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Characterization of *Bean common mosaic virus* strain blackeye cowpea mosaic infecting cowpea (*Vigna unguiculata* (L.) Walp.) in Egypt

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ABSTRACT

Bean common mosaic virus strain blackeye cowpea mosaic (BCMV-BICM), a unique and commercially significant virus that naturally infects cowpea plants and displays a range of symptoms such as mosaic or mottling, vein clearing, vein banding, blistering, necrosis, yellowing, and leaf malformation was obtained from the experimental farm of the Agricultural Research Center in Giza, Egypt during the 2018–2019 growing season. The goal of this research is to identify BCMV-BICM utilizing biological, serological, histological, cytological, and molecular methods. By studying the host range, BCMV-BICM infects just eight plant species and cultivars out of twenty-four plant species and cultivars belonging to four families. The proportion of aphis transmission of BCMV-BICM was 40%. Seed transmission varied from 5% to 8% in the three cultivars evaluated. BCMV-BICM only responded favorably with specific antisera against BCMV in a direct enzyme-linked immunosorbent assay. Light microscopy revealed amorphous cytoplasmic inclusions generated by BCMV-BICM in cowpea leaf strips. The findings clearly established structural differences between cowpea plants infected with BCMV-BICM and healthy plants (control). The electron microscopy of cleared infected cowpea extract stained negatively with 2% phosphotungstic acid exhibited filamentous flexuous particles with a length of 720–750 nm. Reverse transcription-polymerase chain reaction (RT-PCR) analysis and sequencing of RT-PCR products were used to determine the identity of the virus. This is the first report of a BCMV-BICM isolate found spontaneously in Egyptian cowpea plants.

1. INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is a necessary and universally adaptable nutritional legume raised for grain and fodder all over the world. It provides a range of meals as well as revenue and fodder for livestock [1,2]. With a mean yield of 7216.29 tons of dry seeds, the total area of cowpea plants grown in Egypt was estimated to be 1968 hectares [3].

Viral diseases are significantly reducing cowpea yields in Asia, Africa, and Latin America. Worldwide, more than 20 viruses have been reported to naturally infect cowpea, and some are seed-transmitted [4]. *Bean common mosaic virus* strain blackeye cowpea mosaic (BCMV-BICM) has been found to naturally infect cowpeas and to be transmitted through seed [5-7]. Seed-borne viruses have been disseminated to most cowpea-producing regions of the world through the exchange of seed [8]. In most cases, legume seeds infected by viral pathogens are considered the primary source of infection and the resulting field infection may rise to 100% within 2 months, depending on the abundance and activity of the vector [9].

The (BCMV-BICM, equivalent or another scientific name *Blackeye cowpea mosaic virus* (BICMV)) [10] and *Cowpea aphid-borne mosaic virus* (CABMV) are both economically significant and universal seed-borne viruses of cowpea. Analysis of BCMV-BICM coat protein composition [11] and sequencing of the coat protein gene [12] proved the close relationship of the BCMV to what was once considered a separate virus, BCMV-BICM. The identification of strains is supported by serology with strain-specific antibodies and sequence analysis [13,14].

BCMV-BICM, which infects cowpea, is one example of a seed-borne virus that can negatively affect germination, infect seedlings and harm mature plants. To characterize BCMV-BICM infecting cowpea plants,

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the current study used biological, serological, histological, cytological, and light and electron microscopy approaches. In addition, molecular analyses were used to establish the identification of the viral isolate and to compare the coat protein genes' nucleotide sequences with some standard isolates recorded in GenBank.

2. MATERIALS AND METHODS

2.1. Isolation and Propagation of Virus Isolate

Leaf samples of naturally infected cowpea (*V. unguiculata* (L.) Walp.) plants exhibiting virus-like symptoms consisting of various types of mosaic or mottling, vein clearing, vein banding, blistering, necrosis, yellowing, and leaf malformation were obtained from the experimental farm of Giza Agricultural Research Center (ARC), Egypt with zigzag methods as described by Anderson [15] during 2018–2019 growing season.

The BCMV-BICM was maintained in the greenhouse. It was treated for identification in the subsequent experiments. Mechanical inoculation was carried out in the greenhouse to isolate and identify the causal agents as described by Noordam [16]. The inoculum was prepared to inoculate healthy test plants such as Cowpea cv. Kafer El Sheikh and *Chenopodium amaranticolor*. Inoculated seedlings were kept under observation in an insect-proof greenhouse.

The virus isolate was biologically purified by three consecutive transfers of single local lesions [17] formed on *C. amaranticolor*. Finally, a single local lesion was propagated in cowpea plants of cv. Kafer El Sheikh, which was then used as a source of the virus isolate.

2.2. Identification of Virus Isolate

2.2.1. Host range and symptomatology

Ten healthy 15-day-old seedlings each of twenty-four plant species and cultivars belonging to four different families were mechanically inoculated as described by Noordam [16], using young, systemically infected leaves of cowpea cv. Kafer El Sheikh for BCMV-BICM.

2.2.2. Modes of transmission

2.2.2.1. Aphid transmission

Apterous *Aphids cracivora* Koch identified in Plant Protection Res. Institute, ARC were reared on virus- free faba bean cv. Masr1 and starved for 1 h., then given 2 min acquisition access period on symptomatic cowpea leaves infected with BCMV-BICM. Viruliferous individuals were then transferred to ten healthy 7-day-old cowpea cv. Kafer El Sheikh seedlings (5 individuals/plant) for inoculation-feeding periods of 4–6 h., after which they were killed by spraying with insecticide (Actikil 50%). Plants in the control experiment received aphids from asymptomatic ones. Symptoms and the percentage of transmission were recorded for 30 days after inoculation.

