I. INTRODUCTION

Sugar beet (*Beta vulgaris)* ranks as the second sugar crop in the world, it contains 20 % sucrose. It has recently been grown in Egypt. Its cultivate area and productivity have been doubled several folds, (from 11,000 feddans in 1981 to 170,000 feddans in 2006 with average of 22 tons/feddan, **FAO, 2006**). In Egypt, sugar beet is mostly cultivated in certain Governorates which include Kafer El-Sheikh, Dakahlia, El-Menia and El-Fayoum.

In Egypt several viruses affect sugar beet crop such as CMV (**Abdel-Salam *et al.*, 1997**), BNYVV (**Abdel-Ghaffar** and **Farrag, 2004),** BCTV (**Mahmoud *et al.*, 2005**), and BtMV (**Badr, 1986** and **Abdel-Ghaffar *et al.*, 2003**).

BtMV infects sugar beet plants in Egypt and causes a mosaic disease in many species of genus *Beta* (*B. vulgaris, B. patellaris, B. maritime*) or Spinach (*Spinacia oleracea) a*nd causes significant reduction in sugar beet yield by up to 20 % (**Abdel-Ghaffar *et al.*, 2003** and **Jalali *et al.*, 2003).**

BtMV is a member of family *Potyviridae*, genus *Potyvirus* (**Mayo *et al.* 2005**). It has filamentous particles, monopartite, and positive sense single stranded RNA gnome (**Brunt *et al.*, 1996).** It is transmitted by mechanical inoculation **(Mali, 2000)** and non-persistent by aphid **(Stevens, 2007).** It induces cell inclusions typical for potyviruses subdivision II **(Abdel-Ghaffar *et al.*, 2003**).

In Egypt, relatively little attention has been paid to characterize this virus (**Badr, 1986; Abdel-Ghaffar *et al.*, 2003**). Therefore, this study aimed for isolation and characterization of BtMV *via* the following:

I) Biological:

a) Mode of transmission and host range.

b) Examination of the cytoplasmic cylindrical inclusions.

II) Chemical:

a) Purification and negative staining.

b) Determination of coat protein molecular weight by SDS-PAGE.

c) Determination of size length of its ss-RNA by agarose gel electrophoresis.

d) Determination of *cp*-gene size length by RT-PCR.

III) Serological:

Production of diagnostic antiserum and its evaluation for virus detection *via* TBIA and DBIA.

IV) Molecular:

The effect of BtMV on growth and yield of ten sugar beet cultivars, as well as the relationship between the susceptibility of these cultivars to infection with BtMV and their genetic variations were also investigated using ISS-PCR.

II. REVIEW OF LITERATURE

Sugar beet (*Beta vulgaris* L.) is recorded to infect with many viruses which belonging to different virus families and genera **(Table 1)**. The genus *Potyvirus* contains the largest number of plant virus species, including 91 formal species and 88 tentative species (**Mayo *et al.* 2005).**

BtMV is a member of genus *Potyvirus* (**Brunt *et al.*, 1996** and **Mayo *et al.*, 2005).** BtMV is widely spread in the main growing areas of sugar beet crop (*B. vulgaris* L.) throughout the world (**Watson *et al.*, 1951; Bennet, 1964**; **Shepherd and Till, 1965**; **Shepherd *et al.*, 1966; Rusell, 1971; Rogov *et al.*, 1991; Brunt *et al.*, 1996; Nemchinov *et al.*, 2004; Stevens, 2007** and **Xiang *et al.*, 2007**) and in particularly in Egypt (**Bader, 1986**; **Kassim *et al.*, 1993** and **Abdel-Ghaffar *et al.*, 2003**).

2.1. Biological studies on potyviruses as well as BtMV

2.1. 1. Host range and symptomology

BtMV is mechanically transmitted to *B. vulgaris, Chenopodium amaranticolor, C. quinoa, C. cabitatum, Gompherena globosa, Hibiscus esculentus, Montia perfoliata (Domn), Phaseolus vulgaris, Pisum sativum* and *Tetragonia expansa*

The test plants are varied in their response to infect with BtMV isolate. Data of infection by mechanical inoculation indicated that the *G. globosa, B. maritime, B. patellaris, B. vulgaris, C. amaranticolor, C. quinoa* and *Spinacia oleracea* are infected with symptoms of necrotic or chlorotic lesions on their inoculated leaves, then systemic infection, *i.e.* chlorosis, apical necrosis, mosaic and malformation, while *B. vulgaris* shows small chlorotic lesions on inoculated leaves, then systemic infection . Systemic infection with symptoms mosaic on *Glycin max*, leaf wilting on *P. sativum*, apical necrosis and mottle mosaic are observed on *Vicia faba*. On the other hand, the test plants belonging to families *Cucurbitaceae* and *Solanaceae* are immune to infect with BtMV **(Rogov *et al.*, 1991; Owolabi *et al.*, 1998; Mali, 2000; Abdel-Ghaffar *et al.*, 2003** and **Jalali *et al.*, 2003).**

Table 1. Viruses infecting sugar beet (Brunt *et al.*, 1996).

Viruses name	Mode of Transmission	Diagnostic hosts	Symptoms on sugar beet	Viruses morphology
BCV-1	Seed, pollen	*B. vulgaris*	No-symptoms	Isometric
BCV-2	Seed, pollen	*B. vulgaris*	No-symptoms	Isometric
BCV-3	Seed	*B. vulgaris*	No-symptoms	Isometric
BCTV	Grafting, insect (*Circulifer spp.)*	*B. vulgaris, Cucumis sativus, Phaseolus vulgaris*	Leaf rolling, vein clearing	Geminate, rounded profile
BDMV	Mechanical inoculation, fungus (*Polymyxa betae)*	*B. vulgaris*	Malformed, chlorotic leaves	Flexuous ilamentus
BLCV	Grafting, insect (*Piesma quadratum*)	*B. vulgaris, C. quinoa, Spinacea oleracea, Tetragonia expansa*	Vein deformation, leaves curved towards the apex	Rhabdo or bullet-shaped
BMYV	Aphid	*Lactuca sativus, Pisum sativum, Nicotiana clevelandii*	Golden yellowing of order leaves	Isometric
BtMV	Mechanical inoculation, aphid	*C. quinoa, S. oleracea, Glycin max*	Small local lesions, mosaic mottling , malformation	Flexuous filamentous
BNYVV	Mechanical inoculation, fungus (*P. betae)*	*B. vulgaris, C. amaranticolor, C. quinoa, S. oleracea*	Necrosis, stunting, rootlet from main root	Rod-shaped
BPYV	Insect (*Trialeurodes vaporaiorum)*	*B. vulgaris, Lactuca sativua, C. melo, N. glutinosa*	Yellowing, thickening, brittleness of leaves	flexuous

Table 1. Continued.

