V. DISCUSION

Biological studies:

Samples were collected from sugar beet plants exhibited virus-like symptoms from different open fields, El- Riad district, Kafer El-Sheikh Governorate, Egypt, appear to be suggested having three viruses, i.e., BtMV, BNYVV and BCTV but the symptoms concerning BtMV was observed to be dominant.

BtMV isolate was identified *via* reaction of some differential hosts and host range, mode of transmission and detection of BtMV-characterestics CCI in ultrathin sections using electron microscopy. These methods were used to identify the virus isolate **(Rogov *et al.*, 1991; Abdel-Ghaffar *et al.*, 2003** and **Jalali *et al.*, 2003).**

The results of host range showed that the BtMV isolate was mechanically transmitted since it gave chlorotic lesions on *C. amaranticolor, C. quinoa,* chlorotic lesions and mosaic on *B. maritima*, necrotic lesions, mottling, severe stunting, on *Spinacia oleracea*, the virus isolate produced also mosaic on *B. vulgaris* cvs. LP12 and Pamher, mosaic and malformation on cvs. Gloriuspoly, FD9402 and Desprezpoly (N), Severe mosaic on cv. 9002, severe mosaic and leaf curle on cv. Gazel, mosaic, leaf curle and yellowing on cv. Raspoly, mosaic, leaf curle and blisters on cv. M9651, mosaic, stunting and blisters on cv. LP13, Mosaic and blisters on *B. patellaris*, mosaic on *Vicia fabae* cv. Giza 2, mosaic and malformation on *Glycin max* cv. Clark. *C. amaranticolor and C. quinoa* was found to be a good diagnostic host and this was confirmed with those found by **Abd El-Mohsen *et al.* (2003)** for PVY-N. **Kassim *et al.* (1993) and Abdel-Ghaffar *et al.* (2003)** reported that all varieties of sugar beet tested plants were found susceptible to infect with BtMV.

On the other hand, the test plants belonging to families *Cucurbitaceae*, and *Solanaceae* were found to be immune to be infected with

BtMV. These results agree with those reported by **Brunt *et al.* (1996),** but disagree with **Rogov *et al.* (1991)** who reported that the virus isolate from sugar beet collected from South Kazakhstan was identified as an isolate of BMV-K, it transmitted by sap inoculation to 19 species from 7 families tested and it could not be transmitted to *N. tabacum, N. debneyi, N. glutinosa* and *N. clevelandii*.

BtMV was non-persistently transmitted by *M. persicae* and *A. fabae* from infected *B. vulgaris* cv. Gazel to healthy ones with percentage of 70 and 57.5 %, respectively. These results compatible with those reported by **Peters *et al.* (1990); Yuan and Qiu (1991); Dusi and Peters (1999) and Abdel-Ghaffar *et al.* (2003).**

Using electron microscopic examination, the cylindrical inclusions as pinwheels and laminated aggregates were observed in the BtMV-infected sugar beet mesophyll cells 20 days post mechanical inoculation. This was in agreement with **Kolesnik (1987) and Abdel-Ghaffar *et al.* (2003)** who detected the same kind of BtMV inclusions in ultrathin sections obtained from infected sugar beet leaves. This result characterized BtMV as a member of potyvirus subdivision II, but **Abd El-Mohsen et al. (2003)** showed that the electron microscopic examination of leaf ultra-thin sections of PVY-N infected *Datura metel* revealed the presence of pinwheel, scrolls and short curved laminated aggregates which can be classified as subdivision IV according to **Edwardson (1984).**

The modern classification of *Potyviridae* is depending on cytoplasmic inclusion (*CI*) gene (**Chng *et al.*, 1997**). They used the phylogenetic analysis of *CI* gene as an alternative approach for evolution the *Potyviridae,* and suggested that the phylogenetic relationship among potyviruses can be determined using *CI* gene.

Adams *et al.* (2005) found that the comparison of the complete ORF of *CI* gene showed that this region would therefore, be the best for diagnostic and taxonomic studies if only a sub-portion of the genome is to be sequenced.

Analysis of amino acids sequence of the *CI* genes of eleven potyviruses suggested that epitops are primarily in the C-Terminal domains of the CI protein **(Joch Hammond, 1998).**

This virus particles was not observed in the same electron microscopy, this could be due to that the age of the leaf sample taken for ultrathin sections. **Emilio *et al.*(1997)** found that the non structural CI protein of TVMV during early stages of systemic infections in plant detected (Immunogold method) near plasmodesmatal connection between mesophyll cells perior to the appearance of CP.

