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

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ABASTRACT

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 deterurations of host cell organelles and formed various types of cytoplasmic inclusions, in addition to virions accumulations.

The aggrisiveness of virus isolates seemed to be corolated to their cytopathic effect on host cell organelles and was time dependant. AMV, with the greatest damaging effect on organelles, forming abundant virion accumilations 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 aggrissive. BYMV showed a slight effect on host organelles with poor virions accumilations but formed nomerous types of inclusions, was the lowest aggrissive.

Viruses, with sphaerical 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, ultrastrucul, 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 alfomovirus (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 (PesbMV) were most prevalen (EL- Afifi and EL-Dougdoug 1997, EL- Hammady *et al* 2000, Fegla *et al* 2003, Fortass and Bos1991, 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 (mosaico 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 leave veins of *Capsicum annuun* 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 nucleorlar inclusion in cytoplasm. Cylindrical inclusions appeared as scroll-type, pin wheels, tubes, bunding, short, and laminated aggregates (DeBokx and Watteraus 1971, Lisa *et al.* 1981, Hunst and Tolin, 1982, Edwardson 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 payed 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 Developent Center Farm. The virus was isolated from its original host with typical disease symptoms, indentified on the basis of diganostic hosts and serological reactions. AMV antiserum uaed in this study was locally prepared during the course of her study for Ph.D.

The virus was maintaned on *Necotiana glutinosa* plants, grown in insect proof green house, untell 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 Universittly, at Abees 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 mentained on *Vicia faba* by mechanical transmission and kept under grenhouse 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 michanically inoculated. Inoculated plants were kept under carefull observation in a green house. Two weeks after symptoms development, leaflets of iniected broad bean were collected for further investigations.

Disease Sevirity:

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 vacum, rinsed with the same buffer, then postifixed in 1% osmium tetroxide (O_sO_4) for 2 hr. Sample were prestained in 0.5% uranyle acetat at 4°C, overnight. Tissue dehydration was acheved by runing through an ethanol series, then imbeded in Spurr's epoxy-resin. Ultra thin sections of 10 nm theckness 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 examind 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 coures of this study. The infection percentage as well as disease severity (symptom type) on the host, *faba bean* cv. Giza Blanka 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 standed as brown necrotic spots 10 day after inoculation, then death of whole plant by the end of the month.

BBMV gave moderate infection of 40% , but more sever symptoms, developed as necrotic local lesion in the first inoculated leaves, 5-7 day after inoculation, then vien 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 viens and bright green or yellow mosaic spots on the leaves flowed by severe mosaic.

Electron Microscopy:

The careful examination of ultrathin sections of infected broad bean leaffets 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 deteruration, 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 distinctured (Fig. 2B).

Chloroplast had poor undifferentiated laminar structure. Cell sytoplasms were occasionally occupied with multivaculated 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 dence 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). There fine structure tented 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 proliferal endoplasmic reticulum (Fig. 3C).

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

No cytoplasmic inclusions were be 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 lossing 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: necrtic 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 evaluuate their pathoginicity 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).

Ultrasturctural 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 sipherical viruses. An association of the multivesicualar inclusions to chloroplasts or mitocondria was found in BBMV affected cells. Bunding 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. Is 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 last 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.

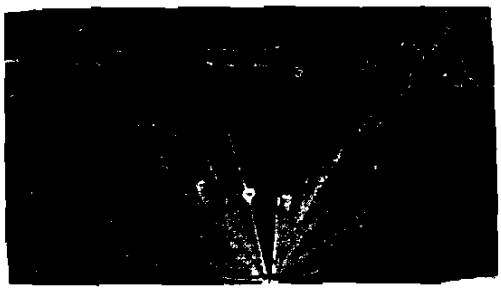
Various aspects of the central portion of potyviruses induced cylindrical inclusions, pinwheels, and bundles appeared in the report of (De Box and Wattereus, 1971 and Edwardson and Chirstie, 1983). Scrolls, laminated aggregates, tubes and bundles were investigated in the actual study (Fig. 4. A, B, C, 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 accur within cell, all the components would not appear in every thin section of that cell. Sections may have nither 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 corolation 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 partied 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 particles 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 lenflets caused by:

A. AMV-(brown neacrosis, 10 days after inoculation).
B. BBMV (systemic blotchy mosaic, 15 days after inoculation).
C. BYMV (severe mosaic15 days after inoculation).

