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**SEROLOGICAL AND MOLECULAR DETECTION OF SUGARCANE
STREAK GEMINIVIRUS
BY**

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ABSTRACT

Sugarcane streak disease (SSD) is the most prevalent virus disease found on most sugarcane cultivars grown in Upper Egypt. Sugarcane streak geminivirus (SSV) is the causal agent of SSD. The virus is a phloem-limited and used to occur in low concentrations, therefore, its detection acquire reliable and sensitive techniques. In this study, the virus was detected at the levels of its protein as well as its nucleic acid using serological and molecular tools, respectively. In the case of serological level, the indirect double-antibody sandwich-ELISA (IDAS-ELISA) using polyclonal antibodies specific to SSV was used for virus detection in sap extracted obtained from 36 sugarcane samples of the cv. G85-37 exhibited virus-like symptoms. Results showed that a number of 13 out of the 36 samples represents positive ELISA values ranged from 1.520 to 1.880, at ratio of 36.11% while, the healthy one showed 0.390 at A₄₀₅ nm. In the case of molecular level, polymerase chain reaction (PCR), using two specific primers designed based on the nucleotide sequence of replicase gene resulting a band with a size of about 846 bp. Results showed that PCR was more sensitive than ELISA, because some samples that showed negative ELISA values were found to be positive when tested by PCR. This result may recommended the use of PCR as a sensitive molecular tool for plant virus detection.

Key words: Sugarcane, Sugarcane streak disease (SSD), Sugarcane streak geminivirus (SSV), Serological and molecular detection, IDAS-ELISA, PCR.

INTRODUCTION

Sugarcane is considered as one of the most important converting industrial crops. Therefore, the improvement of sugar yield is one of the main objectives of the Egyptian agricultural policy. Sugarcane yield has increased up to

51 tons/feddans besides increasing the area cultivated with cane in Upper Egypt now to 323400 feddans represents 73% of the sugar productivity in Egypt (Anonymous, 2003). The yield of sugar noticeably decreased because of accumulation, pests, and systemic bacterial and viral diseases. Bock and Bailey (1989) reported that sugarcane streak geminivirus (SSV) has been found in many countries in Africa as well as in India and Pakistan and it is estimated that viral infection may cause up to 50% decrease in sugarcane yield.

Sugarcane streak disease (SSD), caused by sugarcane streak geminivirus (SSV) is a prevalent virus disease found on most sugarcane cultivars grown in Upper Egypt. Abdel-Hak (1964) noticed that 'streak' was one of the most important diseases affecting yield in Egypt. He indicated this disease as the prime reason for deteriorating the sugarcane cultivar "Jawa105" which was previously the most widely grown in Egypt.

In South Africa, Hughes *et al.* (1993) reported that the viral genome of SSV-N-SA is single stranded circular DNA consists of 2758 nucleotides and has four open reading frames. They also confirmed that the virus is a distinct geminivirus and it is not a strain of maize streak geminivirus (MSV).

On the other hand, SSV is serologically related to maize streak geminivirus (MSV), and transmitted by cuttings and leaf hopper (*Cicadulina bipunctella zae*) (Ammar, 1983). Shamloul *et al.* (2001) developed PCR-probe capture hybridization (PCR-ELISA) system for detection of SSV. They also sequenced the complete genome of SSV from Naga Hammady, Egypt, and revealed that SSV-EG is a new isolate of SSV that shares 66% nucleotide identity with the viral isolate from Natal, South Africa.

Therefore, the improving of sugar production in Egypt is very essential to reverse this trend and sustain the higher cane yield levels (El-Kholi and Esh, 1999), and this could be achieved by cultivating virus-free plants and early detection of such serious disease of SSV *via* some sensitive serological and molecular tools.

The main goal of this study was to detect SSV in SSD-infected sugarcane samples using modern and sensitive serological and molecular tools.

MATERIALS AND METHODS

Source of samples

A set of 36 leaf samples of sugarcane cv. G85-37 exhibited virus-like streak symptoms were kindly collected by Dr. Ahmed Abd El-Fattah, Sugar Crops Institute, ARC, Giza, Egypt, from Naga Hammady Sugarcane Experimental Station (Figure, 1) and stored at -20°C until use.

Serological detection

The indirect double-antibody sandwich-enzyme-linked immunosorbent assay (IDAS-ELISA) as described by Peterschmitt *et al.* (1991) with minor modifications was carried out. These modifications were: incubation for 3 h,

washing three times (five min for each), blocking using 3% bovine serum albumin (BSA), and use the substrate at a concentration of 0.75 mg/ml. The ELISA values were determined as given by Clark and Adams (1977) at A_{405} nm using an ELISA Reader (Bio-Rad Model 3550 Microplate Reader). As controls, healthy and SSV-infected sugarcane leaves were used as negative and positive controls, respectively. Two wells as replicates were used for each sample.

DNA extraction

DNA was extracted from SSV-infected (as a positive control) and 36 sugarcane samples according to the method of Dellaporta *et al.* (1985). Half gram of tissues from both samples were ground into a fine powder using a mortar and pestle with liquid nitrogen, and then 3.5 ml from extraction buffer were added to the powder. The mixture was incubated for 1 h at 65°C in a water bath and the tubes were inverted gently every 15 min to homogenize the mixture. An equal volume of chloroform-isoamyl alcohol (24:1, v/v) was added to the mixture, inverted gently and centrifuged at 14000 rpm for 10 min at 4°C. The aqueous phase was transferred to a fresh tube and an equal volume from isopropanol was added and incubated O/N at -20°C. The nucleic acids were precipitated by centrifugation at 4°C for 10 min at 14000 rpm. The pellet was washed with 70% ethanol and then dried. The pellet was resuspended in 40 µl deionized d.H₂O and then five µl were added to a volume of 95 µl of d.H₂O and subjected to spectrophotometer at 260 and 280 nm and then the nucleic acid concentration was determined and adjusted to 100 ng/µl.

