PRODUCTION AND EVALUATION OF SPECIFIC ANTIBODIES RAISED AGAINST A NEW ISOLATE OF TOMATO YELLOW LEAF CURL GEMINIVIRUS INFECTING SUGAR BEET

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Sugar beet (*Beta vulgaris*) is considered as an important crop in moderate climate regions, which provides nearly third of the world's annual sugar production and considered as an important source for animal feed and bioethanol (Juliane *et al*., 2014). Sugar beet has many advantages over sugar cane, where it needs less water requirements and has better adaptation for sandy soil. Additionally, sugar beet foliage provides rich forage for animals during high temperature of the summer (Megahed *et al*., 2015).

Geminiviruses, family *Geminiviridae*, is the largest group of plant viruses with more than

440 recognized species distributed in warm and moderate climates around the world (Cantú-Iris *et al*., 2019; Zerbini *et al*., 2017).

The main criteria to classify family *Geminiviridae* is the insect vector and the virus genome organization. Due to these criteria, nine genera are recognized: Begomovirus, Becurtovirus, Curtovirus, Capulavirus, Grablovirus, Eragrovirus, Mastrevirus, Turncurtovirus and Topocuvirus (Varsani *et al*., 2014&2017). The begomoviruses constitute the largest genus, including nearly 88% of geminivirus species (Cantú-Iris *et al*., 2019).

Geminiviruses have small genomes composed of one or two circular single-stranded DNA encapsulated into twinned icosahedral virions (Jeske, 2009). This group of viruses can infect a wide range of wild plants and agricultural crops, causing significant reduction of main food supplies and huge economic losses all over the world (Rojas *et al*., 2005). Geminiviruses are transmitted by sap-sucking insects order Hemiptera (Whitfield et *al*., 2015), and the transmission

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process is dependent on the viral coat protein (Briddon *et al*., 1996).

Symptoms of geminivirus infection vary depending on plant age, the kind of virus, plant cultivar, environmental conditions and time of infection. Symptoms include the following in various combinations: a bright yellow mosaic, chlorotic leaf margins, chlorotic mottle, leaf rolling, leaf distortion, puckering and reduction in leaf size, stunting of the infected plants (Polston and Anderson, 1997).

Trapping of plant viruses by ELISA with virus specific antibodies were carried out by Akad *et al*., (2004). In this way, tomato yellow leaf curl virus (TYLCV), a whitefly- Transmitted geminivirus was detected in plant sap in extracts of leaf squashes and in homogenates of individual viruliferous whiteflies.

Detection of viruses is important for preventing the extent of the viral infection. Molecular techniques specifically target the genetic material of plant viruses and the availability of genome sequences for many plant viruses allows molecular techniques to be more sensitive and specific than serologically based techniques. Although ELISA is not as sensitive as molecular techniques, it is still used due to its cost effectiveness, low technical skill requirements and it is ideal for large scale field detection and testing by laboratory methods. Generally serology is a primary requirement for large-scale surveys such as those carried out for healthy selection in the framework of certification programmes (D'Onghia *et al*., 2001).

This study aims to produce an effective diagnostic antibodies specific for a new geminivirus isolate affecting sugar beet in Egypt. This goal will be achieved by examining polyclonal antibodies raised against the viral capsid protein and evaluating its specificity using ELISA and western blotting.

MATERIALS AND METHODS

Virus transmission using whiteflies

Group of 100-200 virus free whitefly (*Bemisia tabaci*) were given an acquisition access period on infected beet plants (Mabrouk *et al*., 2019) for about 48 hrs. Viruliferous whiteflies were transferred to healthy beet plants in insect wooden proof cage at two leaf stage for an additional 48 hrs. A latent period of 6 hrs was required for the *Bemisia tabaci* to retain its ability to infect the test plants. After inoculation, the plants were sprayed with Bremor (1.5 ml per liter distilled water) (Aref *et al.*, 1995) and the plants were tested for the virus presence after 15 days of inoculation using whitefly transmitting geminivirus (WTG) specific primers (HD-1& HD-2). Symptoms were recorded weekly.

