Characterization and molecular studies of *Bean common mosaic virus* isolated from bean plants in Egypt

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ABSTRACT

A sap-transmitted virus was isolated from bean (*Phaseolus vulgaris* L.) plants showing different patterns of mottling and other leaf deformation. Its host rang was very limited to some leguminous plants. Only three bean cultivars, Baldy, Giza 6 and Nepraska were reacted with systemic symptoms which varied among the cvs. tested. It was transmitted by mechanical means, by non-persistent aphid and through seed, in percentage of up to 35%. It was detected from infected immature or mature seed parts but not from healthy ones. The isolated virus was related by its serological affinity to *Bean common mosaic virus,* using ELISA test, and in not being infectious to *Chenopodium amaranticolor and Ch. quinoa.* The virus consists of filaments flexuous virus particles ranged from 730-750 nm in length. Reverse transcription polymerase chain reaction (RT-PCR) was performed using specific primers to amplify the coat protein gene of Qalyobia- Egyptian BCMV isolate. An approximately 404bp fragment was amplified with the specific primers. Sequence analysis and phylogenetic tree among DNA nucleotide sequence showed range of 89 to 90% similarity with the different BCMV isolates in the Gene Bank, whereas, the amino acid alignments for the coat protein showed a range of 80 to 83% similarity with the same isolates.

Key words: *Bean common mosaic virus,* Host range, Transmission, ELISA, RT-PCR, Sequences, Phylogenetic tree**.**

INTRODUCTION

Bean common mosaic virus (BCMV) was first isolated from infected bean *(Phaseolus vulgaris L.)* in New York, USA, by Stewart and Reddic (1917). Pierce (1934) named it *Bean common mosaic virus*. Symptoms induced by BCMV vary greatly depending on the host cultivar, environmental conditions and virus strain (Shukla *et al., 1994).* BCMV is a serious threat to bean cultivation worldwide because it is seed – transmission at a very high frequency. Infection in the field may reach 100%, and yield losses of 35 – 98% have been reported (Schwartz and Galvez, 1980; Larsen *et al*., 2004; Shahreen and Ghotbi., 2005 and Prasad *et al*., 2007).Seed transmission rates ranging from 0 to 93% have been reported in cultivars of different host (Edwardson and Christie, 1991). Seed transmission of the virus is much decreased in plants infected after flowering the virus is located mostly in the embryo (Morals and Bos, 1988). From a long period of time and serologyical diagnostic methods used in the detection of plant viral diseases in infected plant tissue are still used until now ,However, this method is less specific than some other technique such as polymerase chain reaction (PCR) while, are becoming more popular (Matthews, 1993). In 1989, science select polymerase chain reaction (PCR) as the major scientific development of the year (Guyer and Koshland, 1989;Traykova *et al.*, 2008; Abtahi *et al.,* 2009 and Owolabi *et al.*, 2011).The present work has been conducted to : 1) identify the causal agent of the disease biologically and

serologically, 2) confirm the identification of the virus by using RT-PCR , 3) determine the nucleotide sequence of the Egyptian isolate of BCMV as well as the amino acid translation of the coat protein gene and 4) elucidated the genetic relationship with the other BCMV isolates in GenBank.

MATERIALS AND METHODS

I: Isolation and identification

1-1-Isolation of the virus isolate:

Naturally infected bean (*Phaseolus vulgaris L.*) plants showing virus- like symptoms consisted of various types of mottling and intensity, dark- green bands adjoining major veins, interval chlorosis, puckering, blistering, curling and other deformation were collected from different fields of Qalubya Governorate. Mechanical inoculation was carried out in the greenhouse to isolate and identify the causal agent. The inoculum was prepared to inoculate healthy been plants and other tested hosts (*Chenopodium album, Ch. Quinoa* and *Ch. amaranticolar)* as described by Noordam (1973). The inoculated plants were kept under observation in an insect – proof greenhouse.Ten seedling at the two-leaf stage of each test plant unless otherwise stated were inoculated. An equal number of healthy seedlings of the same cultivar and age were left without inoculation to serve as controls. After successive transfers in bean plants , the resulting virus isolate was propagated in bean cv. Baldy.

