated vacuoles giving reason for autophagosome development, (Fig. 2D).

Cell wall was, almost, normally developed in most of examined cells.

AMV virions were present in cytoplasm as bacilliform, parts and strands of virus multiparticels. Inclusion bodies occurred as pinwheel and tube structures, Fig. 2 (C&E).

BBMV Infected Broad Bean Leaflet Tissues:

Nucleus showed abnormal shape with indistinct envelope. The high density heterochromatin was irregularly distributed with tend to be peripheral. Moreover, nucleuses get deteriorated, i.e. pycnotic nucleus giving rise to complete lysid content, (Fig. 3A).

Mitochondria had reduced size, but occasionally became abnormally larger (up 5-6 folds). Their fine structure tended to be vacuolar with no sharp differentiation of plate like cristae, (Fig. 3A). Some cells had abundant amount of reduced size mitochondria. Small vesicle, delimited by single membrane, was mostly free in cytoplasm, similar vesicles were, also, found occasionally within mitochondria between the outer and inner membrane, (Fig. 3B). Masses of virus like particles in cytoplasm were associated with the plasmalemma and proliferated endoplasmic reticulum (Fig. 3C).

Chloroplasts were, in general, poorly developed. Most of them lost their typical structure, i.e. having opaque content with no lamellar arrangement, and extremely, elongated form, Fig. 3 (E&D). Well developed viral spherical particle accumulations, which could occupy a whole cell. Moreover BBMV particles were associated with degenerated plastids (Fig. 3D).

No cytoplasmic inclusions were detected.

ByMV Infected Broad Bean Leaflet Tissues:

Nucleus had deformed shape with poorly differentiated structure. It was absent in the majority of examined cells, (Fig. 4A).

Mitochondria were highly deformed losing their structure, having vascular content. Occasionally, viral rod shape particles could be observed, (Fig. 4B), virus particles were sometimes observed near the pinwheel, (Fig. 4C).

Inclusions occurred as scroll-type. Pinwheels and tubes were observed in all cell types. ByMV produced pinwheels in masses that were easily detected within the cytoplasm at low magnification, Fig. 4 (B&C).

Table 1. Percentage of Infected Plants and Type of Expressed Symptoms on Broad Bean Plants cv. Giza Blanka inoculated by AMV, BBMV and BYMV .

Virus	Infection % *	Disease severity**
AMV	75	BNS, SM, D
BBMV	40	NLL, SBM, D
BYMV	65	MM, SM

*4 replicates 20 plants each -** BNLL: brown necrotic spot; - D: death of whole plant; MM: mild mosaic;-NLL: necrotic local lesion;-SM: sever mosaic;-SMB: systemic mottle blotchy.

DISCUSSION

Three viruses, i.e., AMV, BBMV and BYMV representing three different viral groups, were tested on the same host, cv. Giza Blanka (*Vicia faba*L.), to evaluate their pathogenicity and study their effect on cells of affected host tissues. All viruses were, variously, pathogenic and affect the host cells giving rise to different levels of organelles deformation and deteriorations. The actual results agreed with those previously reported by other authors (Lastra and Gil 1981; Moorman and woodbridge 1983 and Thomas *et al* 1997).

Ultrastructural observation on the invasion of AMV into faba bean parenchyma showed hypertrophied nucleus with marginal distribution of heterochromation (Fig. 2b) as previously described for tomato yellow mosaic, Lastra and Gil (1981). Mitochondria appeared with ellipsoid to spherical shape, and mass of virus like partical were found in cytoplasm and chloroplast, in contrast to that to BBMV and ByMV which were found in cytoplasm only.

Symptoms severity on susceptible Essex soybean plants was associated with cytoplasmic strand development, Hunst and Tolin (1982). Even though cytoplasmic strands were reported for several potyviruses, their function has never been determined (Weintraub and Ragetli. 1970, and Begtrup, 1976). The cytoplasmic strands produced by AMVon susceptible broad bean cultivar, might act as possible defense reaction of the infected cells.

In later stages of infection, cytoplasmic cylindrical inclusions were observed in cytoplasm as scrolls and tubes.

The cytopathic changes induced by BBMV were all important details typical for alteration induced by spherical viruses. An association of the multivesicular inclusions to chloroplasts or mitochondria was found in BBMV affected cells. Bundling of the chloroplasts was not observed. The internal material of the inclusions resembled that of microbodies although this organelle was not observed in infected tissue in contrast to its presence in healthy faba bean. It seems possible, therefore, that the multivesicular inclusions present altered microbodies, such similar consideration already has been discussed by Russo and Matelli (1972).