BSBV	Mechanical inoculation, fungus (*P.beta)*	*B. vulgaris, C. quinoa, C. amaranticolor, S. oleracea*	Chorotic rings	Rod-shaped
BWYV	Aphid	*Gomphrena globosa, L. sativa, G. max*	Yellowing, brittleness of older leaves	Isometric
BYNV	Aphid	*B. vulgaris*	Conspicuous veinal chlorosis	Isometric
BYSV	Aphid	*B. vulgaris, Souchus oleraceus*	Yellowing, stunting	Filamentous
BYV	Aphid	*B. vulgaris, Montia perfoliata, S. oleracea*	Vein clearing , yellow with necrotic spots	Flexuous filamentous
CMV	Mechanical inoculation, seed, aphid	*C. amaranticolor, C. quinoa, C. sativus*	Systemic mosaic, vein clearing	Isometric

2.1.2. Mode of transmission

BtMV is easily transmitted by sap inoculation to many test plants (**Rogov *et al.*, 1991; Staniulis, 1995; Abdel-Ghaffar *et al.*, 2003; Jalali *et al.*, 2003** and **Stevens, 2007).**

The aphid transmission of potyviruses is characterized by short acquisition and transmission access periods. The aphids usually loose their inoculativity rapidly, although the virus can, in some cases, be retained for 24 hour **(Zeyen and Berger, 1990).** BtMV, as well as several other non-persistent aphid-borne viruses, is transmissible by several aphid species with varying degrees of efficiency. BtMV is transmitted in a non-persistant manner by more than 28 aphid species (**William and James, 1988** and **Friesen *et al.*, 2006**) include the green peach aphid *Myzus persicae*, *Aphis fabae*, *Acyrthosiphon pisum*, *Macrosiphum euphorbiae*, *Metopolophium dirhodum*, *Aphis spiraecola*, *Toxoptera citricidu*and and *Rapalosiphum padi* (**Rogov *et al.*, 1991; Owolabi *et al.*, 1998; Dusi, 1999; Dusi and Peters, 1999; Mali *et al.*, 2000; Abdel-Ghaffar *et al.*, 2003** and **Stevens, 2007).**

2.1.3. Inclusion bodies

The CI bodies are formed by virus-encoded protein and can be considered as the most important phenotypic criteria for assigning viruses to potyviruses group **(Shukla and Ward, 1989).**

Formation of inclusion bodies and their structure are properties for plant viruses identification (**Allam *et al.*, 2000**). Four types of CCI for potyviruses group were observed: Type I: pinwheels and scrolls, Type II: pinwheel and laminated aggregates, Type III: pinwheels with scrolls and laminated aggregates and type IV: pinwheels, scrolls and short curved laminated aggregates **(Edwardson *et al.*, 1984; Edwardson and Christie, 1996).** BtMV is inducing CCI typical for potyviruses subdivision II, i.e., pinwheels and laminated aggregates **(Juretic, 1998; Riedel *et al.*, 1998** and **Abdel-Ghaffar *et al.*, 2003).**

2.2 Chemical studies of potiviruses as well as BtMV

2.2.1. Virus purification

BtMV is purified from freshly harvested sugar beet leaves (*B. vulgaris*) displaying characteristic light vein-clearing, green mottle symptoms and mosaic by modification procedure is based on extraction of infected tissue in salt buffer in the presence of various reducing agents such as ammonium acetate, EDTA, sodium acetate, urea and DIECA followed by low centrifugation, clarification with Triron X-100, precipitation with high-speed centrifugation by 30% sucrose cushion containing 4.5% PEG.6000 (**Fujisawa *et al.* 1983, Rogov *et al.*, 1989; Rogov *et al.*, 1991**; **Owolabi *et al.*, 1998; Glasa *et al.*, 2000 ; Abdel-Ghaffar *et al.*, 2003**), and some cases an additional step of purification involving a 10-40% sucrose density gradient **(Hammond and Lawson, 1981; Avgelis and Katis, 1992; Glasa *et al.*, 2000** and **Abdel-Ghaffar *et al.*, 2003).**

2.2.2. Spectrophotometer

Ultraviolet absorption spectrum of the purified virus showed a typical curve of nucleoprotein with an A260/280 ratio ranged from 1.21-1.44. The yield of purified virus ranged from 15-30 mg/kg virus-infected leaves based on the extinction coefficient of 2.4 for a 1 mg/ml solution. (**Rogov *et al.*, 1989;**

Rogov *et al.*, 1991; Ghorbani *et al.*, 2001; Okhoyvat *et al.*, 2001 and **Abdel-Ghaffar *et al.*, 2003**).

2.2.3. Morphology of particles

Particles of potyviruses are flexuous filaments, non-enveloped with length 680-900 nm and width of 11-13 nm (**Brunt *et al.*, 1996; Van Regenmortel *et al.*, 2000; Prito *et al.*, 2001; Sadik *et al.*, 2003** and **Mayo *et al.*, 2005**). BtMV is characterized as flexuous filamentous, non-enveloped particles with long 725-750 nm and wide 13 nm when negatively staining and examined by transmission electron microscope **(Edwardson, 1974; Rogov *et al.*, 1991; Owolabi *et al.*, 1998; Glasa *et al.*, 2000** and **Abdel-Ghaffar *et al.*, 2003).**

2.2.4. Viral coat protein

SDS-PAGE is commonly used for determinating of viral coat protein molecular weight, and it can also used for detecting of plant viruses in clarified sap extracted from infected plants (**Hill and Shepherd, 1972; Yao *et al.*, 1993; Kwon *et al.*, 1997** and **Salama *et al.*, 2003**). BtMV is contain a single protein-species of molecular weight about 30-34 kDa when estimated by SDS-PAGE (**Rogov *et al.*, 1991; Owolabi *et al.*, 1998; Glasa *et al.*, 2000** and **Abdel-Ghaffar *et al.*, 2003).**

2.2.5. Viral RNA

Potyviruses are contained positive-sense RNA genome with size length about 10 kb (**Van Regenmortel *et al.*, 2000**), contains a genome linked protein (VPg) at 5'terminus and a poly A tail at 3'terminus (**Revers *et al.*, 1999**).

The ssRNA of BtMV is isolated and its size length measured of about 9591-10000 base, when determined by agarose gel electrophoresis (**Abdel-Ghaffar *et al.*, 2003** and **Nemchinov *et al.*, 2004).** BtMV genome excluding the 3'terminal poly (A) sequence, and contains a single ORF that begins at nt 166 and terminates at nt 9423, encoding a single polyprotein of 3086 amino acid residues. A 3'untranslated region of 168 nucleotides follows the ORF. The deduced genome organization (**Figure 1)** is includes 10 proteins: P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, Nib and CP (**Nemchinov *et al.*, 2004**).