PCR detection

Plants were tested by PCR using WTG specific primers (Abdallah *et al*., 1993).The primers position; direction and sequence are listed in Table (1).

١٩٥
١٥٠

Total plant DNA was extracted using Qiagen DNA extraction kit according to the protocol supplied. The PCR mixture (25 µL) contained primers (2 µM/L each), 1X PCR buffer [750 mM/L Tris HCL (pH 8.8 at 25°C), 200 mM (NH4)2SO4, 0.1% Tween-20], 1.5 mM/L MgCl2, 160 µM/L dNTPs and 1.5 units Taq DNA polymerase and 5 µL of extracted DNA as template. Thermal cycling parameters were 94°C for 5 minutes and 35 cycles of each 1 minute at 94°C, 1 minute at 52°C and 1 minute at 72°C followed by final extension at 72°C for 10 minutes. As a negative control in molecular characterization, healthy beet plant was used.

Sequencing and phylogenetic analysis

Virus was characterized by studying the nucleotide sequence of part of its genome. The amplified PCR products resulted from using WTGs specific primers (HD1& HD2) were sequenced directly using the previous mentioned PCR primers. DNA band was excised from the agarose gel and DNA fragment was purified using High Pure PCR Product Purification Kit (Cat# 1 732 676, Roche). Purified DNA was determined by cycle sequencing using the fluorescent dideoxy chain terminator technology Sequencing was carried out for both strands of the viral genome fragments using WTGs specific primers (HD1& HD2).The sequence was blasted to the GenBank database of the National Centre of Biotechnology Information to build up the phylogenetic trees

Analysis of the viral capsid protein using SDS-PAGE

Inoculated beet plants 40 days old (after symptoms appearance and PCR detection) were used for SDS polyacrylamide gel electrophoresis and performed as described by Laemmli (1970).

Raising antiserum

Viral protein band was cut from SDS polyacrylamide gel. It was used in ratio 1 protein: 2 PBS, followed by pippeting and vortexing till it dissolve. The eppendorf of the protein was kept in liquid nitrogen till freezing then transferred to water bath 37 for 3 min. Freezing and thawing were repeated for 5 times followed by centrifugation and supernatant used for mice injection.

Raising antiserum against the virus was carried out according to the method described by (Ball *et al*., (1990) and Salem *et al*., 2019) using white mice ' Balb-C' (6-8 weeks-old). A volume of 0.1 mg/ml was used as a primary response; antigen is administered in about 0.1 ml intravenous injection, followed by five intraperitoneal injections of 0.2 mg/ml containing 0.1 ml incomplete Freund's adjuvant, and injection was given every 7 days for one month. Finally, blood was collected after 3 weeks post injection.

The collected blood was left to clot at 37 °C for 1 hr, then the separated anti-

serum was centrifuged at 3000 rpm for 1 min at 4°°C using Eppendorf centrifuge model 5415°C. The serum was filtered through a 0.2 µm Millipore, and 0.025% sodium azide was added and stored at 4°C until use.

Detection of virus by (ELISA)

The indirect ELISA was used for virus detection as described by Banttari and Goodwin (1985).

SDS – PAGE and western blot analysis

The test was carried out using 12% SDS-PAGE which was prepared as described by Laemmli (1970) and Salem *et al*. (2019). The gel was blotted on nitrocellulose membrane (immobilon® PVDF membrane, millipore cooperation, Bed ford, MA 01730) using electrophoretic blotter apparatus (Bio-Rad). The blotted membrane was blocked in TBS containing 1% w/v BSA, then the blocking buffer was replaced with the TBS containing raised antibodies diluted 1:10000 at 37 for 1 hr. After incubation, membrane was washed 5 times (5 min each) with TBST, and incubated in TBS containing anti-mouse universal antibodies and detection was done by NBT/BCIP in alkaline phosphatase buffer followed by agitation until color was observed.