Degeneration of chloroplasts, one of the most conspicuous ultrastructural symptoms caused by BBMV infection, was similar to those reported for other viruses eg. CMV (Gerola *et al.*, 1965; Misawa and Ehara, 1966, and Honda *et al.*, 1973). The plastids remained, however, quite normal in BBMV protoplasts at least for 4 days after inoculation, during which time the virus multiplied substantially. It was likely the chloroplast abnormalities in infected leaves represented a cytopathic effect which appears only in later stages of infection.

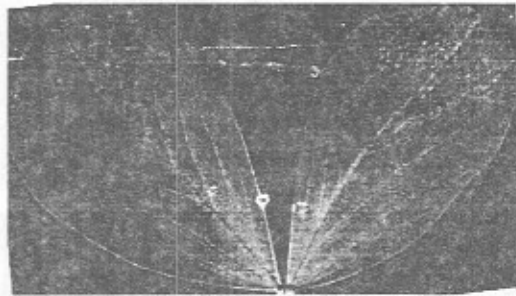
Various aspects of the central portion of potyviruses induced cylindrical inclusions, pinwheels, and bundles appeared in the report of (De Box and Wattereus, 1971 and Edwardson and Chirstie, 1983). Scrolls, laminated aggregates, tubes and bundles were investigated in the actual study (Fig. 4. A, B, C, D). We attribute the differences in cylindrical inclusion morphology, which appear in this reports to differences in sampling. While all of the components of cylindrical inclusions may occur within cell, all the components would not appear in every thin section of that cell. Sections may have either laminated aggregates, and bending, Fig. (4A), pinwheels and laminated aggregates, (Fig. 4B), only scrolls (Fig. 4C) or only tubes, (Fig. 4D) ByMV induced many more scrolls than pinwheels.

Fifteen potyviruses exhibiting similar configurations in the proteinaceous plates constituting their cylindrical inclusions had recently been separated from other members of the group into subdivision-IV, **Edwardson (1974)**. On the basis of its cytoplasmic inclusion, ByMV became member of subdivision IV.

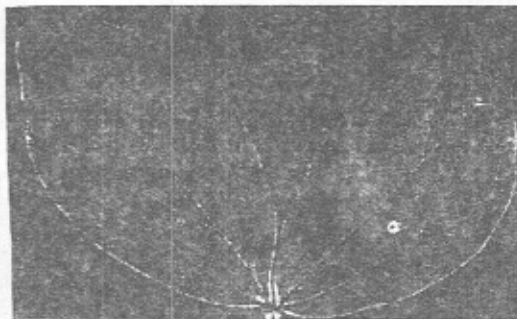
Collected results pointed out to a correlation between the degree and type of infection and the virus isolate aggressiveness. The most aggressive isolate was that of AMV, had the greatest destructive effect on host organelles and formed more abundant viral particle accumulations, but poorly developed inclusion bodies (tubes and scrolls). The less aggressive isolate of BBMV had considerable cytopathic effect on host organelles and abundantly formed viral particle accumulations, but no specific inclusions. The isolate of ByMV, showed to be the lowest aggressive, had a slight effect on cell organelles, meanwhile resulted in formation of abundant inclusion of different types but poor accumulation of viral particles.

The degree of host organelles damage and severity of disease symptoms were time dependant.

(A)



(B)



(C)

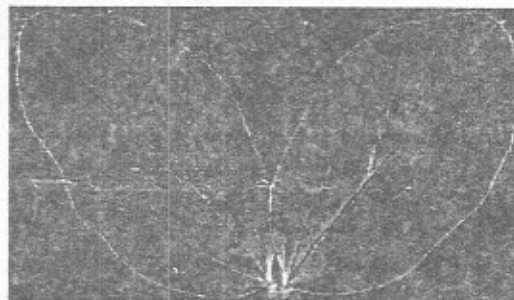


Fig 1. Broad bean infected leaflets caused by:

- A. AMV-(brown necrosis, 10 days after inoculation).**
- B. BBMV (systemic blotchy mosaic, 15 days after inoculation).**
- C. BYMV (severe mosaic 15 days after inoculation).**