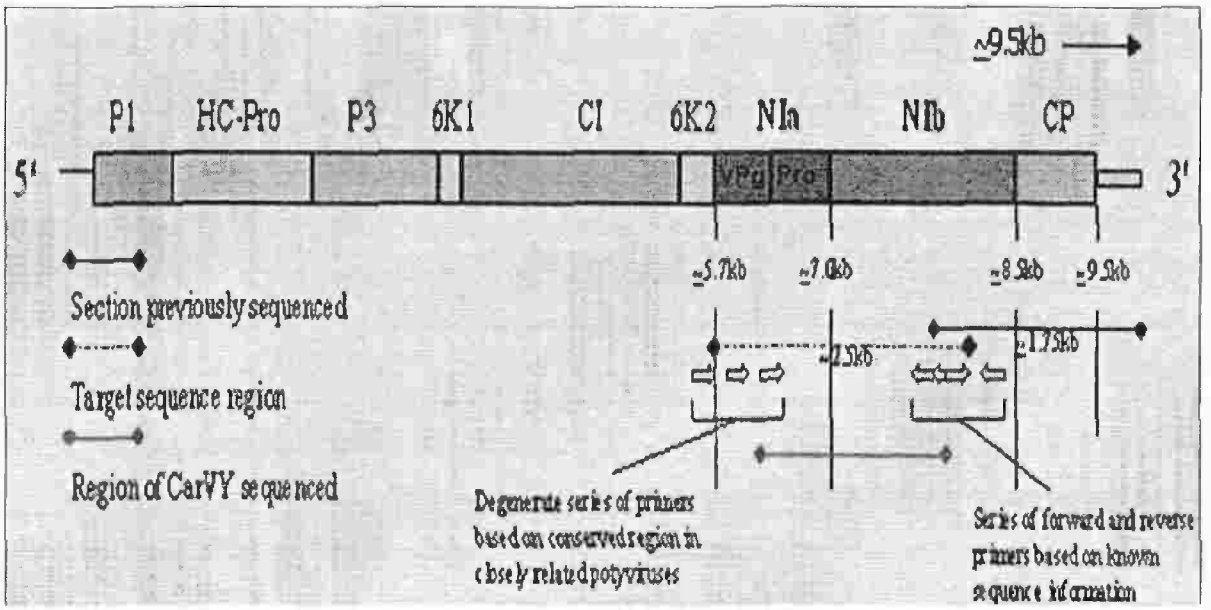

Figure 1. Proposed genomic map of BtMV and sequencing strategy.

Becker-Andre and Hahlbrock (1989) indicated that detecting of RNA-characteristics sequences by RT-PCR is accomplished by first synthesized cDNA molecule using reverse transcriptase, which is subsequently used as a template for PCR. RT-PCR is applied successfully to enhance detection sensitivity of sugar beet viruses, i.e., BSBV and BNYVV (**Mahmoud and Rush, 1999**), BtMV (**Moran *et al.*, 2000; Abdel Ghaffar *et al.*, 2003**; **Glasa *et al.*, 2003** and **Nemchinov *et al.*, 2004**) and BCTV (**Mahmoud *et al.*, 2005**). RT-PCR reaction confirmed the specificity of the synthesized primers that used to amplify the complete BtMV-*cp* with size length (**Abdel Ghaffar *et al.*, 2003** and **Glasa *et al.*, 2003**).

2.3. Serological studies on potyviruses as well as BtMV

2.3.1. Antisera production

Antisera are produced by immunizing rabbits using intramuscular injections with purified virus preparations (from infected leaves) emulsified in adjuvant as an efficient scheme for some potyviruses (**DeLourdes *et al.*, 1981**). Other investigators applied intraveinous injections using adjuvant-free antigens (**Makkouk and Gumpf, 1976**). It is found that using scheme depending on combination between intravenous and intramuscular injections is more

efficient in producing high antiserum titer.

An immunization scheme for BtMV is studied by **Rogov *et al.* (1991)** in which rabbits are injected with 1 mg purified virus with intramuscularly injection, after 2 weeks, another 2 mg of purified virus emulsified in incomplete adjuvant (1:1 v/v) are injected intramuscularly and subcutaneously. Post 2 weeks, a series of intraveinous injections of 2, 2.5 and 3 mg were weekly done, respectively. The rabbits are bleeding after 2 weeks post last injection.

Abdel Ghaffar *et al.* (2003) used the another scheme for BtMV, the first injection with 1 mg /ml of purified virus is emulsified with 1ml of freund's incomplete adjuvant and injected subcutaneously, followed after 4 weeks by five intramuscular booster injections; 1 mg/ml of purified virus for each was emulsified in an equal volume of incomplete adjuvant at intervals of 2 weeks. The rabbits are bled three times, started one, two and three weeks after the last injection.

2.3.2. Serological detection

2.3.2.1. Using ELISA

ELISA is one of the most widely used as a serological technique for sero-diagnosis of plant viruses (**Clark and Adams, 1977; Flegg and Clark, 1979; Rogov *et al.*, 1991; Jordan and Hammond, 1991; El-Afifi, 1997** and **Zimmermann *et al.*, 1998).** ELISA procedures are among the most sensitive serological techniques for diagnosis of plant viruses (detection range is 1-2 ng virus/ml) (**Wilson and Nakane, 1978**; **Van Regenmortel, 1982; Rogov *et al.*, 1990; Jordan and Hammond, 1991; Ismail, 1997).**

Wiesner and Krause (1990); Rogov *et al.* (1992); Owolabi *et al.* (1998); Subikova and Kollerova (1999); Abdel Ghaffar *et al.* (2003) and **Chod and Chodova (2004)** used ELISA to detect BtMV in crude extracts of virus preparations from artificially infected leaves of different sugar beet cultivars

3.2.2. Using TBIA

TBIA was used to detect for many viruses include AMV, BYDV and BBSV (**Makkouk and Kumari, 2002** and **Njukeng *et al.*, 2005**). Various modifications of the TBIA were compared for detecting of BYDV. Similar

results were obtained by using three different labelled molecules, i.e., goat anti-rabbit antibodies conjugated with alkaline phosphatase, protein A conjugated with alkaline phosphatase and goat anti-rabbit antibodies conjugated with colloidal gold. Blocking the nitrocellulose membrane with polyvinyl alcohol for 1 min was effective and allowed the procedure to be shortened by one hour. TBIA was sensitive enough to detect BYDV in old dry tissue which had been soaked in water for 1 h (**Makkouk and Comeau, 1994**).

2.3.2.3. Using DBIA

DBIA on nitrocellulose using MAbs or PAbs as used to detection of many viruses (**Makkouk and Comeau, 1994; Schenck *et al.*, 1997; Abdel-Salam, 1999; D'Onghia *et al.*, 2001; Fitch *et al.*, 2001; Kamenova and Adkins, 2004** and **Kumari *et al.*, 2006**).

2.4. Effect of potyviruses and BtMV infected plants

The mild strains of BtMV do not cause significant economic reduction in the yield of sugar beet crops (about 10%), but the severe strains usually cause a significant yield losses (about 20%), and reduced the total sugar content and fresh weight, depending upon the age of the plant when infection began and virus strain virulence (**Watson and Watson, 1953; Bennet 1964; Shepherd *et al.*, 1964**; **Shepherd and Till, 1965; Kassim *et al.*, 1993; Gadysiak and Peczynski, 1998** and **Dusi and Peters, 1999**). Infected plants with BtMV at early stages of development suffer the heaviest losses; late infections (4-6 weeks before harvest) may not cause significant yield loss (**Kaffka and Lewellen, 2001**). **Stevens (2007)** reported that the root weight rather than sugar concentration was affected by BtMV infection.