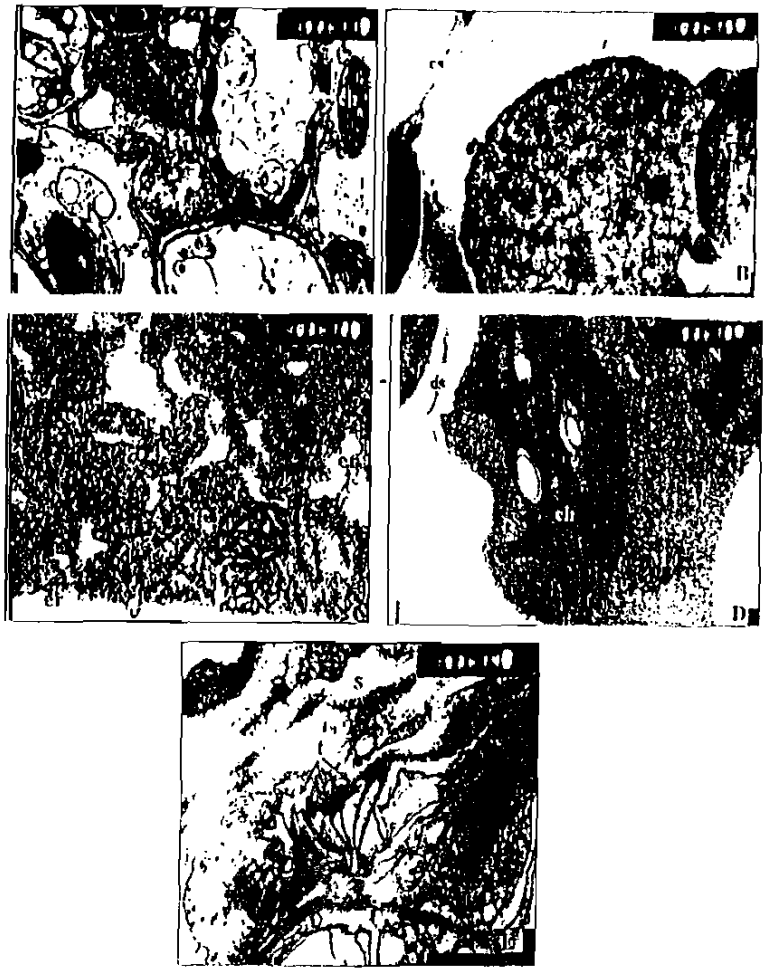


Fig. 2: Ultrastructural effects due to infection of faba bean leaflet 4 and 15 days after inoculation with alfalfa mosaic virus (AMV). Ultrathin sections through young phloem tissue: A. phloem parenchyma cell with deteriorated nucleus (N) containing a large masse of virus like particles and deformed 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 cytoplast (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 inocuiation.