2.2.2.2. Seed transmission

An experiment was carried out to study the transmission of the virus isolate through cowpea seeds. Cowpea seeds from the three cultivars tested (Tiba, Kafer El Sheikh, and Dokii 331) were seeded separately in 20-cm-diameter pots (5 seeds/pot) containing sterilized peat-sand-clay (1:1:1) mixture and grown under virus-free glasshouse compartments before inoculation. Two weeks later, the resultant seedlings were mechanically inoculated, either separately with the isolate or with healthy crude sap (the control). Virus-infected plants showing typical symptoms as well as healthy plants were left to mature. Subsamples composed of two hundred seeds, harvested from plants representing different treatments, were sown in clay pots, as mentioned. Grown seedlings were observed for symptom development for 30 days and tested by enzyme-linked immunosorbent assay (ELISA). The percentage of virus seed transmission was estimated in cowpea cultivars tested. Seeds from healthy plants were used as controls and received similar treatments.

2.2.2.3. Virus incidence in seed parts

Another experiment was carried out using a double antibody sandwich ELISA (DAS-ELISA) to assess the virus isolate infected seed parts of cowpea cv. Balady or not. One hundred cowpea seeds raised from infected plants with BCMV-BICM were soaked individually in water for 2 days, avoiding seed-to-seed contamination. The coats were removed manually, whereas individual embryos were aseptically dissected with a razor blade into two cotyledons and embryo axis, then they were washed and triturated separately with a mortar and pestle in seed antigen buffer (0.04 M KH_2PO_4, 0.46 M Na_2HPO_4, 0.14 M NaCl, 0.003 M NaN_3, 0.003 M KCl, 0.05 [v/v] Tween 20, PH 7.5) at the rate of 1 mL for each coat and cotyledon sample and 0.5 mL for each embryo axis [5]. Each extract was used as an antigen in DAS- ELISA.

2.2.3. Serological diagnosis of the virus isolate

BCMV-BICM was tested by direct-ELISA [18] using obtainable antisera specific to CABMV, BCMV, *Bean yellow mosaic virus* (BYMV). Results were assessed by measurement of absorbance at 405 nm in a Vinskan ELISA reader. Reading greater than twice the value of healthy controls was considered positive.

2.2.4. Histological and cytological studies

2.2.4.1. Virus-induced inclusions by light microscopy

Epidermal strips obtained from the lower surface of healthy and systemically infected cowpea leaves with BCMV-BICM were treated with 5% Triton X-100 for 10 min [19]. Finally, the treated strips were examined under a light microscope (model Olympus Japan) and photographs were taken with an Olympus C-35AD-4 camera from Japan.VICZ, Germany, after being kept on glass slides.

2.2.4.2. Histological study

Semithin leaf sections were prepared to check the histological and cytological changes induced by BCMV-BICM infection in cowpea leaves. The midrib and small parts of the leaf blades of healthy and infected cowpea leaves were examined histologically. Semithin sections (1 m thick) were prepared using the Leica Ultra Cut UCT ultramicrotome and stained with toluidine blue for 90 s before being examined using the previous light microscope model M-200M at Cairo university research park faculty of agriculture (CURP).

2.2.4.3. Cytological studies

Ultra-thin sections of cowpea leaf cells were investigated to detect the effect of virus infection using the following procedure: the samples were fixed overnight in cold 2.5% glutaraldehyde prepared in 0.1 M potassium phosphate buffer (PH 7.4) and post-fixed in 1% osmium tetroxide (OSO_4) in the same buffer for 3 hr. After staining overnight in 1% uranyl acetate, the specimens were dehydrated in the ethanol-acetone series and embedded in Spurr's medium according to the method described by Rocchetta *et al*. [20] and adopted by Udayashankar *et al*. [2] and El-Banna *et al*. [21]. Ultrathin sections were cut with an ultramicrotome, stained with 5% uranyl acetate for 20 min, and stained with Reynolds' lead citrate for 10 min. Images were recorded by a JEOL electron microscope (JEM-1400 TE, Japan), as mentioned above.

2.2.5. Particle morphology

To study the morphology of virus particles, a drop of clarified, extracted sap infected with BCMV-BICM as described by Noordam [16], was applied to carbon-reinforced, formvar-coated copper grids for 2 min. A drop of 2% potassium phosphotungstate (a negative stain), pH 6.5, was added. One minute later, the excess stain at the edge of the grid was drained with the aid of filter paper, and the stained grids were then examined in the electron microscope unit of the faculty of agriculture at Cairo University. At the candidate magnification, virus particles were photographed.

2.3. Molecular Detection and Characterization of BCMV-BICM

2.3.1. Reverse transcription-polymerase chain reaction (RT-PCR)

In order to detect BCMV-BICM, total RNA was extracted from collected cowpea cv. Dokii 331 infected and uninfected samples using the Total RNA Mini Kit plant (Geneaid, New Taipei City, Taiwan) following the manufacturer's instructions and the purified RNA was used as a template for RT-PCR amplifications. Primers of BCMV (F-`3-CCA ATG GTT GAA AAT GCA AAG CC-5`) and (R- `5-CGA CGC GAG ATG CTA ACTG-3) El-kady *et al.* [22] were used to amplify 404bp of its coat protein gene.