Chemical studies:

The purification method developed was found to be satisfactor method for the extraction and purification of BtMV particles free from host protein contaminats. Using 0.1 M ammonium acetate buffer, pH 7.2 containing 0.02 M EDTA, 1 % sodium sulfate and 1M urea as an extraction buffer improved the purification procedure. Clarification with chloroform-N-butanol and low speed centrifugation, then precipitating the virus particles by 20% sucrose cushion in high-speed centrifugation, and concentration through sucrose density gradient centrifugation were found to be satisfactory without much loss of virus particles. The purified virus preparation showed a typical curve of nucleoprotein with Amax/Amin and $A_{260/280}$ ranged from 1.6-1.7 and 1.3-1.4, respectively. BtMV was purified from freshly harvested sugar beet leaves by many methods; most of them depended on using differential and density gradient centrifugation to precipitate the virus particles (**Hammond and Lawson, 1981; Rogov *et al.*, 1989; Ghorbani *et al.*, 2001; Okhoyvat *et al.*, 2001**). The average yield of the purified virus was estimated to be 20-30 mg of virus / Kg infected leaf tissue based on the extinction coefficient of 2.4 for a 1-mg/ml solution at 260 nm. **Rogov *et al.*, (1991)** and **Abdel-Ghaffar *et al.* (2003)** reported that the estimated yield of BtMV was 15-30 mg/kg infected tissues.

Electron micrographs of virus particle negatively stained with 2% uranyl acetate showed minimum amount of host components, and a large number of flexuous filamentous particles of 750 X 13 nm were detected in the BtMV-purified preparations when examined by the transmission electron microscope. Similar results were reported by **Rogov *et al.* (1991)**; **Glasa *et al.* (2000); Abdel-Ghaffar *et al.* (2003) and Mayo *et al.* (2005)** who confirmed that the BtMV particles were ranged from 700-750 nm in length and typical of the potyvirus group.

SDS-PAGE was used for determinating of virus coat protein molecular weight in purified virus preparation and extracts of sugar beet leaf tissues. BtMV-bands with molecular weight of 34 kDa were clearly observed from purified preparations and sap extracted from BtMV-infected leaves. This result was consistent with that found by **Rogov *et al.* (1991)** and **Abdel-Ghaffar *et al.* (2003)** and relatively agrees with **Glasa *et al.* (2000)** who reported that the molecular weight of BtMV-coat protein was approximately 30 kDa.

BtMV-ssRNA was extracted from the purified virus particles and migrated in 1% agrose gel eletrophoresis. Results showed that a single component with estimated size length of about 10 kb. This result is in compatible with that reported by **Brunt *et al.* (1996)** and **Abdel-Ghaffar *et al.* (2003).**

RT-PCR was successfully used for amplification of complete *cp*-gene from the BtMV-RNA full genome using two oligonucleotide specific primers. Results indicated that the size length of BtMV-*cp* gene estimated about 755 bp. Similar results were reported by **Abdel-Ghaffar *et al.* (2003); Glasa *et al.* (2003)** and **Nemchinov *et al.* (2004)** who confirmed that the size length of BtMV-*cp* gene determined for about 750-800 bp.

Serological studies:

In this study, BtMV-antiserum was successfully raised, and three bleedings were obtained one, two and three weeks post last injection. Results

showed that DEP of BtMV-antiserum of the 2nd bleeding was the highest one and estimated to be 1/4096 using I- ELISA. The resultant antiserum was able to detect the BtMV in infected leaf tissue. Since ELISA test detect viral proteins, it is considered as a sensitive technique that can detect virus proteins in an early stage of infection before virus assembly and formation of intact virion when compared with biological detection techniques **(Matthews, 1970).** This test was found to be easy and efficient for detection many samples at the same time in one ELISA plate. The same procedure was used by **Jordan and Hammond (1991)** used indirect antigen-coated plate ELISA and a panel of potyviral-specific MAbs.

TBIA was successful used as a serological technique for detection of BtMV in clarified sap extracted from infected sugar beet leaves as well as virus purified preparation, since it gave always purple color with the infected tissues, while no color was obtained with healthy ones. Some investigators used the same procedure for detection of many viruses such as AMV, BYDV and BBSV (**Makkouk and Kumari, 2002** and **Njukeng *et al.*, 2005**).