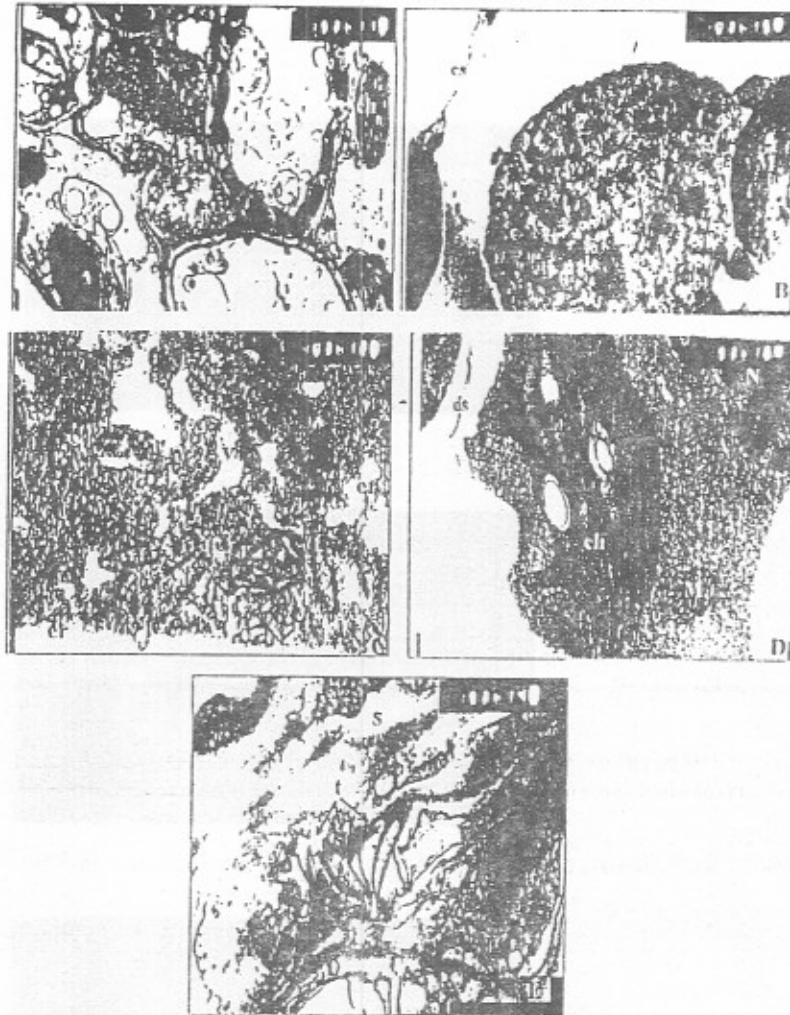


Fig. 2: Ultrastructural effects due to infection of faba bean leaflet 4 and 15 days after inoculation with alfalfa mosaic virus (AMV). Ultrathin sections through young phloem tissue: A. phloem parenchyma cell with deteriorated nucleus (N) containing a large masse of virus like particles and deformed chloroplast (ch), (x13.000). B. hypertrophied nucleus (N) with marginal distribution of heterochromatin. Mitochondria (M) with ellipsoid to shape, indistinct membranes and cristae in structure (x10.000) (4 days after inoculation). C. Endoplasmic reticulum (er) proliferated mass of virus particles (v) in cytoplasm (X40.000). D. Chloroplast (ch) with hypertrophy, poor undifferentiated laminar structure and cytoplasmic strand (cs). Virus like particles found in the chloroplast and cytoplasm (X25.000) 15 days after infection. E. Portion of cylindrical inclusions exhibiting scrolls (s) and tubes (t), (x 15.000) 15 days after inoculation.