2.3.2. One-step RT-PCR

One-step RT-PCR was performed using Verso TM one-step RT-PCR kit (Thermo Scientific) in a 25 µL reaction mixture containing 3 µL RNA sample, 12.5 µL of one-step PCR master mix (×2),1.5 µL 10 µM of each primer,0.25 µL Verso enzyme mix, 2.5 µL RT Enhancer and 3.75 µL of nuclease-free water. The reaction conditions were as follows: Synthesis of cDNA was done at 50°C for 15 min and denaturation at 95°C for 2 min followed by 35 cycles at 95°C for 1 min, 55°C for 1 min and 72°C for 1 min and final elongation step at 72°C for 10 min for BCMV. PCR products were loaded in 1% agarose gel with 100 bp DNA ladder (BIOMATIK) in ×0.5 TBE buffer, then visualized on 1% UV illumination using the Gel Documentation system (Gel Doc 2000, Bio-Rad, USA). RNA extractions from healthy plants were used as the negative control.

2.3.3. DNA Purification and sequencing

RT-PCR fragments were cut from the gel and purified using the Gel/PCR DNA Fragments Extraction Kit (Geneaid, New Taipei City, Taiwan) following the manufacturer's instructions. Purified DNA was used for direct sequencing at the Macrogen Company (South Korea). Sequencing results for both viruses were analyzed using DNAMAN Sequence Analysis Software and compared with the isolate of BCMV available in GenBank.

3. RESULTS AND DISCUSSION

3.1. Isolation and Propagation of the Virus Isolate

From naturally infected cowpea plants grown in the experimental farm of Giza ARC, Egypt, one distinct virus isolate representing one different host and morphological virus particles were obtained. Infected plants exhibited the characteristic symptoms of BCMV-BICM, consisting of mosaic or mottling, green vein banding, vein chlorosis, vein yellowing, yellow spots, blistering and distortion of leaves. The virus isolate was distinguished by its susceptibility to infect the indicator test seedlings. The virus was identified based on host range, symptomatology, modes of transmission, serological typing, histological and cytological studies, as well as particle morphology. The identity of the virus isolate was further supported by molecular studies. The isolated virus after identification (as shown later) is BCMV-BICM (synonym, BICMV).

3.2. Identification of BCMV-BICM

3.2.1. Host range and symptomatology

The reactions of twenty-four plant species and cultivars belonging to four different families to virus infection are summarized in Table 1. Among twenty-four plant species and cultivars belonging to four families, it has a restricted host range and infects only eight species [Figure 1] and cultivars belonging to the *Chenopodiaceae* and *Fabaceae*. The virus caused local lesions in inoculated *C. amaranticolor* and *Chenopodium quinoa*. BCMV-BICM produced yellow local lesions on these hosts. Additionally, cowpea plants infected with BCMV-BICM developed chlorotic local lesions followed by vein clearing, vein banding, and mosaic or mottling. On the other hand, it did not infect pea and faba beans; it produced systemic symptoms only on bean cv.

Table 1: Reaction of selected host range mechanically inoculated with Bean common mosaic virus strain blackeye cowpea mosaic.

Family species cultivar	English name	Reaction	
		L	S
Chenopodiaceae			
Ch. amaranticolor Cost and Reyn	Ghost foot	YLL	-
Ch. quinoa Wild		YLL	-
Cucurbitaceae			
C. sativus L. cv. Mayadeen	Cucumber	-	-
C. melo L. cv. Balady	Melon	-	-
C. pepo L. cv. Balady	Squash	-	-
Fabaceae			
G. max L. cv. Giza 21	Soybean	-	-
P. vulgaris L. cv. Giza 6	Green bean	-	-
cv. Nebraska		-	-
cv. Contender		-	M or Mo+VB
cv. Mont calm		-	-
cv. Bronco		-	-
cv. Paulista		-	-
cv. Karnak		-	-
P. sativum L. cv. Master B	Pea	-	-
cv. Snow wind		-	-
V. faba L. cv. Masr1	Broadbean	-	-
cv. Nobaria		-	-
V. unguiculata (L.) Walp cv. Kareem	Cowpea	CLL	VB+M or Mo
cv.Qaha1		CLL	VB+M or Mo
cv.Dokii331		CLL	VB+M or Mo
cv. Kafer El-Shiekhe1		CLL	VB+M or Mo
cv. Balady		CLL	VB+M or Mo
Solanaceae			
N. tabacum L. cv. White Burley	Tobacco	-	-
L. esculentum Mill cv. Nada	Tomato	-	-

LL: Local lesions, S: Systemic infection, CLL: Chlorotic local lesions, NLL: Necrotic local lesions, VB: Vein banding, -: No symptoms, M: Mosaic, Mo: Mottle, YLL: Yellow local lesions, *Ch. quinoa: Chenopodium quinoa, Ch. amaranticolor: Chenopodium amaranticolor, C. sativus: Cucumis sativus, C. melo: Cucumis melo, C. pepo: Cucurbita pepo, G. max: Glycine max, P. vulgaris: Phaseolus vulgaris, P. sativum: Pisum sativum, V. faba: Vicia faba, V. unguiculata: Vigna unguiculata, N. tabacum: Nicotiana tabacum, L. esculentum: Lycopersicon esculentum*

Table 2: Transmission of Bean common mosaic virus strain blackeye cowpea mosaic by *Aphis craccivora* Koch.