RESULTS AND DISCUSSION

Virus transmission

Plants were tested for virus presence after 15- 20 days of inoculation by PCR using WTGs specific primers. Positive PCR amplicon of 674bp fragment were detected and no signals appeared with DNA extracted from healthy one as shown in (Fig. 1).

Inoculated beet plants exhibited typical infection symptoms including curling and upward cup shape of the leaves after 40-45 days post infection compared with healthy one, which didn't develop any symptoms during the same period (Fig. 2).These data agreed with Ghanim *et al*. (2001) who reported that whiteflies (*Bemisia tabaci*) were able to transmit tomato yellow leaf curl virus (TYLCV) 8 hrs after being caged with infected tomato plants. PCR was used by Abou-Jawdah *et al*., (2006) to confirm the presence of TYLCV in tomato plants in Lebanon. Elgaied *et al*., (2014) and Mabrouk *et al*. (2019) successfully isolated new geminivirus isolates from pepper and sugar beet using whiteflies and characterized them using molecular and serological methods. Eui-Joon *et al*., (2016) used different methods including whitefly mediated inoculation and agro-inoculation to transmit TYLCV. They checked its presence by PCR using different sets of primers.

Sequencing of PCR product

Virus was characterized by studying the nucleotide sequence of a specific part of its genome. The amplified PCR product (674bp) resulted from using WTGs specific primers (HD1& HD2) were sequenced directly.

Sequencing was carried out for both strands of the viral genome fragments. The sequence was blast to the GenBank sequences to build the phylogenetic tree (Fig. 3).

Tree produced from blasting the viral genome fragment (part of V1 and V2 viral genome) showed that the new isolate is very near at the molecular level to TYLCV isolates although it is located in different clusters from the TYLCV Egyptian isolate. These results agreed with Salem *et al*., (2018) who used phylogenetic relationships to determine the relationship of TYLCV isolate with selected begomoviruses in Saudi Arabia. Results also were in accordance with Shirin and Reza, (2019) who study different isolates of beet black scorch virus (BBSV) belonging to the genus Betanecro virus, family Tombusviridae in Irain.

Analysis of viral protein using SDS-PAGE for raising antiserum

Results from electrophoretic technique (Fig. 4) indicated that an extra band appeared with samples from infected plants and absent with healthy one. This band has a molecular weight approximately 30 KD which match with the geminiviruses coat protein molecular weight Aref *et al*., (1995).

Raising antiserum

Blood was collected 3 weeks post injection. Antigenicity of the raised antibodies was measured using indirect ELISA and western blotting. Indirect ELISA was used to measure the antigenicity of the raised antibodies. Infected and healthy beet plants were investigated using 1/1000 dilutions of the antiserum against 1/10 of tested plants (Fug.(5). Akad *et al*., (2004) measured the trapping of plant viruses by ELISA with virus specific antibodies. In this way, tomato yellow leaf curl virus (TYLCV) a whitefly-transmitted geminivirus was detected in plant sap, in extracts of leaf squashes and in homogenates of individual viruliferous whiteflies. Shirin and Reza (2019) used ELISA to detect the presence of beet black scorch virus in beet plants in Iran.

Western Blot analysis was carried out following the ELISA test to confirm that the antiserum produced is specific for the viral Cp which supports our results.

Results showed a sharp band produced with the extracted viral protein (lanes 1) at a molecular weight of approximately 30KD. This band was absent with healthy beet plant sample (Fig. 6). This test was carried out for confirming the antigenicity of the produced antiserum., Aref *et al*. (1995) reported that TYLCV preparation has a major protein of 30 KD which was detected in the purified preparation of TYLCV particles from *Nicotiana benthemiana*. Elgaied *et al*., (2017) used western blot analysis to confirm efficacy of raised antibodies against geminvirus isolates. Zing *et al*. (2019) analyzed the quantity of TYLCV coat protein and another protein abundantly expressed in whitefly midgut at protein level, to find a

good way for molecular mechanisms of the transmission of the virus by whiteflies

Our obtained results molecularly characterized the new beet infected geminivirus isolate by sequencing part of its genome and verify the efficiency of the raised polyclonal antibodies in the new isolate detection by different serological methods.