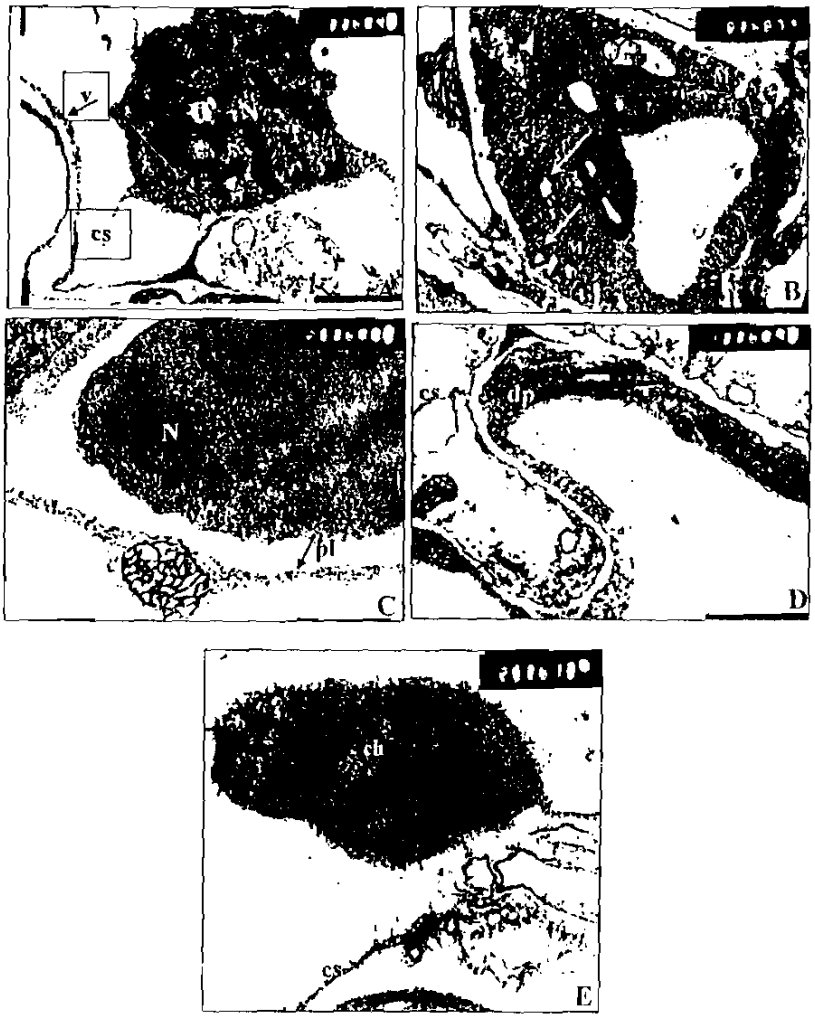


Fig.3: Broad bean mottle virus (BBMV) in phloem tissue of faba bean. A, Nucleus of abnormal shape with indistinct envelope(N), luacunose nucleolar inclusion (i). Mitochondria (M) of reduce size and cytoplasmic strands (x13.000). B, large numbers of reduced size mitochondria (M) and small vesicle, delimited by single membrane (arrows). C. mass of virus like particles (v) in cytoplast 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).

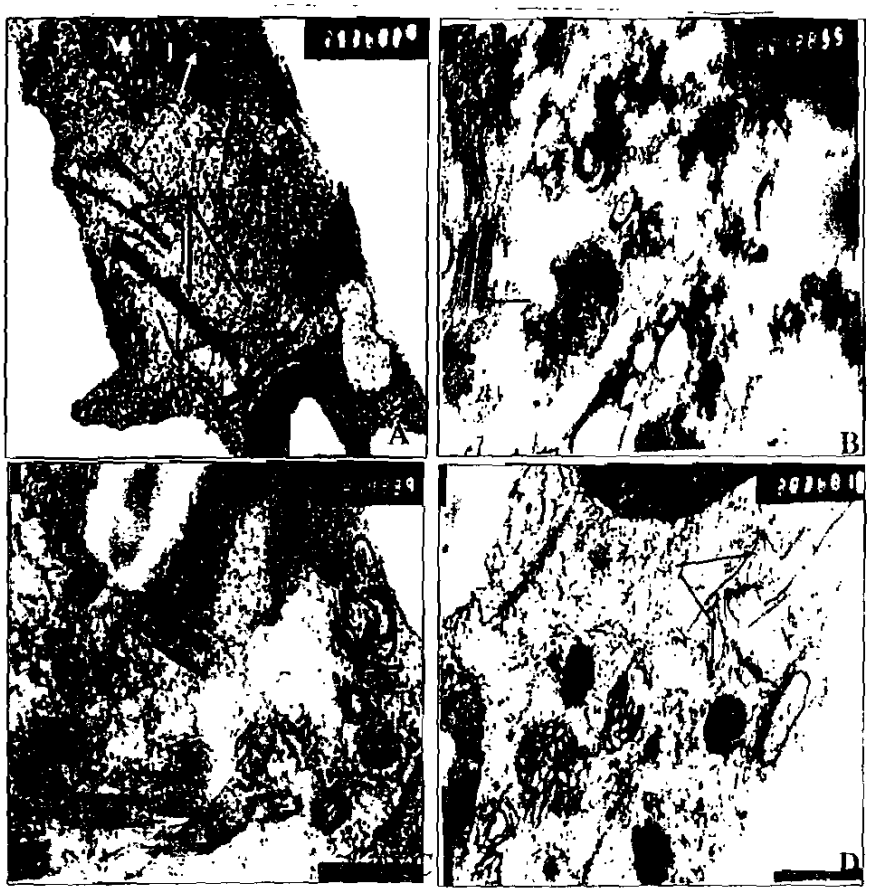


Fig. 4: Electron micrographs of faba bean cells infected with ByMV induced: A, Portions of cylindrical laminated aggregates (L) inclusions, bundle (b) and virus iike particies in cytopiasm (arrow). Mitochondria (M) highly deformed losing their structure (x25.000). B, cylindrical inclusions pinwheels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

The three different viruses reacted diffirently on the same host concerning lesions formation and type. Where ByMV with filamentous visions formed various types of inclusions (5 types) in considerable amounts, BBMV with spherical virions did not form any. In contrast Moorman and (Woodbrige 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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