1-2: Identification of the virus isolate:

Identification of the isolated virus was based on host rang and symptomatology, modes of transmission, serological reaction, morphology of virus particles, and molecular characterization.

1-2-1: Host range and symptomatology

Twenty-seven different plant species and cultivars belonging to six families were mechanically inoculated by the virus under study. Five weeks later, symptomless plants were checked for virus infection by back inoculation onto the test plant (bean cv. Baldy) and/or ELISA technique.

1-2-2- Modes of transmission:

A- Insect transmission:

Individuals of *Myzus persicae* Sulz and *Aphis craccivora* Kock reared on red pepper plants *(Capsicum annum L.* were tested as vectors. Non-viruliferous aphids were starved for 1-2 hr, then transferred to infected bean plants (cv. Baldy) for 10 min. virus acquisition access period before being transferred in groups of five to each of ten virus-free bean (cv. Baldy) seedlings and allowed a 24 hr. inoculation access period. The same procedure was applied to serve as control, except that virus-free aphids were used. After the inoculation period, aphids were killed with the insecticide. Symptoms and percentage of transmission were recorded for a period of 30 days.

B- Seed transmission:

Bean seeds of three cultivars (Baldy, Giza 6 and Nepraska) tested obtained from Field Crop Res. Institute, ARC were sown in 20 cm-diameter pots containing steam sterilized soil mixture and grown under insect-proof greenhouse conditions. Two weeks later, the emerged seedlings assayed biologically and/or serologically by ELISA were sap inoculated either with the virus isolate or with distilled water (control). Virus-infected plants showing typical symptoms as well as healthy plants (assayed negatively in infectivity tests) during the growing season were harvested. Sub samples composed of four hundred seeds from each bean

cultivar were also sown in 20 cm diameter pots (5 seeds/pot). The resulting seedlings were observed for symptoms development (or assayed by ELISA) and percentage of virus seed transmission was estimated in bean cultivars tested. Seeds from healthy plants were used as controls and received similar treatments. The seeds were treated with 0.1 N HCl for 0.5 hr. and rinsed in running tap for 5-8 hr. before planting to eliminate surface contamination.

B-1: Virus incidence in mature seed parts:

Another experiment was carried out to detect BCMV in various parts of the seed. A hundred mature seeds of bean cv. Baldy raised from infected bean plants and had not been acid-treated were soaked individually in distilled water for two days. The seeds aseptically dissected into seed coats, cotyledons and reminder of the embryos, the dissected seed parts were washed in running water for 20-25 min. homogenized individually in PBS-tween 20 and 2% PVP in a ratio of 1 : 10 (g tissue/ml) by pressure-crushing in polyethylene bags. The bags were then shaken for one hour at room temperature. Each extract was used as antigen in ELISA test.

B-2: Virus incidence in immature seed parts:

The same test as mentioned above conducted with immature bean seeds infected with the virus isolate. Fifteen green pods (before striation) were chosen at random and 2 or 3 immature seeds were removed and mixed. Four samples of six seeds each were tested. The samples were dissected into seed coats, cotyledons, and remainder of the embryo and were washed decontaminated by washing for 20-25 min. in running water and tested by ELISA.

1-2-3. Serological diagnosis:

Bean plants showing distinct symptoms were tested by DAS-ELISA (Clark and Adams, 1977) using ELISA kit (provided by Sanofi, Sante Animal, Paris, France) for detection of BCMV and by indirect ELISA (Converse and Martin, 1990) using antisera specific to some bean viruses such as *Bean yellow mosaic virus* (BYMV), *Bean pod mottle virus* (BPMV), *Cucumber mosaic virus* (CMV) and *Alfalfa mosaic virus* (AMV) which were produced by Virus and Phytoplasma Research Department for routine diagnostic work and routine seed healthy testing.The intensity of the color can be rated visually or measured spectrophotometrically as absorbance intensity by 405 nm light (A_{405}) using Vniskan ELISA reader. Reading greater than twice the value of healthy controls was considered positive.