Test plant	Virus isolate	Number of infected plants/number of inoculated plants	Infection (%)
Cowpea cv. Kafer El-Sheikh	BCMV-BICM	4/10	40

BCMV-BICM: Bean common mosaic virus strain blackeye cowpea mosaic.

Table 3: Seed transmission of Bean common mosaic virus strain blackeye cowpea mosaic by different cowpea cultivars.

Cowpea cultivars	BCMV-BICM	
	Ratio	Seed transmission (%)
Tiba	15/200	7.5
Kafer EL-Sheikh	10/200	5
Dokii 331	16/200	8

BCMV-BICM: Bean common mosaic virus strain blackeye cowpea mosaic.

Table 4: Incidence of enzyme-linked immunosorbent assay-detectable bean common mosaic virus-blackeye cowpea mosaic in seed parts of infected cowpea tested cultivar.

Seed parts	Number of infected/number of tested	Infection (%)
Coat	16/100	16
Cotyledons	3/100	3
Embryo axis	3/100	3

Contender. These BCMV-BICM results appear to be consistent with Hao *et al*. [23], UdayaShankar *et al.* [24] and Purcifull *et al*. [25]. It has been postulated that symptoms produced are dependent on the particular virus, the strain involved, the species and age of the plant, the time of the year, and environmental conditions [7].

3.2.2. Modes of transmission

3.2.2.1. Aphid transmission

Since aphids are important in virus transmission, *A. craccivora* was used to study the transmission of the virus isolated. The percentage of BCMV-BICM transmission was 40% [Table 2]. This is in agreement with Purcifull *et al*. [25], Wall *et al*. [26], and Dijkstra *et al.* [27]. The difference in the capability of the insect that transmits the virus may reflect a difference in the concentration of the virus in the source plant.

3.2.2.2. Seed transmission

Seed transmission is an extremely efficient method of introducing viruses into a crop at an early stage, resulting in randomized foci of infection throughout the plants. Subsequently, when some other method of transmission can spread the virus within a crop, In this work, trials have succeeded in transmitting the virus isolate through the seed of three cowpea cultivars. The percentage of seed transmission varied according to the virus isolate and cowpea cultivar. For BCMV-BICM, the percentage ranged from 5 to 8% [Table 3]. These results are in agreement with Hao *et al*. [23], UdayaShankar *et al*. [24], and Bashir *et al*. [28]. Diversity in viral seed-transmission rates would be a consequence of interactions involving host genotype, age of plant infection, viral isolate characteristics, plant vigor and nutrition, temperature, and possible virus-pathotype interaction [22,29,30].

3.2.2.2.1. Virus incidence in seed parts

Results in Table 4 showed that BCMV-BICM infected the coat, cotyledons, and embryo axis of the tested cowpea cv. Blady [Figure 2].

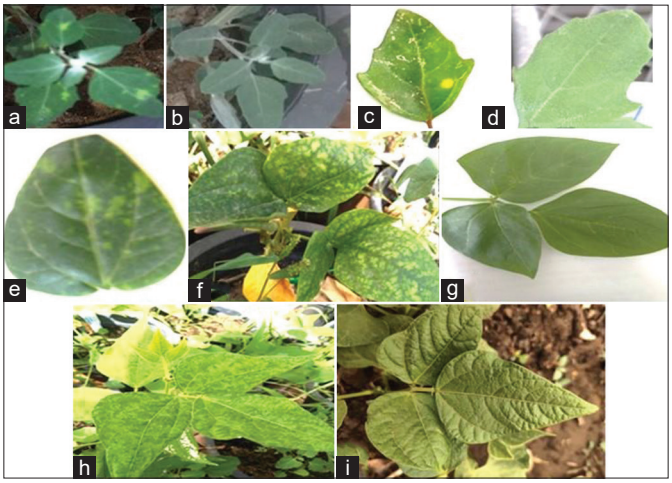

Figure 1: Plants infected with BCMV-BICM: (a) yellow local lesion produced on either *Chenopodium amaranticolor* (b) healthy control or *Chenopodium quinoa* (c and d) Healthy plant. (e) chlorotic local lesions exhibited on cowpea cv. Kafer El Sheikh (cotyledons leaf) followed by systemic mottling and vein banding on trifoliate (f and g) Healthy one. (h) systemic mosaic and vein banding on bean cv. Contender and (i) Healthy plant.

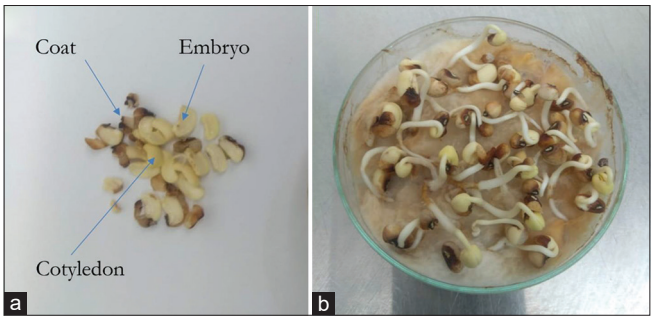

Figure 2: (a) Seed parts (coat, cotyledon and embryo) of infected cowpea cv. Balady, (b) germinated seeds.