DBIA was found to be a simple and rapid method for serological detection of BtMV. The test gave positive results in the form of purple color with the purified virus preparations and with the infectious sap dilutions up to 10^{-6}. Negative result was obtained with sap extracted of healthy sugar beet leaves. This test was used for detection of many viruses include BNYVV (**Abdel-Salam and El-Shazly, 2001),** CCDV **(Kumari *et al.*, 2006)** and SCYLV (**Schenck *et al.*, 1997).**

Effect of BtMV on sugar beet plant:

The results of effect BtMV on different cultivars of sugar beet showed that the virus infection was reflected in the significantly reduction in fresh and dry weight of shoots and roots which ranged from 21-51, 25-49 % in shoots and 27-57, 29-56 % in roots, respectively. BtMV was caused significantly reduction of sucrose content which ranged from 29-50%, as reflected in raised of amino nitrogen, in storage roots of different cultivars of

sugar beet. **Shepherd *et al.* (1964)** and **Shepherd and Till (1965)** reported that the virulent strains of BtMV cause a loss on root yield of 20% depending upon the age of the plant. **Stevens (2007)** reported that the root weight rather than sugar concentration was affected by BtMV infection. Our results disagree with **Wintermantel (2000)** which reported the infected sugar beet with BtMV alone has little impact losses on the yield of sugar beet roots

Stevens *et al.* (2004) reported that both BMYV and BYV caused significantly decreased the root weights and sugar yield of field-grown sugar beet.

ISSR-PCR analysis:

The results of mechanical inoculation showed that all sugar beet cultivars had been infected with BtMV with different percentage of infection. It was shown that the cultivars Desprezpoly (N), 9002, Pamher, LP12, Gloriuspoly, Rasploy, M9651, LP13, Gazel and FD9402 were susceptible with percentage of 40, 50, 55, 60, 70, 75, 80, 85, 90 and 100 , respectively.

Therefore, this experiment aimed to determine the relationship between the susceptibility of some sugar beet cultivars to infection with BtMV and their genetic variations using (ISSR-PCR). Six primers used in the present investigation resulted a total of 87 DNA bands. The unique markers for sugar beet accessions generated from ISSR-PCR analysis are as high as 22 out of the 87 (25%). ISSR-PCR markers were found to be useful as accession-specific markers. The largest number of ISSR-PCR unique markers was scored for the cutivar Glorius poly (4 markers). A number of 11 unique markers were scored for the presence of a unique band for a given accessions (positive marker), while 11 were scored for the absence of a common band (negative marker). The largest number of ISSR-PCR accession-unique markers was generated by primer 17899B (8 markers) followed by primers 17899A and primer ISSR16 (4 markers). On the other hand, the least number of ISSR-PCR unique markers was generated by primer 17898A and primer ISSR17.

ISSR dendrogram obtained from cluster analysis of genetic distances and similarity index for sugar beet cultivars are revealed that the strongest relationship was scored between Desprez poly (N) and 9002 (90%), while cultivars FD9402 and Pamher were shown to be the most genetically distant accessions (79%). This result is compatible with that obtained with biological results at which, cultivars Desprezpoly (N) and 9002 gave 60 and 50 % resistance to BtMV, while cultivars FD9402 and Pamher gave zero and 45 % resistance to BtMV, respectively. The ISSR-based coefficients of genetic similarity among the ten cultivars of sugar beet resulted in dendrogram, where comprised into three clusters, the first one was included four sugar beet cultivars, i.e., Desprez poly(N), 9002, Pamher and LP12 (60, 50, 45 and 40 % resistance to BtMV); the second cluster includes four cultivars, Gloriuspoly, Raspoly, M9651 and LP13 (30, 25, 20 and 15 % resistance to BtMV); the third cluster includes cultivars Gazel and FD9402 (10 and zero % resistance to BtMV).

Schmidt and Heslop-Harrison (1996); Dent *et al.* (2004) and Laurent *et al.* (2007) reported that the specific markers were isolated from *B. vulgaris* generated from ISSR-PCR analysis are as about 40-60 ISSR-PCR markers were found to be useful as accession-specific markers. Many investigators **Collins *et al.* (1998); Yamamoto *et al.* (2000); Quint *et al.* (2003); Redinbaugh *et al.* (2005); Frisen *et al.* (2006)** and **Xing *et al.* (2006)** used ISSR-PCR to dermine the molecular markers linked to resistante gene to many viruses such as SCMV, MDMV, BtMV and sequence characterized amplified region markers were also developed for use in marker-assisted selection for the interogression of these genes into new plant germplasm.