SUMMARY

Sugar beet is considered as the second important crop for sugar production worldwide. The major problem associated with sugar beet production, its high vulnerability to at least sixteen different viruses. Therefore, production of efficient and specific diagnostic tools is highly required for rapid and convenient virus detection. This study aimed to better characterization of a new isolate of the tomato yellow leaf curl geminivirus infecting sugar beet plants in Egypt, followed by producing of specific diagnostic antibodies to facilitate the viral detection in infected plants. The presence of the virus isolate in infected beet plants were tested using whitefly transmitting geminivirus specific primers. *Viruleferous* whiteflies were used to infect healthy beet plants to obtain sufficient amount required for analysis. Virus monitoring in beet plants was characterized by PCR using whitefly transmitting geminivirus (WTG) specific primers which confirmed the viral presence in DNA extracted from infected beet plants. Further molecular characterization was performed by sequencing the PCR product and it was blasted to the GenBank for generating phylogenetic tree, which proved that the obtained virus sequence is close to the TYLCV isolates. This suggested that it could be new isolate related to the published TYLCV isolates. Furthermore, Serological studies were performed using total proteins extracted from the infected Beet plants after symptoms manifestation including curling and upward cup shape of the leaves in 40-45 days post infection. Virus capsid protein band was extracted and used for mice injection to raise viral specific antibodies. Efficacy of the obtained viral antiserum for virus infection diagnoses was tested and confirmed the use of ELISA and western blotting for this purpose.

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Abdallah *et al*. (1993). Table (1): Nucleotide equences of the primers of what used according to.

Primer	Nucleotides Sequence 5'-3'	Additional coding site	Position in viral genome	Product expected size (bp)
HD-1	CGGAATTCGCCCACCAATAACTGT AGC	EcoRI	855-2528	674
HD-2	CGGGATCCGCAGTCCGTTGAGGA AACTTAC	BamHI		

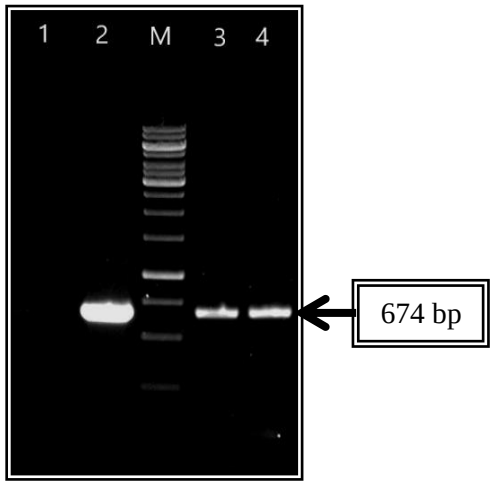

Fig. (1): PCR analysis using WTGs (HD1and HD2 primers) for infected beet plants (Lanes 3-4). Lane 2 represents PCR produced from cloned TYLCV genome at 674bp. Lane M represents 1 Kb ladder. Lane 1 represents healthy plant.

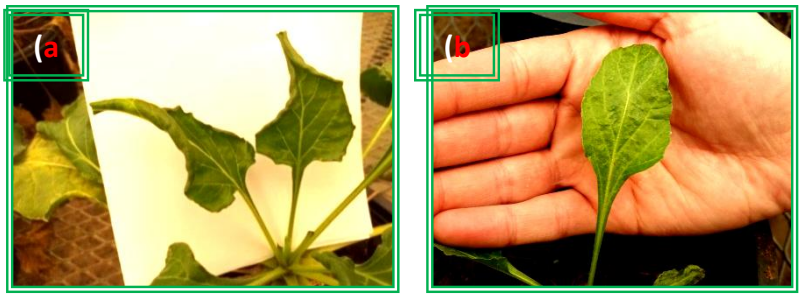

Fig. (2): Symptoms appeared on beet plants 40-45 days post inoculation with viruliferous white-flies. (a) Symptoms include deformation and curling of leaves compared with healthy one (b).