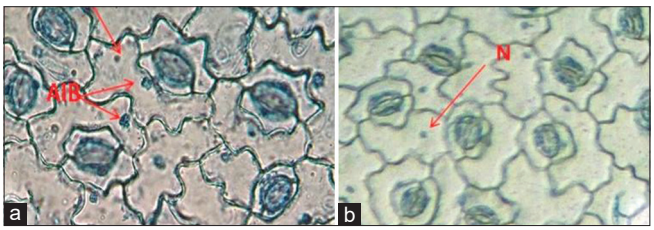

Figure 3: (a) Light microscopy of epidermal strips of cowpea cv. Dokii 331 leaf infected by BCMV-BICM,14 days post-inoculation showing amorphous inclusion bodies (AIB). (b) Healthy epidermal cells. N-Neculos. (X=350).

The incidence of positive BCMV-BICM tests in these parts was recorded at 16, 3 and 3%, respectively. These results agree with the previous results obtained by Gillaspie *et al*. [5], who worked on BCMV-BICM. On the other hand, Sekar *et al*. [31] mentioned that infectious BCMV-BICM was present in cotyledons and embryo axis in mature seeds of two infected cowpea cultivars, whereas the virus was absent from the seed coats.

3.2.3. Serological diagnosis

Serological tests provide rapid and convenient methods for the identification and definition of plant viruses [30-32]. Positive

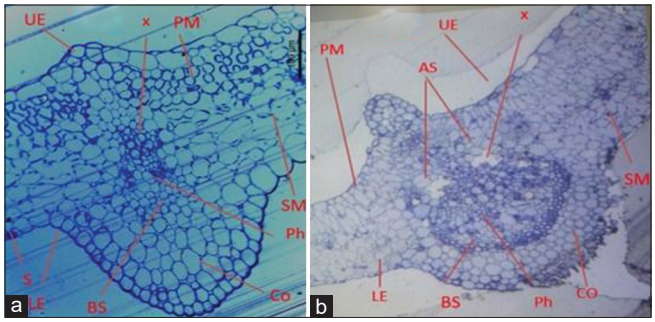

Figure 4: Infected tissues and healthy tissue (a), light microscopy of semi thin section of BCMV-BICM (b) UE: upper epidermis, LE: Lower epidermis, PM: Palisade mesophyll, SM: Spongy mesophyll, AS: Air space, VB: Vascular bandle (X: Xylem, Ph: Phloem), Co: Collenchyma, S: Stomata. (X=400), BS: bundle sheath cells

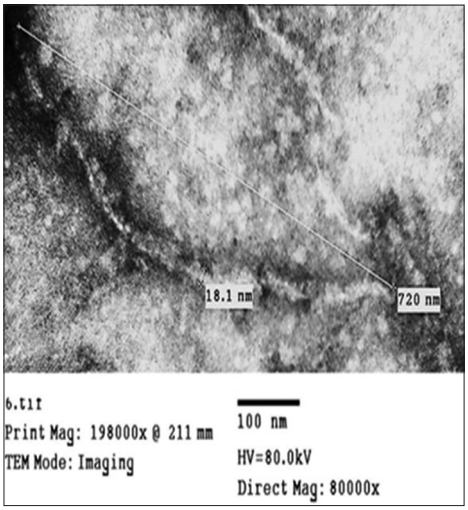

Figure 6: Filamentous flexuous virus particles of BCMV-BICM ranged from 720 to 750 nm in length (Magnification X 50.000).