Infected sugar beet with BtMV alone has little impact losses on the yield of sugar beet roots. However, mixed infection with the BYV causes severe stunting in sugar beet, compared to a single infection with either virus, infections with complexes of aphid-borne viruses, such as BtMV, BYV and BWYV can result in considerable crop losses ranging up to 2% or more per week of infection (**Wintermantel, 2002**).

2.5. ISSR-PCR analysis

ISSR takes advantage of simple sequence repeats or microsatellites that are abundant in all eukaryotic genomes. ISSR are short, tandem repeats that are useful as genetic markers. However, use of ISSR requires a knowledge of the sequence of the region flanking the tandem repeats. ISSR, in contrast, does not require any prior knowledge of genome sequence. ISSR uses primers that are anchored at the 5' or 3' end of a repeated region and extend into the flanking region. This analysis allows amplification of the genomic segments between inversely oriented repeats.

ISSR markers have recently become widely used in population studies because they have been found to be highly variable, require less investment in time, money and labor than other methods (**Wolfe and Liston, 1998**), and have the ability to be inherited (**Gupta *et al.*, 1994**; **Tsumura *et al.*, 1996; Rae *et al.*, 2000; Laurent *et al.*, 2007**). The largest number of ISSR-PCR specific markers was scored for many viruses specially potyvirus group (SCMV , MDMV and BtMV) to determine of resistant genes (**Melchinger *et al.*, 1998; Lubberstdt *et al.*, 1999; Dussle *et al.*, 2000; Friesen *et al.*, 2006** and **Lubberstedt *et al.*, 2006**).

III. MATERIALS AND METHODS

3.1. Materials:

3.1.1. Source of virus:

Sugar beet plants exhibited virus-like symptoms, i.e., mosaic, leaf curl, blisters, stunting, vein clearing, mottling, leaf roll, and rootlet were collected from naturally infected sugar beet (*Beta vulgaris* cv. Gazel) plants cultivated in El-Riad region (El-Thabet, Abo Ghallab and El-Hageen), Kafer El-Sheikh Governorate, Egypt.

3.1.2. Plant materials:

Sseeds of a number of 10 cultivars of *B. vulgaris* (Gazel, LP12, LP13, FD9402, M9651, 9002, Ras poly, Glorius poly, Pamher and Desprez poly (N)) were kindly provided by Sugar Crops Research Institute, ARC, Giza, Egypt, but *B. patellaris, B. maritima, Spinacia oleracea, Glycine max* cv. Clark, *Vicia faba* cv. Giza 2, *Phaseolus vulgaris* cv. Giza 3, *Pisum sativum* cv. Lincoln and *Cucumis sativus* cv. Atlas seeds were purchased from ARC, Giza, Egypt.

3.1.3. Greenhouses:

Insect proof-greenhouses belonging to Virology Lab, Department of Agricultural Microbiology, Faculty of Agricultue, Ain Shams University, Shoubra El-Kheima, Egypt, were used for growing the plants and maintaining virus isolate in this work under a temperature range from 26 to 30°C.

3.1.4. Source of insects:

Two aphid species called, *Aphis fabae* (Glover) and *Myzus persicae* (Sulz) were used in this study. Aphids were collected from healthy sugar beet plants grown in the field and identified in the Insect Laboratory, Department of Plant Protection, Faculty of Agriculture, Ain Shams University, Shoubra El-Kheima, Egypt. Aphids of *A. fabae* were colonized on healthy faba been plants, while *M. persicae* were colonized on healthy cabbage plants. All aphid cultures were started with a single aphid and kept in an isolated cage in the greenhouse.

3.1.5. Electron microscopy:

The ultrathin sections and virus purified preparations were examined in a Jeol JEM-1010 Transmission Electron Microscope at Center of Mycology and the Regional Biotechnology, Al-Azhar University, Cairo, Egypt.

3.1.6. Animals used for antiserum production:

White New Zealand rabbits, in cages in the animal room belonging to Virology Lab, Department of Agricultural. Microbiology, Faculty of Agricultue, Ain Shams University, Shoubra El-Kheima, Egypt, were used for producting of specific antiserum against the virus isolate through rabbits immunization.

3.1.7. Centrifuges:

Low-speed (T 23), High-speed (Beakman) and Ultra-speed (XL-90 Beakman) centrifuges were used throughout virus purification.

3.1.8. Spectrophotometer:

Spectrophotometer model Du series Beakman was used for measuring absorbance values of purified virus and RNA preparations.

3.1.9. Serological studies:

ELISA plates (Dynatech Immunobon 2^{TM}, manufactured by Dynatch Labs., USA), ELISA reader (Bio-Tec Instruments) and 0.45 µm nitrcellulose membrane (Millipore Corporation, Bedford, MA, USA) were used throughout serological techniques.

3.1.10. Primers:

For RT-PCR of BtMV-*cp* gene, two oligonucleotides specific primers, one Forword : 5' GTG CCA CAA CAA GTT GAT GCT GGA 3' and another Reverse: 5' TGA TGC ATG TGA GCG TTG ACA TCT 3' were used for amplificating of complete BtMV-*cp* gene from BtMV-RNA full genome according to **Nemchinov *et al.* (2004).** These primers were synthesized in ABI 392 DNA/RNA synthesizer (Applied Biosystems, USA), at the AGERI, ARC, Giza, Egypt.

Concerning the ISSR-PCR fingerprinting of the sugar beet cultivars, a number of 6 ISSR-PCR primers were purchased from Clinilab Company, El-Maadi, Cairo, Egypt, and the nucleotide sequences shown in **Table (2).**

Table 2. Names and nucleotide sequences of the primers used for ISSR-PCR

*Primers number	*Names	Nucleotide sequences (5'.....3')
P1	17899B	(CA)6GT
P3	17898A	(CA)6AC
P6	17899A	(CA)6AG
P27	ISSR3	(CA)8AT
P29	ISSR16	CGTC(AC)7
P30	ISSR17	CAGC(AC)7

*Primer numbers and names according to the Gene Bank Database (www.ncbi.nlm.gov).

3.1.11. Buffers and solutions:

3.1.11.1. Virus propagation:

0.1M Tris-HCl (pH 7.2):

Tris base	3.05 g
2-ME	0.25 ml
In 250 ml distilled H_2O	

3.1.11.2. Ultrastructure

2.5%-glutaraldehyde

2.5 ml glutraldehyde in 100 ml 0.1 M cacodylate buffer

2% paraformaldehyde

2 ml paraformaldehyde in 100 ml 0.1 M cacodylate buffer

0.1 M cacodylate buffer, pH 7.2

10.7 g Na (CH3)2 AsO3. 3H2O (Sodium cacodylate), pH was adjusted to 7.2 with 1 N HCl, and then completed to 500 ml with d.H_2O	1 g
and storage at 4°C.	100 ml

1% osmium tetra-oxide

Osmium tetra-oxide	5 g
cacodylate buffer	100 ml

5% Uranyl acetate

Uranyl acetate

50 % Ethanol

3.1.11.3. Purification:

Extraction buffer (pH 7.2):

Ammonium acetate	3.85 g
EDTA	2.9 g
Na_2SO_3	5 g
Urea	30.3 g

In 500 ml d.H_2O.