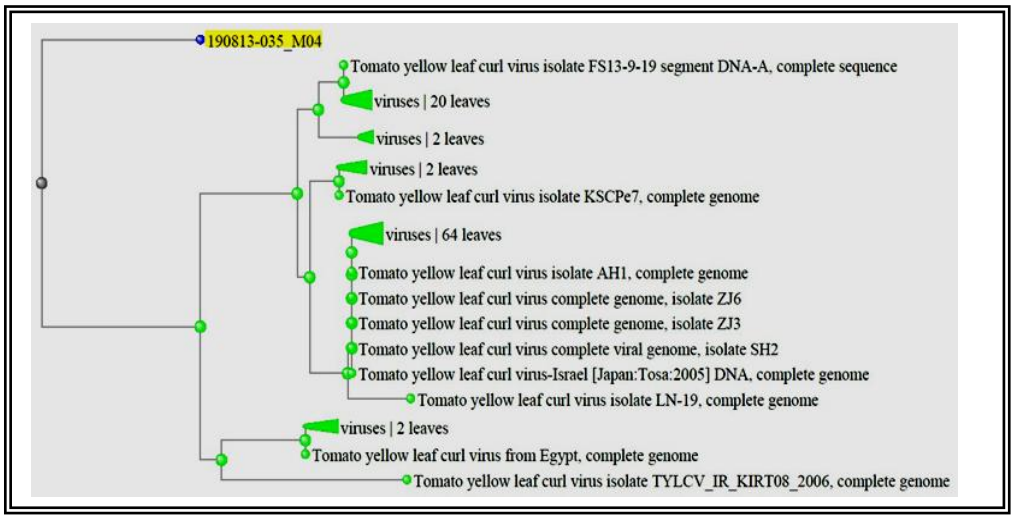

Fig.(3): Phylogenetic Tree produced from the cluster alignment of the nucleotide sequence of the cloned fragment obtained from infected beet plants with other WTGs presented in the GenBank showing the close relation with other begomoviruses .

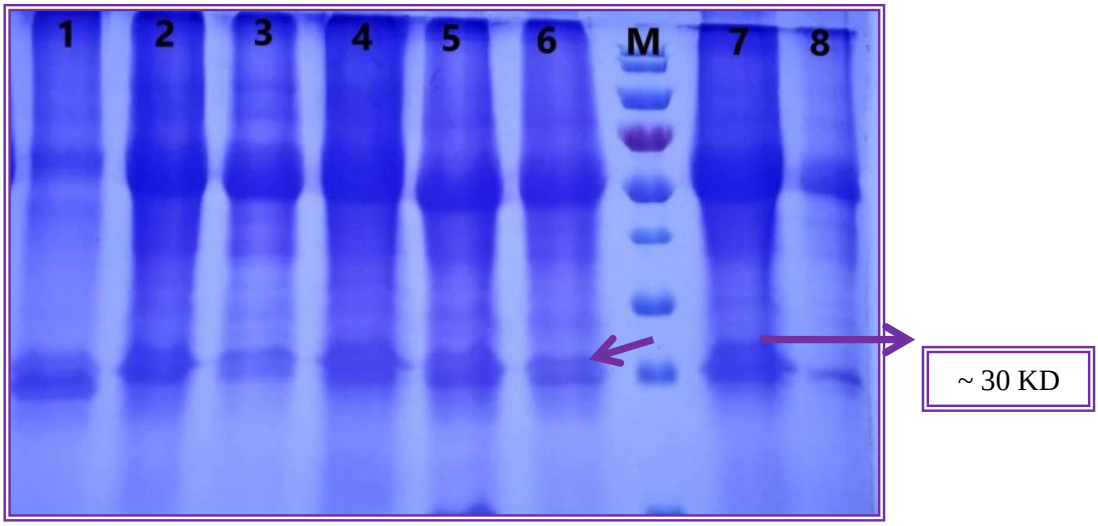

Fig. (4): SDS polyacrylamide gel electrophoreis for infected beet plants (1-7) compared with healthy one, lane 8, while M represents page ruler plus prestained protein ladder marker.