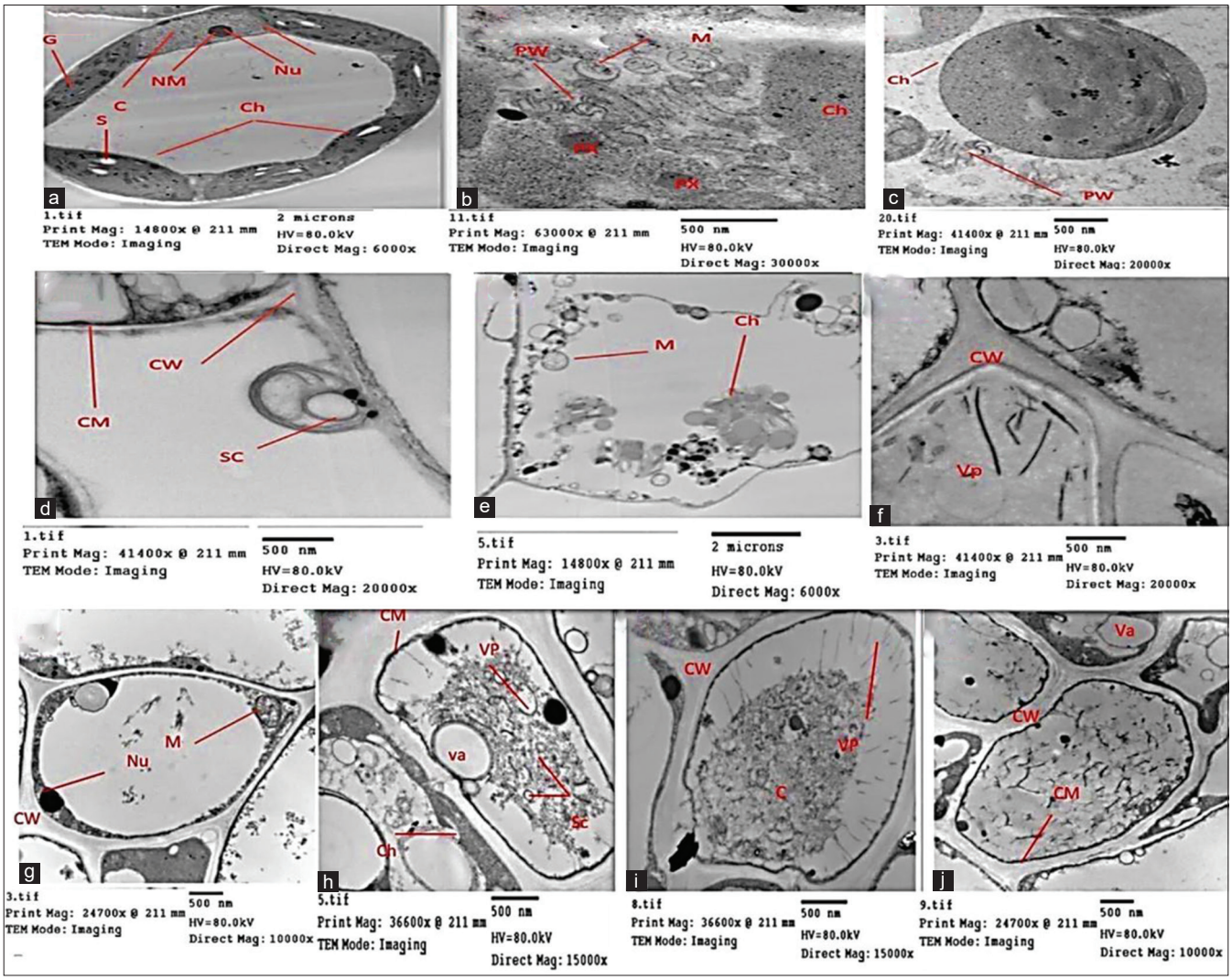

Figure 5: (a-j) Ultrastructure of cowpea leaf tissues infected with BCMV-BICM compared with healthy ones. CW: Cell Wall, Ch: Chloroplasts, S: Stroma, G: Grana, Nu: Nucleus, NM: Nuclear Membrane, C: Cytoplasm, CM: Cytoplasm Membrane, PW: Pinwheels, PX: Peroxisomes, M: Mitochondria, Sc: Scrolls, VP: Virus Particles, Va: Vacuoles.

Table 5: Comparison of the nucleotide sequences of the *bean common mosaic virus* strain blackeye cowpea mosaic coat protein gene isolated in the present study with the corresponding sequences of other isolates available in the Genbank.

Isolate name	Accession Number	Country	Original Host Plant	Identity%
BCMV-BlCVN/BB2-6	DQ925423.1.seq	Vietnam	Black bean (*P. vulgaris*)	98.9
DH-C	MH795801.1.seq	India	*V.unguiculata*	98.4
BCMV-BlC-VN/RB2	DQ925421.1.seq	Vietnam	Red bean (P. vulgaris)	98.1
BCMV-BlC-VN/RB1	DQ925420.1.seq	Vietnam	Red bean (P. vulgaris)	97.8
BCMV-BlC-VN/BB1	DQ925417.1.seq	Vietnam	Black bean (*P. vulgaris*)	97.8
BICMV	AY575773.1.seq	Taiwan	Not available	97.8
BICMV	AF395678.1.seq	China	Not available	97.8
Mysore-BCMV strain blackeye cowpea mosaic	HQ864644.1.seq	India	*V. mungo* cv. LBG-623	97.8
BCMV Hempur strain blackeye cowpea mosaic	HQ864645.1.seq	Moscow	*V. mungo* cv. PU-35	97.8
BICMV	Y17823.1.seq	Florida	Cowpea	97.5
Y strain: “blackeye cowpea mosaic”	AJ312438.1.seq	China	Cowpea	96.5
R strain: “blackeye cowpea mosaic”	AJ312437.1.seq	China	Cowpea	96.5
BCMV-BlC-VN/YB1	DQ925424.1.seq	Vietnam	Yard long bean (*V. unguiculata*)	96.2
BCMV-MB	KC832502.1.seq	China	*M. bean*	96.2
BCMV-MS1	EU761198.1.seq	Australia	*M. atropurpureum*	95.9
BCMV Northern Western Australia 1	AH015028.2.seq	Australia	Not available	95.6
BCMV NY15	AF083559.1.seq	USA	Not available	95.4
BCMV-NL4	L21766.1.seq	Not available	Not available	95.1
BCMV-PR1	L21767.1.seq	Puerto Rico	Not available	95.1
Mexican BCMV	L11890.1.seq	Mexican	Not available	92.1

P. vulgaris: Phaseolus vulgaris, M. atropurpureum: Macroptilium atropurpureum, M. bean: Mung bean, V. unguiculata: Vigna unguiculata, V. mungo: Vigna mungo, BICMV: Blackeye cowpea mosaic virus, BCMV: Bean common mosaic virus, BCMV-BICM: Bean common mosaic virus strain blackeye cowpea mosaic.

serological reactions obtained with the virus isolate using direct ELISA indicated the identity of the virus. In the absence of a specific antiserum against BCMV-BICM, we used an antiserum specific to BCMV. It was found that our BCMV-BICM isolate reacted with BCMV antiserum. BCMV-BICM is serologically related to numerous other potyviruses [33], a circumstance that is common within the group. Lana *et al*. [10] reported that serological tests showed a close, but dissimilar relationship between BCMV and BCMV-BICM. In the description of BCMV-BICM (BICMV) [25], the fact that BCMV is not recorded among the viruses that cause similar symptoms in cowpea indicated that, when found in cowpea, BCMV is classified as BICMV [11].