Sodium borate buffer (pH 8.2):

Sodium borate	0.61 g
Borax	3.81 g
EDTA In 100 ml d.H_2O.	0.1 g

3.1.11.4. Negative staining:

2% Uranyl acetate

Uranyl acetate	2 g
d.H2O	100 ml

3.1.11.5. SDS-PAGE :

30% acrylamide 0.8% bisacrylamide:

30 g acrylamide + 0.8 g bisacrylamide were dissolved in 100 ml d.H_2O, the solution Was filtered through filter paper and storage at 4°C in dark.

Separating (resolving) gel buffer: (4 x Tris-HCl/SDS, pH 8.8)

45.5 g Tris base and 1 g SDS in 150 ml H_2O, pH was adjusted to 8.8 with 1 N HCl, then completed to 250 ml with d.H_2O and storage at 4°C.

Stacking gel buffer: (4 x Tris-HCl/SDS, pH 6.8)

6.05 g Tris base and 0.4 g SDS in 40 ml H_2O, pH was adjusted to 6.8 with 1 N HCl, then completed to 100 ml with d.H_2O and storage at 4°C.

2 x SDS sample (laemmlli) buffer:

To 40 ml distilled H_2O, 1.52 g Tris base, 20 ml glycerol, 2.0 g SDS, 2.0 ml 2 mercaptoethanol, 1 g bromophenol blue were added, pH was adjusted to 6.8

with 1 N HCl, volume was completed to 100 ml with d.H_2O.

5X SDS electrophresis buffer:

15.1 Tris base, 72.9 glycin, 5.0 g SDS were added to 500 ml distilled wat pH

was adjusted to 8.3 with 1 N HCl, then completed to 1000 ml with d.H_2O.

Staining solution:

35 ml acetic acid (96%), 100 ml methanol, d.H_2O 400 ml, 0.2%.

Comassie Billiant Blue R-250.

Distaining solution:

32.5 ml Glacial acetic acid,100 ml methanol, complete to 500 ml d.H_2O.

Ammonium persulfate (10%):

100 mg in 1 ml d.H_2O.

3.1.11.6. Extraction of total RNA buffers:

TE buffer (pH 7.0):

Tris	20 g
EDTA	0.073 g
SDS	2.5 g
2-ME	2.5 ml

In 250 ml d.H_2O.

10X TBE (Running buffer, pH 8.0):

Tris	25.2 g
Boric acid	13.7 g
EDTA	1.86 g

In 250 ml d.H_2O.

10X Stop solution (pH 7.2):

Glycerol	125 ml
SDS	2.5 g
EDTA	292.25 g
Bromophenol blue	0.25 g

Sodium acetate buffer (pH 5.2)

40.8 g Sodium acetate in 40 ml H_2O, pH was adjusted to 5.2 with acetic acid, then completed to 100 ml with d.H_2O.

10X Stop solution (pH 7.2):

Glycerol	125 ml
SDS	2.5 g
EDTA	292.25 g
Bromophenol blue	0.25 g

In 250 ml d.H_2O.

3.1.11.7. ELISA : (PBS, pH 7.4):

NaCl	8.00 g
KH_2PO_4	0.20 g
Na_2HPO_4	1.15 g
KCl	0.20 g

In 1000 ml d.H_2O.

PBS-Tween (PBS-T):

PBS+0.5 ML Tween 20/liter

Coating buffer (PH 6.9):

Na_2CO_3	1.59 g
$NaHCO_3$	2.93 g

In 1000 ml d.H_2O.

Extraction buffer (pH 7.4):

The follwing were dissolved in 1 liter PBS-T buffer:

PVP	20.0 g
Sodium sulfite	10.0 g

Conjugate buffer (pH 7.4):

To 1 liter PBS the following were added:

Ovalbumin	2.0 g
PVP	20.0 g
Tween 20	0.5 ml

Substrate buffer (pH 9.8):

Diethanolamine	9.7 ml
Distilled water	80.0 ml

The pH was adjusted to 9.8 with a concentration HCl. These were increased to 100 ml using d.H_2O.

Substrate:

1 mg of *p*-Nitrophenyl phosphate in 1 ml substrate buffer.

3.1.11.8.TBIA and DBIA:

TE buffer (pH 8.0):

Tris	0.3 g
EDTA	0.073g

In 250 ml d.H_2O.

Substrate buffer (pH 9.6):

Tris	0.1 M
NaCl	0.1 M
MgCl2	5.0 M

pH was adjusted to 9.6 with 5 N HCl.

Blocking buffer (pH 7.5):

Tris	20 m M
NaCl	500 m M
Tween-20	0.1 %

Substrate:

14 mg of NBT, 7 mg (BCIP) in 40 ml substr buffer.

3.1.11.9. RT-PCR :

5X first strand cDNA buffer:

250 mM Tris-HCl, pH, 8.3, 37 mM KCl and 15 mM MgCl2.

Reaction solution:

4 µl of 5X first strand cDNA buffer, 5 µl of 2-ME, 2.5 µl of 10 mM dNTP (2.5 mM each dGTP, dATP, dTTP, dCTP).

10X PCR buffer:

100 mM Tris-HCl, pH, 9.0, 500 mM KCl and 1% Triton x-100.

TBE buffer:

89 mM Tris, 89 mM borate and 2.0 m M EDTA, pH, 8.3.

TE buffer:

10 mM Tris-HCl, 1mM EDTA, pH, 8.0.

3.1.11.10. Chemical constituents :

5 % basic lead acetate	59 g
Basic lead acetate	1000 ml
d.H_2O	

3.1.11.11. ISSR-PCR:

1 X PCR buffer	
KCl	50 mM
Tris-HCl (pH, 8.3)	10 mM
$MgCl_2$	1.5 mM
1% agarose	
Agarose powder	1 g
1X TAE buffer, pH, 7.8	100 ml
TAE buffer 50X	
Tris-base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA, pH, 8.0	100 ml

Dilute 20 ml stock solution with 980 ml double d.H_2O to obtain 1 X concentration.

3.2. Methods:

3.2.1. Biological identification

3.2.1.1. Virus isolation

3.2.1.1.1. Preparation of virus inoculum

Virus inoculum was prepared by triturating leaf tissues of sugar beet (*B. vulgaris* cv. Gazel) plant exhibited virus-like symptoms in a mortar and pestle using 0.1 M tris buffer, pH 7.2 containing 0.1% 2-ME (1:2 w/v). The sap was filtered through two layers of cheesecloth and used for mechanical inoculation of differential hosts.