1-2-4: Particle morphology:

To study the morphology of virus particles, the virus was first purified and then the purified preparation was examined by an electron microscope at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University.

1-2-4-1: Virus purification:

BCMV was partially purified as described by Meiners *et al.* (1978). Inoculated bean cv Balady plants were used as a source for virus purification.

Electron microscopic examination was done as described by Noordam (1973).

1-2-5: Molecular characterization:

1-2-5-1: Total RNA isolation:

Total RNA was isolated from leaves of infected and uninfected (control) bean plants, using gene jet ™ RNA purification kit (fermentas, USA) according to manufacturer's instructions.

1-2-5-2: Reverse transcription-polymerase chain reaction (RT-PCR):

The one step RT-PCR was performed using Verso ^{TM} one step RT-PCR kit (Thermo scientific). The extracted total RNA was submitted to one step RT-PCR in a final volume of 25 µl containing 3 µl RNA (4 ng/µl), 12.5 µl of one step PCR master mix (2x) ,3 µl 10 µM of each primer,0.5 µl Verso enzyme mix, 1.25 µl RT-Enhancer and 4.75 µl of nuclease - free water. Oligodeoxynucleotide primers specific for BCMV coat protein gene were used. The sequence of the forward primer; BCMV-F is`5-CCA ATG GTT GAA AAT GCA AAG CC -3`, while the sequence of the reverse primer ,BCMV-R, is`5- CGA CGC GAG ATG CTA ACTG -3`.

PCR reactions were performed by a first denaturation of the samples at 50 ºC for 15 min, 95 ºC for 2min followed by 35 cycles at 95 ºC for 1 min ,55 ºC for 1 min and 72 ºC for 1 min and final elongation step at 72 ºC for 10 min. The RT-PCR products were stained with gel star (Lonza, USA) and analyzed on 1% agarose gels in 0.5X TBE buffer then visualized by UV illumination using Gel Documentation System (Gel Doc 2000, Bio-Rad, USA).

1-2-5-3: Gene cleaning and DNA purification:

The fragment of the RT-PCR product that amplified form the Qalyobia BCMV- CP gene was extracted from the agarose gel and purified using QIAquick Gel Extraction Kit from QIAGEN.

1-2-5-4: DNA sequencing

The purified DNA fragment that extracted from the agarose gel was directly sequenced using Automated DNA sequencing. To observe genetic distance, phylogenentic tree was constructed and the sequencing results were analyzed using DNAMAN Sequence Analysis Software (LynnonBioSoft. Quebec, Canada) and compared with sequences of other selected BCMV isolates available in Gen Bank.

RESULTS

I-Identification of the virus isolate:

1-1- Host range and symptomatology:

Reaction of twenty- seven plant species and cultivars belonging to six families to virus infection is shown in Table (1) and Fig.(1). Infection was confirmed by back inoculation to bean cv. Baldy and by ELISA technique. It was shown that, bean cvs Baldy, Giza 6 and Nepraska were only reacted with systemic symptoms which varied among the cultivars tested. These symptoms were ranged from mild and severe mottling, associated with blisters, curling, distortions and other leaf deformation. No symptoms could be observed; neither could virus recovered in the other host plants.

1-2- Modes of transmission:

A- Insect transmission:

It was found that insect transmission rates were low. Nevertheless, *Myzus persicae* was more efficient (50%) as a vector than did *A. craccivora* (20%).

B- Seed transmission:

The percentage of seed transmission of BCMV was varied according to the bean cultivars tested. It was 35, 11 and 15% for Balady , Giza 6 and Nepraska , respectively.