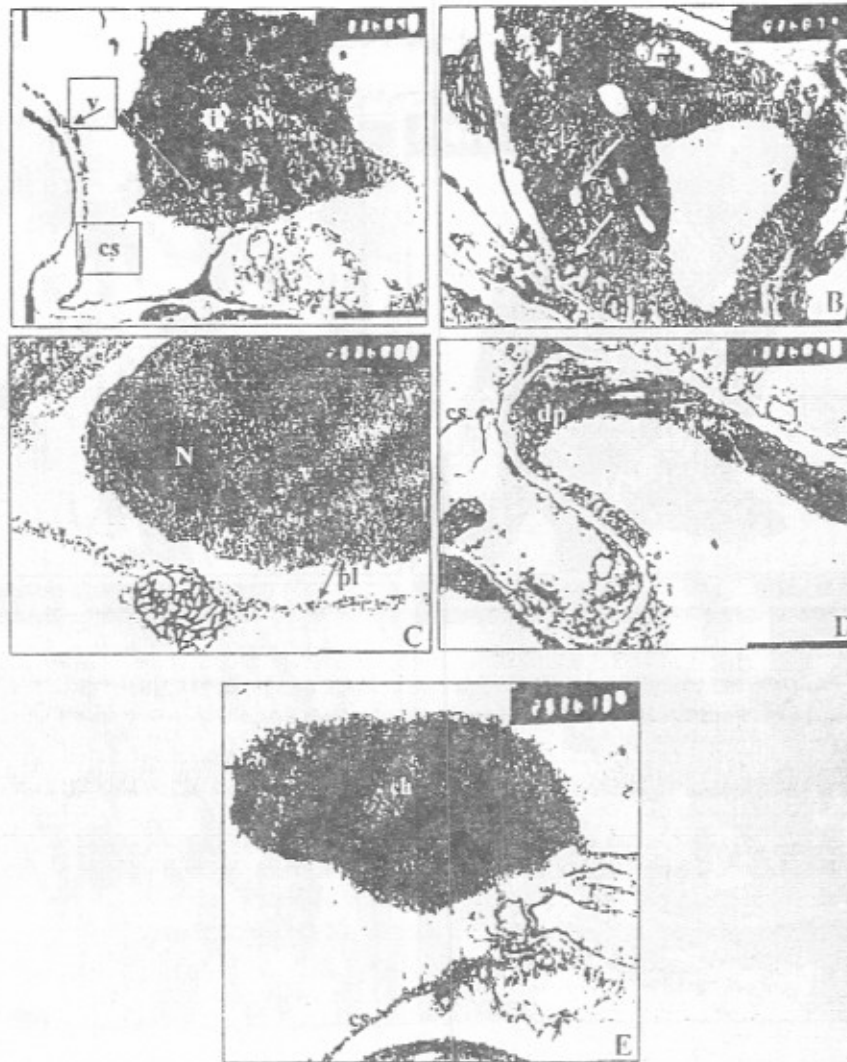


Fig.3: Broad bean mottle virus (BBMV) in phloem tissue of faba bean. A, Nucleus of abnormal shape with indistinct envelope(N), luacunose nucleolar inclusion (i). Mitochondria (M) of reduce size and cytoplasmic strands (x13.000). B, large numbers of reduced size mitochondria (M) and small vesicle, delimited by single membrane (arrows). C. mass of virus like particles (v) in cytoplasm associated with plasma lemma (pl) and proliferal endoplasmic reticulum (er) (x25.000). D. virus particles associated with degenerated plastid (dp) and cytoplasmic strands (cs)(x13.000). E. degeneration of chloroplast (ch) (x15.000).