3.2.1.1.2. Mechanical inoculation

Mechanical inoculation was performed according to **Walkey (1985)** by dusted plant leaves of *C. quinoa* (as diagnostically hosts) with

carborandom and rubbing with forefinger dipped in the infectious sap. After 30 minutes, plants were sprayed with water and kept in under greenhouse conditions (28°C±2). The inoculated plants were examined daily for visual symptoms up to 7 days. The virus isolate was biologically purified by single local lesion method according to **Sidek and Sako (1996),** then maintained in *B. vulgaris* plant (as a propagation host), and kept under aphid-free greenhouse conditions and all subsequent work was done with material from this source.

3.2.1.2. Host range

Different hosts were mechanically inoculated with BtMV-infectious sap. These hosts included *B. patellaris, B. maritima, B. vulgaris* cvs. Gazel, LP12, LP13, FD9402, M9651, 9002, Ras poly, Glorius poly, Pamher, Desprez poly (N), *Chenopodium amaranticolor, C. quinoa, Spinacia oleracea* L. (*Chenopodiaceae*), *Glycine max cv.* Clark, *V. faba cv.* Giza 2, *Phaseolus vulgaris* cv. Giza 3 , *Pisum sativum* cv. Lincoln (*Fabaceae), Cucumis sativus* cv. Atlas (*Cucurbitaceae), Datura metel, D. stramonium, N. glutinosa, N. rustica* and *N. tabacum* cvs. Samsun and White Burley (*Solanaceae*).

3.2.1.3. Insect transmission

Aphids were tested for their ability to transmit the virus isolate in non-persistent manner according to the method of **Zeyen and Berger (1990)** with some modifications. Aphids were transferred to Petri dishes containing dry filter papers where they starved for one hour. Single aphid of each of *A. fabae* and *M. persicae* were allowed to feed on virus-infected sugar beet (*B. vulgaris* cv. Gazel) leaves (obtained from previous systemically infected plants 12-15 days after virus inoculation) for 10 minutes. The aphids were observed by a hand lens for the required acquisition period. Single aphid from each species was then placed on a healthy leaf of the test plant. Twenty healthy seedlings (with 2-3 leaves) of *B. vulgaris* cv. Gazel were used for each aphid species. The aphids were killed with Malathion insecticide one day after feeding on healthy plants. This experiment was twice repeated.

3.2.1.4. Types of inclusions

To identify types of inclusions induced by BtMV, systematically BtMV-infected leaves of *B. vulgaris* cv. Gazel plant 20 days post virus mechanical inoculation were subjected to electron microscopic examination using the method of **Abdel-Ghaffar *et al.* (1998).** Leaves were cut into 1x1 mm pieces, then fixed in cold 2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M cacodylate buffer, pH 7.2 overnight at 4°C. The tissue pieces were washed three times for 15 minutes, each with 0.1 M cacodylate buffer, pH 7.2 containing 3 % sucrose. Post fixation was carried out in cold 1% osmium tetraoxide in the same buffer for 40 minutes at 4°C, and then washed 3 times for 10 minutes each with the same washing buffer. Tissues were then dehydrated in graded series of ethanol as follows: Two changes of 15 minutes each in 30, 50, 70, and 85% ethanol, then 2 changes of 30 minutes each in 95% and 3 changes of 30 minutes each in 100% ethanol at RT. After dehydration, samples were infiltrated with undiluted Propylene oxide solution 2 times of 15 minutes each at RT, then in a 1:1 mixture of propylene: plastic (plastic mixture: DDSA, Epon 812, Arldite 6005, Dibutyl phthalate and DMP-30) overnight at RT and then in a pure plastic for 2 hours at RT. Samples were then embedded in pure plastic in emdedding capsules and placed at 45°C overnight, then at 60°C for 24 hours for curing. Ultrathin sections were prepared using glass knife of ultramicrotom and the sections were taken on copper grid and stained with 5% uranyl acetate in 50% ethanol and examined in the transmission electron microscope.

3.2.1.5. Virus purification and spectrophotometer

This method was carried out according to **Glasa *et al.* (2000)** with some modifications. Systematically infected leaves of *B. vulgaris* cv. Gazel were collected 20 days post virus mechanically inoculation and stored at -20°C overnight. Two-hundred grams of infected leaves were ground in a blender in 2.5 volumes of extraction buffer and the extracted sap was filtered through double layers of cheesecloth, and then was centrifuged at 9000 rpm for 10 minutes. Chloroform and n-butanol in a ratio of 1:1 (final

concentration 8%) were added to the sap for clarification and stirred at 4°C for 15 minutes. Then the sap was centrifuged at 8000 rpm/15 min. Pellets were discarded and supernatant was subjected to two ultracentrifugation steps (28000 rpm/90 minutes at 4°C), one on a 20% sucrose cushion and another on a 10-40% Sucrose density gradient. Fractions were separated manually and subjected to spectrophotometering at 254 nm. The fractions showed virus-like peak were collected and pelleted by ultracentrifugation at 45,000 rpm for 90 min. Pellets were resuspended in the 0.01 M sodium borate buffer, pH 8.2.

Purified virus preparations were diluted in 0.01 M ammonium acetate, pH 7.2 (1/10 dilution) and measured at serial wave lengths ranged from 220 to 320 nm. The same buffer was used as a blank. Virus concentration was calculated according to **Noordam (1973)** using extinction coefficient of 2.4 for a 1 mg/ml solution.

$$\text{Virus concentration (mg/ml)} = \frac{\text{Optical density (OD) at 260}}{\text{Extinction coefficient (2.4)}} \text{ X dilution factor}$$

The percentage of virus nucleic acid was calculated according to an equation that mentioned by **Gibbs and Harison (1976)** using the value of A260/280 for the virus nucleoprotein.

3.2.1.6. Negative staining:

Purified virus preparation was negatively stained according to the method of **Griffin (1990)**. Carbon coated grids were kept floating on the surface of purified preparation for 10 seconds, then dried using filter paper (from the grid side) and transferred onto the surface of 2% Uranyl acetate. Grids were then left to dry on filter paper in a Petri dish and examined by transmission electron microscope.

3.2.2. Chemical identification:

3.2.2.1. Determination of molecular weight of BtMV coat protein:

This was carried out by using SDS- PAGE with 4% stacking gel and 12% separating gel based on method of **Laemmli (1970).** Aliquots of purified

virus preparation was denaturated for 5 min in boiling water-bath after mixing with 2X sample Laemmlli buffer with ratio of 1:1 (v/v). Gels were prepared by mixing rates of stock solutions as shown in **Table (3).** In addition, after 15 days of mechanical inoculation, 1.0 gram from BtMV-infected leaves and healthy leaves were extracted in 2X sample Laemmlli buffer at ratio of 1:2 (w/v), then denaturated in a boiling Water-bath for 10 minutes and centrifuged at 10000 rpm for 10 minutes, supernatant was again denaturated for 5 minutes and centrifuged at 10000 rpm for 1 minutes. Aliquots of 50 µl from each of denaturated purified virus preparation, infected and healthy samples as well as stander protein marker were loaded onto slab gel and electrophoresed at 100 V for 30 min, then at 200 V for 4 h. Protein bands were observed after staining in 0.2% Comassie Brilliant blue R-250 for 8 h and destained with destain solution until background became clear.