Table (2): Comparison of ELISA test with BCMV in various parts of mature and immature cv. Baldy bean seeds:

Seed parts	Mature seed	Immature seed	Healthy seed (control)
coat	0.198	2.040	0.125
cotyledon	2.870	2.142	0.130
embryo	2.780	2.130	0.127

Average A 405 nm values of four samples of six seeds each (about 35% seed transmission).

B-1: Virus incidence in mature and immature bean seeds:

Data tabulated in Table (2) indicated that the concentration of the virus isolate was higher in immature seed coats than in mature seed coats. On the countrary its concentration was much higher in mature cotyledons and embryos than did in immature seeds.

1-2-3- Serological diagnosis of the virus isolate:

The virus was detected by DAS-ELISA and thus, confirmed the identity of the virus under study. Positive reaction was obtained only with BCMV-specific antiserum whereas, it was failed to react with the antisera of the other bean viruses (BYMV, BPMV, CMV and AMV), using indirect ELISA.

1-2-4- Particle morphology:

Electron micrograph of partially purified virus preparations, negatively stained with 2% uranyl-acetate obtained from BCMV-infected bean leaves showed filamentous flexuous virus particles ranged from 730-750nm in length.

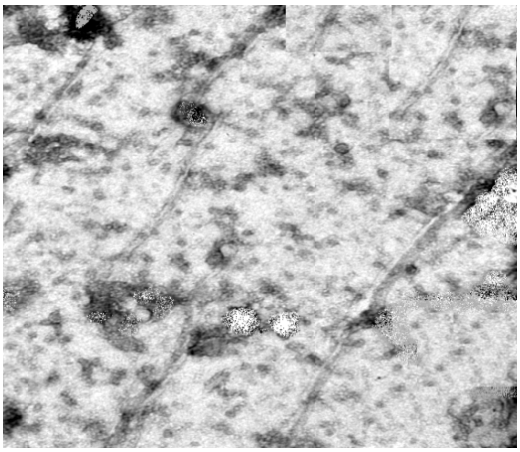

Fig (2): Electron micrograph of partially purified BCMV, negatively stained with 2% uranyl acetate(Magnification X 50.000).

(A) **(B)**

Fig.(1): Naturally healthy(A) and infected (B) bean *cv.* Baldy showing mosaic , blisters and curling.

Table (1): Reaction of different hosts inoculated mechanically with the virus isolate:

Host Plant	Cultivar	Symptoms, induced by the Virus isolate
(1) Family: *Chenopodiaceae:* *Chenopodium album L.* *Ch. amaranticolor cost &Reyn* *Ch. quinoa wild*		 NR NR NR
(2) Family: *Cucurbitaceae:* *Cucumis sativus L.*	 Baldy	 NR

Cucurbita pepo L.	Eskandrani	NR
(3) Family: *Compositeae:* *Zinnia elegans Jacq.*		 NR
(4) Family: *Amranthacea:* *Gomphrena globosa L.*		 NR
(5) Family: *Fabaceae:* *Vicia fabae L.* *Vigna unguiculate L.* *Glycine max L.* *Pisum sativum L.* *Phaseolus vulgaris L. cvs.*	 Giza 843 Clark Mster B Baldy Bolesta Ekzera Narena Bronko Giza 6 Nepraska	 NR NR NR NR ST+M+BL+CR NR NR NR NR M + ST M + BL
6) Family: *Solanaceae* : *Capsicum annum L.* *Datura staramonium L.* *Datura metel L.* *Lycopersicom esculentumMill.* *Nicotiana tobacum L. cvs.* *Nicotiana glutinosa L* *Nicotiana rustica L.*	 California wonder Nema 1400 White Burley Sumsum Turkish	 NR NR NR NR NR NR NR NR NR NR

NR = No reaction M = Mosaic
ST = Stunting BL = Blister Cr = Curling.