3.2.4. Histological and cytological studies

3.2.4.1. Virus-induced inclusions by light microscopy

In the present study, amorphous cytoplasmic inclusion bodies induced by BCMV-BICM were observed in infected epidermal strips of cowpea cv. Dokii 331, 14–21 days after inoculation using light microscopy [Figure 3a], compared to healthy epidermal strips [Figure 3b]. Similar results for BCMV-BICM were obtained by Purcifull and Gonsalves [25], Allam *et al*. [34] and Badr *et al*. [35].

3.2.4.2. Histological study

Clear histological differences were identified between infected BCMV-BICM and healthy plants [Figure 4a and b] at this study point. These changes are mostly limited to the lowering of epidermal thickness, either in the upper or lower epidermal layers. Furthermore, these findings revealed a reduction in the midrib zone, palisade, and spongy tissues, leaf blade thickness, and the length and width of both protoxylem and metaxylem vessels. These findings are consistent with

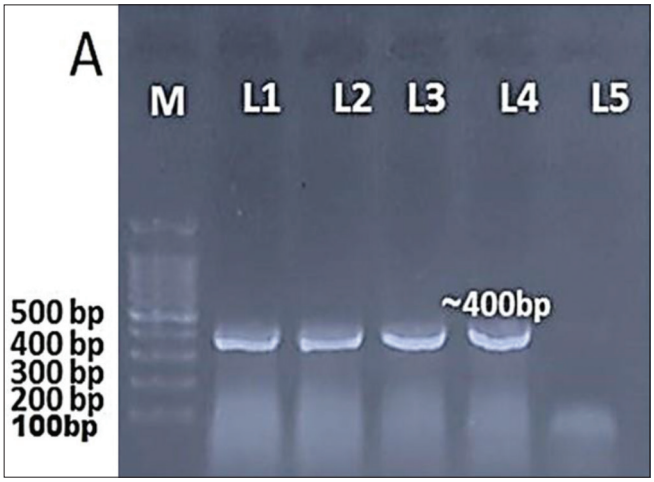

Figure 7: Agarose gel electrophoresis of RT-PCR amplified products. M: 100 bp DNA ladder (BIOMATIK); L 1, 2, 3, 4: Infected leaf samples with BCMV; L5: 1: Healthy sample.

Badr *et al*. [35] on the BCMV and El-Abhar *et al*. [36] on the *Alfalfa mosaic virus*.

3.2.4.3. Cytological studies

In Figure 5, BCMV-BICM-infected plants showed alterations in host cells, either in cytoplasmic components or cell organelles, in ultrathin sections. The cell wall, chloroplasts, mitochondria, nucleus, and virus-related inclusion materials are among the abnormalities. The mesophyll tissue of healthy cowpeas was rich in chloroplasts with normal parenchyma cell organization [Figure 5a] and healthy Phloem