Table 3. SDS-PAGE solutions for preparing gels.

Stock solutions	**Separating gel 12%**	**Stacking gel 4%**
Acrylamide	12 ml	1.5 ml
Separating gel buffer (pH 8.	7.5 ml	--
Stacking gel buffer (pH 6.8)	--	2.5 ml
d.H_2O	10.5 ml	6.1 ml
Ammonium persulfate 10%	100 µl	50 µl
TMED	25 µl	20 µl

3.2.2.2. Determination of BtMV-RNA size length

3.2.2.2.1. Extraction of BtMV-RNA

RNA was extracted from purified virus preparations according to the procedure described in Tripure Isolation Reagent Manual (Roche Diagnostics Coporation, IN, USA). Plastic wares and solutions were RNase- free by using DEPC to 0.1% (v/v) and then incubated overnight at RT, glasswares were heated-sterilized at 200°C overnight. DEPC traces were removed by autoclaving for 30 min. Aliquots of 50 µl purified virus preparation (5 mg/ml) were transferred to a sterile microcentrifuge tube, then added 1 ml tripure

isolation reagent after homogenization incubate for 5 min at RT to ensure the complete dissociation of nucleoprotein complexes, then 0.2 ml chloroform was added to the initial homogenization and shake virigously for 15 sec. Then the tube was incubated at RT for 15 min then the tube was centrifuged at 14,000 rpm for 15 min at 4°C to separate the solution into three phases, then the colorless upper aqueous phase transferred to a new tube and 0.5 ml isopropanol was added. The tube was inverted several times and the mixture incubated for 10 min at -20°C, then centrifuged at 14,000 rpm for 10 min at 4°C. The pellet was washed by 1 ml of 75% ethanol by vortexing and centrifugation for 5 min at 8,000 rpm at 4°C. The supernatant was discarded and the excess of supernatant was removed by the air drying and finally the RNA-pellet was resuspended in 50 µl of DEPC-treated deionized autoclaved H_2O, and stored at -70 °C until use. The concentration was estimated using spectrophptometer, assuming an extinction coefficient of 25 cm^{-1} mg^{-1} at 260 nm.

$$\text{RNA concentration (µg/µl)} = \frac{\text{Optical density (OD) at 260}}{\text{Extinction coefficient (25)}} \text{ X dilution factor}$$

3.2.2.2.2. Electrophoresis of BtMV-RNA

The size length of BtMV-RNA was electrophoretically estimated through 1.0 % agarose gels as described by **Tamada *et al.* (1989).** The gels were stained with ethidium bromide **(Sambrook *et al.*, 1989).** The bands were then visualized under UV-transilluminator.

3.2.2.3. Estimation of BtMV *cp* gene size length

In this experiment, protocol of the AccessQuick™ RT-PCR System was successfully used for estimation of BtMV *cp* gene size length using the extracted BtMV-RNA, the *Taq* DNA polymerase, the forward and reverse primers, *AMV* reverse transcriptase and nuclease-free water. The reaction components used were shown in **Table (4).** The mix was subjected to one cycle at 48°C for 1 hr; one cycle at 95°C for 2 min and 30 cycles, each consists of 95, 50 and 72 °C for 45 sec, 45 sec and 1 min, respectively. The

final cycle was extended for 10 min. The PCR product was analyzed through 1.0% agarose gel electrophoresis in TAE buffer then stained with ethidium bromide and the product was then visualized by transillumination as recommended by **Sambrook *et al.* (1989).**

Table 4. RT-PCR reaction components for amplification of complete BtMV-*cp* gene.

Components	**µl**
Nuclease free water	18
AMV/Taq 5X reaction buffer	10
dNTPs mix (10 mM each dNTPs)	1
BtMV-F primer (50 pmol)	1
BtMV-R primer (50 pmol)	1
25 mM $MgSO_4$	2
AMV reverse transcriptase (5U/ µl)	1
Taq DNA polymerase (5U/ µl)	1
RNA sample (3.2 ng/ µl)	15
Total volume	**50**

3.2.3. Serological studies:

3.2.3.1. BtMV-Antiserum production

Two adult of White New Zealand rabbits (2-4 kg) were used for such purpose. Before starting the injection schedule, the rabbits were bled to obtain the normal serum. Rabbits were injected with BtMV purified preparation four times at one week intervals according to the method described by **Makkouk and Gumpf (1976)** as shown in **Table (5).** Bleeding was carried out one, two and three weeks post last injection. Rabbit ear was shaved and wiped with 70% ethanol; small longitudinal cut was made by a new blade along the external marginal vein. Blood was collected in sterilized glass tube, which was left to coagulate at 37°C for 2 hours and then was placed in refrigerator overnight. Clot was removed and the serum was centrifuged at 6000 rpm for 15 minutes, serum was kept at -20°C till used.

Table 5. Injection schedule for raising BtMV-antiserum.

Injection #	Positions	mg/ml of virus	Freund's adjuvant	
			ml	Type
1	Im	2.0/0.5	0.5	Complete
2	Iv	1.0/0.5	---	---
3	Im	2.0/0.5	0.5	Incomplete
4	Im	2.0/0.5	0.5	Incomplete

Im = Intramuscular. **Iv = Intraveinous.**

3.2.3.2. Determination of DEP of BtMV-antiserum

DEP was determined with indirect ELISA according to **Koening (1981)** as follows: BtMV-antiserum was diluted with coating buffer to two-fold dilutions, i.e., 1/2, 1/4, 1/8. 1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024, 1/2048, 1/4096, 1/8192 and 1:16384. Samples of infected and healthy tissues were ground in sample extraction buffer at a ratio of 1:10 (w/v), while purified virus preparation were diluted to 10^{-1} using coating buffer. Two-hundred µl of purified virus (10 ng/100 µl) were added to 60 wells (4 wells/dilution) of the microwells ELISA plate, as well as 4 wells from each of clarified extracts of BtMV-infected and heathy leaves. The plates were incubated at 4°C for 2 h. The plates were washed 3 times with PBS-Tween (PBS-T) buffer till wells became clear. Two-hundred µl of serial dilutions of BtMV-antiserum were added to each well (4 wells/dilution), while 200 µl of undiluted BtMV-antiserum were added to wells coated with infected and healthy preparations (4 wells/each), and then the plates were incubated at 37°C for 3 h. The plates were washed 3 times with PBS-T buffer. Two-hundred µl of anti-rabbit IgG conjugated with alkaline phosphatase (diluted to 10^{-4} with conjugate buffer) were added to each well, then incubated at 37°C for 2 h. The plates were washed 3 times with PBS-T buffer. To each well, 200 µl of freshly prepared substrate solution (containing 1 mg *p*-nitrophenyl phosphate per 1 mg substrate buffer) were added, and then incubated at 37°C. The plates were read at 405 nm using ELISA reader each half an hour up to 2 h, and the average reading values were calculated.