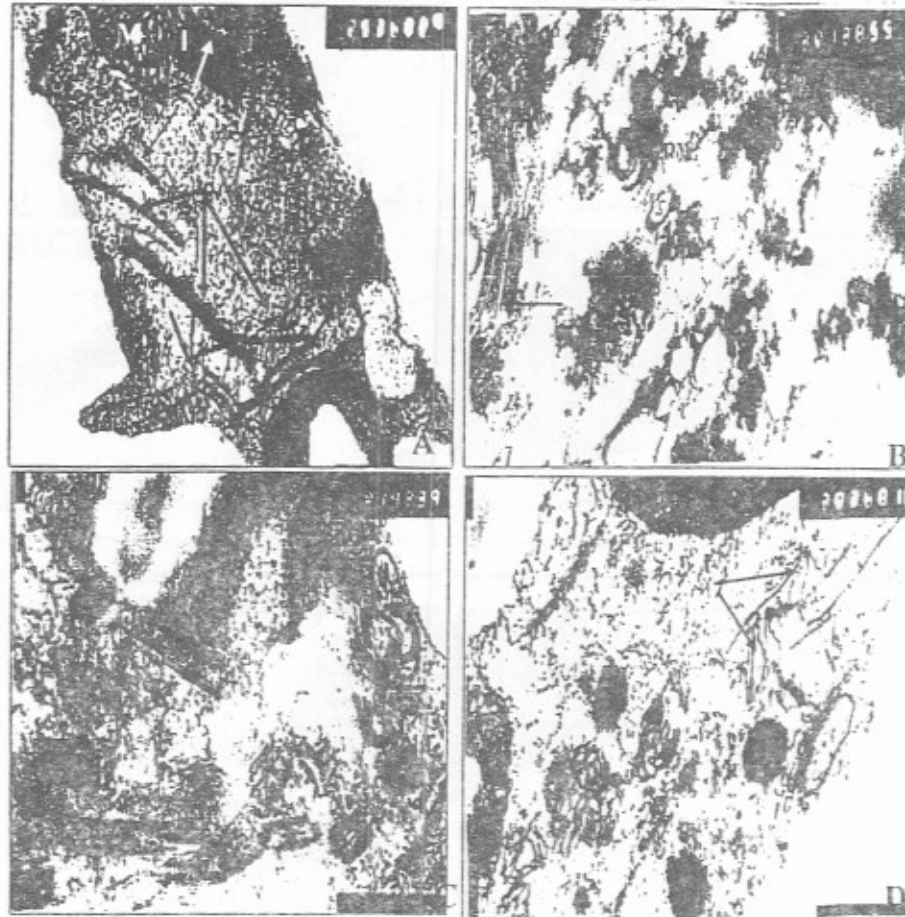


Fig. 4: Electron micrographs of faba bean cells infected with ByMV induced: A, Portions of cylindrical laminated aggregates (L) inclusions, bundle (b) and virus like particles in cytoplasm (arrow). Mitochondria (M) highly deformed losing their structure (x25,000). B, cylindrical inclusions pinwheels (pw). C, cylindrical inclusions exhibiting pinwheels (PW) and scrolls (S). d, center portions of cylindrical inclusions tubes (T).

The three different viruses reacted differently on the same host concerning lesions formation and type. Where ByMV with filamentous inclusions formed various types of inclusions (5 types) in considerable amounts, BBMV with spherical virions did not form any. In contrast Moorman and (Woodbridge 1983 and Moorman 1982) reported that two types of crystalline inclusion were observed in the epidermis covering the abaxial surface of the vein of the inoculated leaves. Consequently, AMV having multi virions (rod and spherical) formed few types of inclusions (2-types) in small amounts.

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التركيب الخلوي الدقيق المقارن لانسجة الفول المصابة بفيروس تبرقش البرسيم
الحجازي وفيروس تبرقش الفول وفيروس تبرقش الفاصوليا الأصفر
مرفت مصطفى فتح الله
معهد بحوث أمراض النباتات ، مركز البحوث الزراعية - الجيزة

درس تأثير ثلاث عزلات كل لفيروس يتبع مجموعة فيروسية مختلفة وهى فيروس تبرقش البرسيم الحجازي الفامو فيروس متعدد الجسيمات (كروية الى عصوية باطوال مختلفة) ، وفيروس تبرقش الفول برومو فيروس (كروي الجسيمات) ، وفيروس تبرقش الفاصوليا الاصفر بوى فيروس (خيطة الجسيمات) ، على التغيرات المرضية فى خلية العائل المصاب (فول صنف جيزة بلانكا) . اثرت الفيروسات المختبرة على خلايا العائل المصاب مسببة تشوهات وتدميرا لعضيات هذه الخلايا . كما انه نتج عن غزو الفيروسات تكشف طرز مختلفة من الاجسام المحتواه، وتجمعت فريونات كل نوع فيروسي بدرجات مختلفة فى مواقع مختلفة من خلية العائل . ظهر ان مدى القوة الامراضية للعزلة الفيروسية يرتبط بمدى تأثيرها المرضي على خلية العائل وعضياتها ، كما يرتبط طرديا مع مضي الوقت بعد التفريح بالفيروس .

سبب فيروس تبرقش البرسيم الحجازي اكبر تأثير تدميري على عضيات العائل وكون تجمعات كبيرة من الفيريونات مع تكشف القليل من الاجسام المحتواه كما ونوعا ، وكانت هذه العزلة هي الاشد مرضية على الفول . سبب فيروس تبرقش الفول ضررا اقل نسبيا لعضيات العائل ، وكون تجمعات كثيرة من جسيمات الفيروس ، ولكنه لم يكون اي اجسام محتواه ، وكان هو اقل امراضية ، نسبيا ، من الفيروس السابق . اما فيروس تبرقش الفاصوليا الاصفر فكان ذا تأثير ضعيف على عضيات خلايا العائل ، وكون تجمعات فقيرة من جسيمات الفيروس بينما احدثت تكشفيا جيدا لطرز متعددة من الاجسام المحتواه ، وكان هو الاقل شدة امراضية .

وقد لوحظ ان عزلة الفيروس ذى الفيريونات الكروية تميل الى عدم تكوين اجسام محتواه وان عزلة الفيروس ذى الفيريونات الخيطية تميل الى تكوين الكثير من الاجسام المحتواه وبطرز متعددة ، بلغت خمسة ، فى حين ان عزلة الفيروس ذى الجسيمات المتعددة (الكروية والعصوية) كانت متوسطة فى هذا الصدد، حيث كونت طرازين فقط من هذه الاجسام .