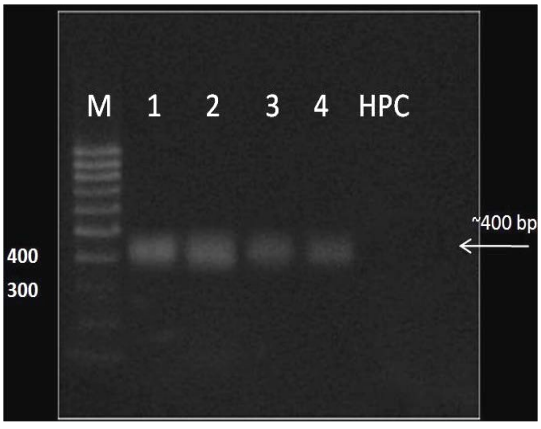

Fig.(3): Agarose gel electrophoresis analysis of RT-PCR amplified products. M: 100 bp DNA ladder (Fermentas); L1 to L4: different bean samples infected with BCMV; HPC (healthy plant control**).**

1-2-5-Molecular characterization:

A-Amplification of BCMV coat protein gene:

RT-PCR amplification of viral RNA was carried out on the total RNA isolated from infected plants using specific primers to amplify the coat protein gene of BCMV. Electrophoresis analysis of RT-PCR products showed a single amplified fragment of 404 bp while no fragments were amplified from the RNA extracted from healthy plants (Fig. 3).

B-Sequence analysis:

Nucleotide sequencing of the purified RT-PCR fragment for the BCMV coat protein gene was performed (MacroGene; Korea) and compared with sequences of other

isolates of BCMV available in GenBank. The nucleotide sequence and translated amino acids for the BCMV coat protein gene shown in Fig.(4). The obtained data were analyzed using DNAMAN software. Multiple sequence alignment was completed to compare the sequence of 404 nucleotides of our findings with the corresponding sequences of different BCMV CP genes available in Gene Bank : USA-AY863025, Viet Nam (BlC-VN) DQ925417.1, India - HM776637, C-6 India- GQ850881.1, strain NL1- - L15331.1, Strain NL.7- JN692257, isolate NY15 - AF083559, Australian EU761198 and China AJ312438 . The coat protein gene was translated into 134 amino acid according to the universal codon as shown in Fig.(4). The translated amino acids were compared with the other coat proteins available in the GenBank. Fig (5a + b) showed phyligenetic tree for the coat protein alignments based on the nucleotide sequences of their CP genes (5a) and the amino acid translation (5b). Sequence analysis and the phylogenetic tree among the DNA nucleotide sequences showed range of 89 to 90% similarity with the different BCMV isolates in the GeneBank(Fig.5a). While the amino acid alignments for the coat protein showed a range of 80 to 83% similarity with the same isolates (Fig.5b).

DISCUSSION

Bean common mosaic virus (BCMV) has been recognized as a major constraint on bean production . A study conducted to assess the status of bean – borne diseases in beans found that BCMV is among the most important factors leading to low bean production (Marvic and Sustar- Vaslic, 2004).

In this work BCMV was isolated from naturally infected bean plants showing mottling and various types of malformation and other alterations in the shape of the leaves. The virus was identified as such on the bases of symptomatology, host range, modes of transmission, serological reaction, particle morphology and molecular characterization.

Fig. (4): DNA nucleotide sequence as well as the amino acid translation of the BCMV coat protein gene.