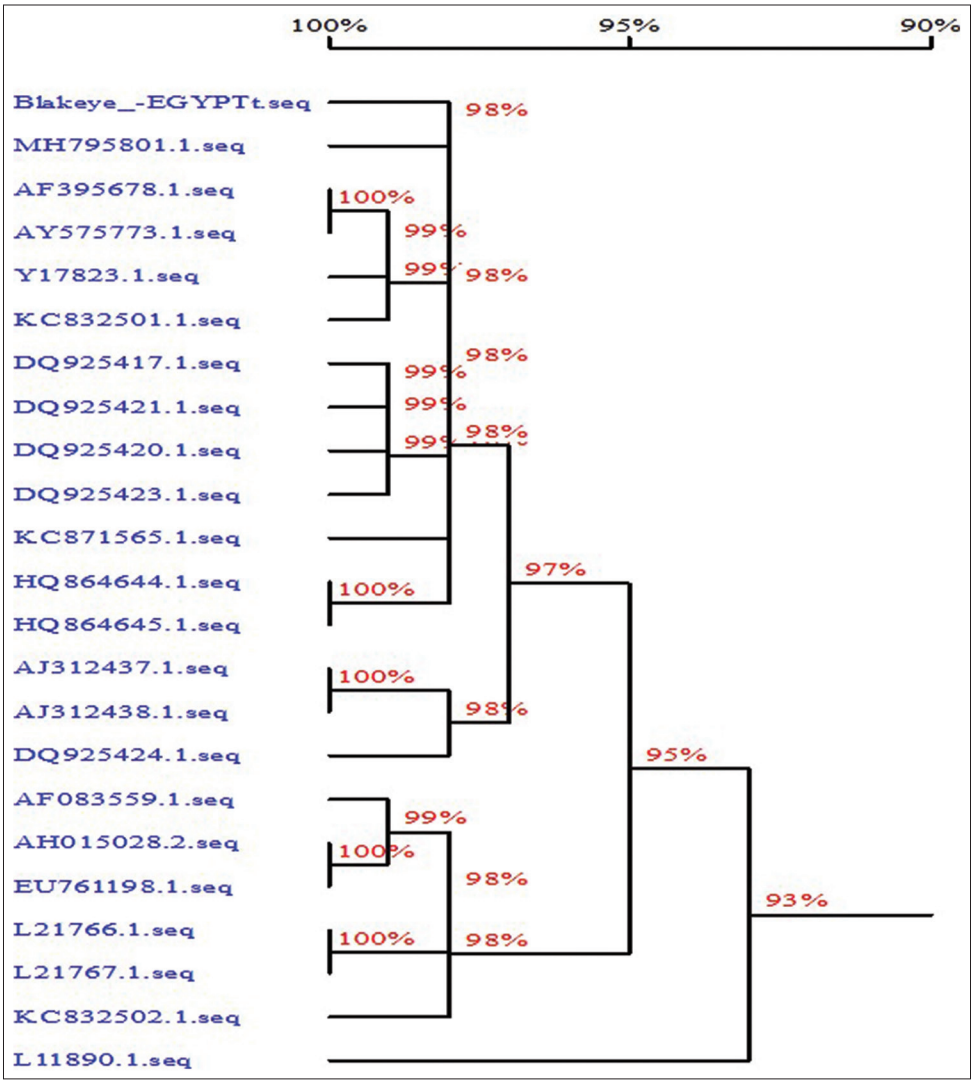

Figure 8: The phylogenetic relationships tree among the multiple sequence alignments of the Egyption isolate of BCMV-BICM and other isolates referenced in GenBank.

tissue [Figure 5j], but in BCMV-BICM-infected cultivars, the cell wall was changed and became wrinkled, and the parenchyma cells appeared destructed and arranged irregularly [Figure 5e]. The findings are consistent with Khalil *et al*. [37], El Banna *et al.* [38] and Rizk *et al*. [39]. The cell wall was thicker than it should have been [Figure 5f]. In infected cells, the chloroplasts become spherical and clump together with irregular rows of grana [Figure 5c], whereas the latter becomes disordered and degenerate [Figure 5g]. These findings coincide with Badr *et al.* [35]. The presence of cytoplasmic cylindrical inclusions such as pinwheels, scrolls, and laminated aggregates is visible [Figure 5b and c]. In epidermal cells and trichomes, the cytoplasmic inclusion bodies were predominant in the parenchyma cells adjacent to the epidermis. Infected cells had typical pinwheels, scrolls [Figure 5d and h], laminated clumps, and endoplasmic reticulum in their cytoplasm. Pinwheel inclusions in the cytoplasm of infected cells are produced by all potyviruses that have been investigated so far by Dougherty and Carrington [40] and Bello *et al*. [41]. Pinwheels are constructed at the plasmalemma and subsequently moved into the cytoplasm, according to Lawson *et al*. [42] and Alexandre *et al*. [43]. The most useful instrument for investigating plant cell ultrastructure as well as direct virus particle detection and its influence on cell organelles [Figure 5i], which are strongly related to the kind of plant-host response to viral infection, this is in agreement with Otulak *et al*. [44] and Rizk *et al*. [39], is transmission electron microscopy.

3.2.4.4. Particle morphology

For examination of the virus particles in crude extracts or purified preparations, a negative-staining procedure is now used extensively [45,46]. In the present investigation, examination of a clarified infected extract with BCMV-BICM negatively stained with 2% phosphotungstic acid using an electron microscope revealed filamentous viral particles 720–750 nm in length [Figure 6], typical of the *Potyvirus* group [23,41,47,48].

3.3. Molecular Detection and Characterization of BCMV-BICM

3.3.1. Reverse transcription-polymerase chain reaction (RT-PCR)

The BCMV-BICM isolate was further identified by RT-PCR amplification using specific primers for its coat protein genes.

The BCMV-BICM isolate was amplified by RT-PCR from fresh cowpea-infected plants, but no products were amplified from RNA extracted from asymptomatic leaf samples when subjected to agarose electrophoresis [Figure 7]. These results for BCMV, cowpea isolate were noted by El-kady *et al*. [22] and Dada *et al*. [49].

3.3.2. DNA purification and sequence analysis

The amplified products for the virus isolate were gel purified and sequenced using DNAMAN Sequence Analysis Software, and the data were evaluated for a consensus sequence and submitted to the GenBank database for BCMV-BICM (OM 891556 seq.). The phylogenetic relationships [Figure 8] were determined. Blast analysis of the coat protein gene of the BCMV cowpea isolates revealed the highest (98.9%) nucleotide identity with BCMV-BICM isolates from Vietnam (DQ925423.1), followed by 98.4% identity with India (MH795801), and 98.1% identity with Vietnam (DQ925421), compared with lower (96.5%) identity with China (AJ312437.1) and India (AJ312438.1) isolates [Table 5]. The close relationship of BCMV-BICM to a previously unknown virus, BICMV demonstrated by Higgins *et al*. [12], McKern *et al.* [13] and UdayaShankar *et al*. [24].

4. CONCLUSION

Characterization of BCMV-BICM was achieved by traditional identification methods and by RT-PCR. Since the virus isolates are transmitted through cowpea seeds and spread naturally by aphids, which can cause potentially high yield losses, it is necessary to produce virus-free seeds and establish certification regulations for legumes to protect growers from infected stocks. This is the first report of a BCMV-BICM isolate found spontaneously in Egyptian cowpea plants.

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6. AUTHORS' CONTRIBUTIONS

All authors contributed to conception and design, data collection, analysis, and interpretation, authoring and editing the article for intellectual content, agreeing to submit to the journal and approving the final version, and agreeing to be accountable for all elements of the work.

7. CONFLICTS OF INTEREST

There are no conflicts of interest that the authors can disclose with regard to this article.

8. FUNDING

There is no funding to report.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

The data that support the findings of this study are openly available in [repository name] at [URL], reference number [reference number].

The authors confirm that the data supporting the findings of this study are available within the article [and/or] its supplementary materials.

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