3.2.3.3. Evaluation of BtMV-antiserum

3.2.3.3.1. Using TBIA

TBIA was carried out as described by **Lin and Hus (1990)**. Infected and healthy leaves were cut to parts (0.5×0.5 cm) using a new razor blade, then stamped on a nitrocellulose membrane (0.45μm) after wetting the membrane by immersing in PBS buffer solution for few seconds to remove air and left it to dry. Membrane was left for 20 min for sap to be adsorbed, plant tissues were then removed. The mmbrane was placed in blocking buffer on a shaker for 30 min at RT, and then incubated with BtMV-antiserum (dilution 1/2) for 1 h at 37°C on a shaker. After washing 3 times, 10 min each with PBST, the membrane was incubated with conjugated anti-IgGs (dilution 1/5000) for 1 h at 37°C on a shaker and washed as mentioned. The substrate solution consists of 14 mg NBT and 7 mg BCIP dissolved in 40 ml of substrate buffer was prepared. The membrane was incubated in a volume of 20 ml of substrate for 10-20 minutes under dark conditions. When purple color developed clearly, reaction was stopped by placing membrane in TE buffer.

3.2.3.3.2. Using DBIA

The method of **Hibi and Saito (1985)** was performed as follows: infected and healthy sugar beet tissues were extracted in PBS (1/2 w/v), then centrifuged at 10000 rpm for five minutes. Supernatant was diluted to 10 fold dilutions using PBS, as a control purified virus preparation was diluted to 10^{-1} using PBS. Nitrocellulose membrane was placed for few seconds in PBS to remove air and left to dry. By micropipette dilutions were dropped (5 μl) in the center of each circle drawn on the membrane according to a standard cast, then drops were left to dry. Membrane was placed in blocking buffer solution as mentioned and the rest was carried out as previously described before.

3.2.4. Effect of BtMV on growth and some chemical constituents of sugar beet plant

This experiment was carried out under greenhouse condition in sterilized clay pots (30 cm in diameter) contained sterilized mixture of clay and sand soil. Five seeds of ten sugar beet cultivaers were cultivated in the pots (10 pots/cultivar), the cultivated pots were regularly irrigated with tap water. The five pots from each cultivar (2 seedlings/pot) were mechanically inoculated with BtMV whereas; the other five pots were left without inoculation as a control. BtMV-infection was confirmed by external symptoms, mechanical inoculation on *C. quinoa* (as a diagnostic host). The plants were carefully removed from the soil five months post plantation, then washed with tap water and air-dried.

3.2.4.1. Effect of BtMV on fresh and dry weight of shoots and roots

The fresh weight of shoots and roots were estimated separately, then the shoots and roots were dried in a drier at 60°C for 24 h according to **AOAC (1975),** then the dry weight was recorded.

3.2.4.2. Effect of BtMV on some chemical constituents of sugar beet storage roots

3.2.4.2.1. Sucrose determination

Sucrose content in the storage roots was determined by using Automatic Saccharimeter on a lead acetate extract of fresh root according to method of **Le Docte (1927).**

3.2.4.2.2. Potassium and sodium determination

Potassium and sodium contents in the storage roots were photometrically estimated using flame photometer according to **Carruthers and Oldfield (1961)**

3.2.4.2.3. Amino nitrogen determination

Amino nitrogen content was determined according to procedure described by **Detavernier (1979).**

3.2.4.3. Statistical analysis

All data were subjected to statistical analysis according to **SAS (1996),** analysis and means were compared by range tests at the 5% level.

3.2.5. ISSR-PCR analysis:

This experiment was done at Labs of Gene Bank, ARC, Giza, Egypt.

3.2.5.1. Extraction and purification of genomic DNA

About 200 mg of leaf material were collected from the ten cultivars of sugar beet, i.e., *B. vulgaris* cvs. Gazel, FD9402, LP12, M9651, LP13, 9002, Ras poly, Glorius poly, Pamher and Desprez poly (N). Genomic DNA was extracted and purified using the DNeasy Plant Mini Kit following the manual instructions (QIAGEN, Chatsnorth, CA, USA).

3.2.5.2. Estimation of DNA quantity and quality

DNA concentration was determined by as described by **Hoisington *et al.* (1994)**. The DNA samples were diluted to 1:5 in d.H_2O and electrophoresed in 1 % agarose gel in the presence of 10 µl of a DNA size marker (Lambda DNA digested with *Hind* III and ØX174 DNA digested with *Hae III*). This marker covers a range of DNA fragments size between 1800 and 100 bp, and a range of concentration between 95 ng and 11 ng. Thus, estimation of the DNA concentration in a given sample was achieved by comparing the degree of fluorescence of the unknown DNA band with the different bands in the DNA size marker.

3.2.5.3. ISSR-PCR

3.2.5.3.1. PCR amplification

The amplification reaction was carried out in 25 µl reaction volume containing in a final concentration 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 pmol primer, 0.04 U *Taq* DNA polymerase and 2 ng tempelate DNA. PCR amplification was performed in a MJ-research Thermal Cycler programmed to fulfill 35 cycles after an initial denaturation cycle for 4 min at 94°C. Each cycle consisted of a denaturation step at 94°C for 45 sec, an annealing step at 50°C for 30 sec, and an extension step at 72°C for 2:05 min. The primer extension segment was extended to 5 min at 72°C in the final cycle.

3.2.5.3.2. Gel electrophoresis of PCR products and analysis

The edges of a dry clean plastic tray supplied with the electrophoresis apparatus (Bio-Rad mini cell) was sealed with autoclave tape so as to form a

mold, which was set on a horizontal section of the bench. The agarose gel was prepared by adding 0.6 gm of powdered agarose to 50 ml of 1 X TAE buffer in Erlenmeyer flask. The flask was heated in a microwave oven until the agarose was melted and dissolved in the buffer. The agarose solution was cooled to 60°C, and then ethidium bromide (10 mg/ml) was added to a final concentration of 0.5 μg/ml and mixed thoroughly. A comb (1 mm thickness) was placed above the plate so that a complete well is formed when the agarose solution was added to the mold (the comb should not be attached to the plastic plate of the mold to prevent the sample leakage between the gel and the plate). The remainder agarose solution was poured into the mold to make the gel thickness more than 3 mm and air bubbles were avoided under and between the wells of the comb. The gel was allowed to solidify at room temperature. Then, the comb and the tape were carefully removed. The agarose gel was put on the electrophoresis apparatus and the tank buffer (1X TAE) was added to cover the gel to a depth of about 1 mm. The DNA samples were mixed with loading buffer, and then the mixture of each sample was loaded separately into the wells of the gel using an Eppendorf adjustable micropipette. The DNA marker was loaded on the first well of the gel. The lid of the gel tank was closed and the electrodes were attached to the power supply and the samples were allowed to migrate toward the anode at 100 volts for 1 h. Amplified products were visually examined under UV transilluminator and the presence or absence of each sizes class was scored as 1 (present) or – (absent), respectively.