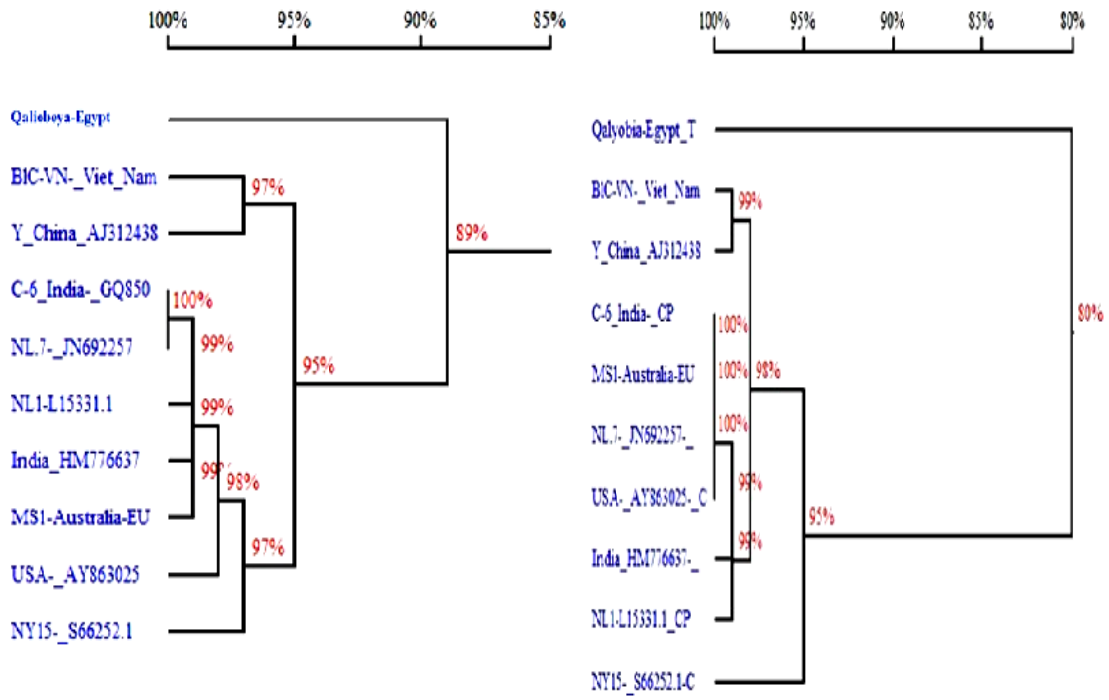

Fig. (5**):** A phylogenetic tree showing relationships among reported isolates of BCMV in GenBank and the Egyptian isolate (Qaliobeya) based on the nucleotide sequences of their CP genes (5a)and the amino acid sequences (5b) . Horizontal distances indicate degree of relatedness.

Among twenty seven species and cultivars belonging to six families, the obtained virus isolate reacted positively with only *Phaseolus vulgaris* cvs. Balady, Giza 6 and Nepraska. Systemic symptoms produced in these cultivars varied among the cultivars tested from mild to severe mottling, associated with blisters, curling, distortions and other leaf deformation. Whereas, plants which did not produced symptoms, the isolated virus was not recovered by using either biological or serological assays. These results indicated that the hosts of the virus isolate are mainly restricted to *Phaseolus* spp., especially *P. vulgaris*. These finding appear to be in the line with others workers (Awad, Maisa, 1988, El- Kady, 1991, Spence and walkey, 1995 and Morales, 1998).

For experimental point of view, the virus under study was found transmitted mechanically and by two aphid species namely: *Myzus persicae*(50%) and *A*phis *craccivora* (20%). *M .persicae* was found more effective vector in transmission the virus under the greenhouse conditions than did *A. craccivora*. Other workers reported the same results (Omunyin *et al*., 1995; Sengooba *et al*., 1997 and Poonam and Gupta., 2010).

In this research, the percentage of BCMV transmission was varied according to the bean cultivars tested (Balady 35% , Giza 6 11% and Nepraska 15%). ELISA test indicated that bean cv. Balady had a higher virus concentration followed by Giza 6 and Nepraska cultivars comparing with the control treatment.

Reddick and Stewart (1919) demonstrated the seed borne nature of BCMV and noted considerable variability in the percentage of virus transmission in the seed. Burkholder and Muler (1926) found that plants affected with the virus seldom give rise to more than 50% diseased plants .Whereas, Ekpoo and Saettler (1974) demonstrated that BCMV has been established in 83% of excised empryos of been seeds. The same results reported by Deif (1983) and Marvic and Sustar-Vaslic (2004).