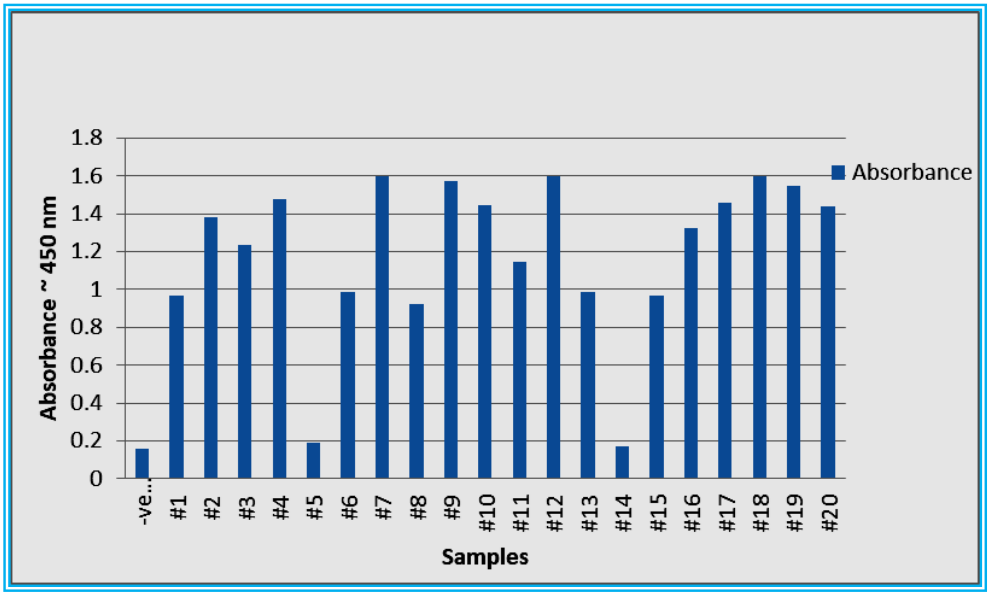

Fig. (5): Indirect-ELISA graph to measure the antigenicity of the raised antibodies using dilution of 1/1000 and 1/10 of samples.

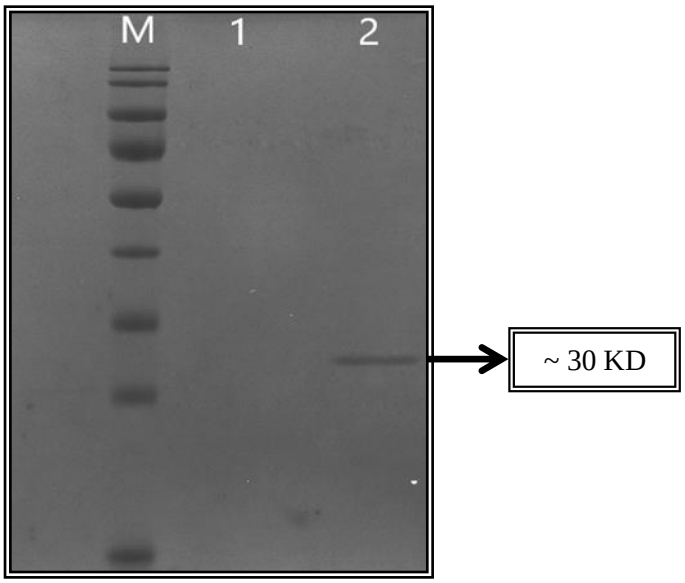

Fig. (6): Western blot analysis shows the positive signals approximately 30KDa developed from the raised antiserum reaction against the new isolate infecting beet plants. M: pre-stained protein marker. 1: healthy beet plant. 2: infected beet plant.