Concerning the distribution of BCMV in bean seeds, our results indicated clearly that the virus isolate is carried manly in embryos and cotyledons .Whereas, very little virus concentration was detected by using ELISA in mature dry seed coats, but higher in mature cotyledons and embryos than in immature ones. However, most of the virus was inactivated or had been difficult to extract from the seed coat. In contract, the relative virus concentration was higher in mature embryos and cotyledons than in immature ones. This indicated that virus is not inactivated in embryos and cotyledons during maturation and drying. A similar results were noted by Abdel Salam *et al*. (1990) using microprecipitin and/ SDS- double diffusion test, by Spence and Walkey (1993) and by Saiz *et al.* (1995) using ELISA test.

Serological assays are the most reliable and sensitive method for detecting BCMV and confirming diagnoses (Forster *et al.,* 1991).Positive reaction was obtained only with BCMV - specific antiserum , whereas , it was failed to react with the antisera of the other bean viruses (BYMV, BPMV, CMV and AMV). This result provided further evidence that the virus under study is indeed BCMV .However, several authers were used ELISA test for BCMV detection (klein, 1992 , Davis and Tsatsia, 2008 , and Dizadja and Shahreaeen, 2011).

Electron microscopy study of the partially purified BCMV preparations showed flexous filamentous virus particles ranged from 730- 750 nm in length. Particles with these dimensions were not seen in negatively stained preparations of healthy plants. This result was in agreement with results obtained by other workers (Sengooba *et al.*, 1997, Roberto *et al.,* 2003 and Naderpour *et al.,* 2010).

The Egyptian isolate of BCMV was detected by RT-PCR using the procedure described above based on sequence – specific primers for the coat protein gene. A product of approximately 404 bp was amplified from viral RNA extracted from infected bean leaves. All the infected bean samples those showed symptoms characteristic for BCMV were exhibited positive reactions , whereas no amplification was detected with the healthy bean extracts. This size was in agreement with those expected from the nucleotide sequence of the BCMV- CP gene previously reported (Bravo *et al.,* 2008 and Sharma, *et al.,* 2011). To confirm the RT-PCR results as well as the incidence of the BCMV, which is one of the main goals of this study, molecular characterization of the amplified BCMV fragment was performed. To achieve that goal, the BCMV coat protein gene was isolated and amplified with RT-PCR utilizing specific primers. Focusing on the analysis and sequencing of the coat protein gene as a confirmatory tool for virus incidence comes from the fact that, the coat protein region seems to exhibit the most substantial variability among all viral proteins, illustrating the divergence of a cluster among different isolates of the virus identified all over the world.

The amplified coat protein gene fragment was purified and prepared for DNA sequencing. The nucleotide sequencing of the BCMV- CP gene was performed and the multiple sequence alignment was completed to compare the sequence of 404 nucleotides of our findings with the corresponding sequences of different BCMV CP genes available in GenBank. The coat protein gene was translated into 134 amino acids according to the universal codon and the translated amino acids were compared with the other coat proteins available in the GenBank.

Sequence analysis and the phylogenetic tree among the DNA nucleotide sequence showed rang of 89 to 90% similarity with the different BCMV isolates in the GenBank, while

the amino acid alignments for the coat protein exihibited 80-83% similarity among themselves. This indicated that our isolate was related to other isolates in the GenBank. Nevertheless, there were a slight difference in nucleotide and amino acid sequences in their CP gene. Therefore, further studies, comprising cloning and complete nucleotide sequences could be performed to clarify relatedness of BCMV subgroup members.

Potyviruses with sequence homology ranging between 38-71% (average 54%) are considered distinet members, while that between strains of the virus ranged from 90 - 99% with average 95% (Shukla and Ward , 1988).

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