PCR detection of SSV

Two specific SSV primers flanking the Rep A gene were designed from published nucleotide sequence of SSV-EG (Shamloul *et al.*, 2001). The primers were synthesized at AGERI, ARC, Giza, Egypt, with the following sequence: P1: 5'CGG GAT CCA TGA CAA CCG TAG GAT CAG3' and P2: 5'CGG ATC CCT AGG CTT CTG GCC CAT GTT3'.

The PCR was conducted in a volume of 50 µl (Sadik *et al.*, 1999) and the PCR amplification was performed in a Perkin-Elmer (Gene Amp PCR System 2400) for 35 cycles after initial denaturation for 4 min at 94°C. Each cycle consisted of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min. The primer extension was extended to 7 min at 72°C in the final cycle.

The PCR amplified product was analyzed by electrophoresing on 1.0% agarose gel in 1X TAE buffer at 80 volts for 1h (Sambrook *et al.*, 1989). The DNA was visualized by staining gel in ethidium bromide (0.5 mg/ml) and photographed under UV transilluminator using a Polaroid camera.

RESULTS AND DISCUSSION

The international exchange of sugarcane germplasm for breeding and commercial production has played a major role in the development of the sugar industry worldwide (Braithwaite and Smith, 2001). To reduce the risk of

introducing new diseases or new strains of pathogens into sugar-growing regions, the early and sensitive detection of such pathogens is important and required.

In the past sugarcane streak geminivirus (SSV) was considered as a strain of maize streak geminivirus (MSV) affecting sugarcane. The disease was identified according to its characteristic streak symptoms. In this study, sugarcane leaves showing streak symptoms were collected from Naga Hammady Experimental Station. Leaf samples were exhibiting streak-virus-like symptoms (Figure 1) similar to that reported by Storey (1925) and Bock and Bailey (1989), i.e., narrow, elongated translucent spots and streaks following the veins and consequently parallel to the length of leaf. These streaks were more or less of even width, and tend to be associated with the veins.



Figure (1): Characteristic symptoms of SSV on sugarcane leaves collected from Naga Hammady Experimental Station, Upper Egypt.

Serological detection

Standard detection methods for viruses include ELISA and electron microscopy; have been used successfully for many years (Hill, 1984). The increased knowledge and advancing the molecular techniques and the requirements of faster, more sensitive detection methods have stimulated research in molecular-based virus detection techniques. SSV was reported to occur in severe form in South Africa and Egypt and considered as a minor disease in areas of Mozambique, Uganda, Sudan, Reunion, Mauritius, Pakistan and India (Bock and Bailey, 1989).

In this study, the virus was detected at the levels of its protein as well as its nucleic acid using both serological and molecular tools. In case of indirect-

ELISA, polyclonal antibodies specific to SSV were used for virus detection in sap extracted from 36 sugarcane samples of the cv. G85-37. Results in Table (1) showed that a number of 13 out of the 36 samples represent a ratio of 36.11% gave positive ELISA values ranged from 1.520 to 1.880, while, the healthy one showed value of 0.390 at A₄₀₅ nm. Bailey (1996) developed both ELISA and immunosorbent electron microscopy (ISEM) assay to detect SSV. ISEM provided accurate diagnosis because of the distinctive appearance of the geminivirus particles.

Table (1): Serological (ELISA) detection of SSV.

SS	ELISA		SS	ELISA		SS	ELISA	
	Value	Result		Value	Result		Value	Result
1	1.82	+	13	0.51	-	25	1.72	+
2	0.38	-	14	0.47	-	26	0.44	-
3	0.38	-	15	1.80	+	27	0.46	-
4	0.44	-	16	0.56	-	28	1.68	+
5	1.83	+	17	0.50	-	29	1.80	+
6	0.42	-	18	0.45	-	30	0.49	-
7	0.45	-	19	1.68	+	31	1.65	+
8	1.84	+	20	0.48	-	32	0.47	-
9	1.88	+	21	0.40	-	33	1.52	+
10	1.73	+	22	1.70	+	34	0.61	-
11	0.54	-	23	0.42	-	35	0.39	-
12	0.50	-	24	0.40	-	36	0.50	-
Cont.	0.39	-	+cont.	1.85			+	

SS: Sugarcane sample. -: Negative. +: Positive.

PCR detection of SSV

PCR has become an indispensable tool in a molecular-based laboratory (Saiki *et al.*, 1988). It is currently one of the most sensitive methods for pathogen detection. The application of PCR for plant disease diagnosis has been reviewed by Henson and French (1993). They listed a number of viroids, RNA and DNA viruses that have been detected by PCR. To detect viruses in plants, total nucleic acid extractions (Braithwaite *et al.*, 1995) are often used as the PCR template, although rapid crude extraction methods can also be used (Thomson and Dietzgen, 1995).

Data in Table (2) and Figure (2) show that 14 out of the 36 sugarcane samples were positive, as a fragment with a size of about 846 bp representing Rep A open reading frame (ORF) which amplified using the two SSV-specific primers designed by Shamloul *et al.* (2001). Sample (No. 34) that showed a negative ELISA value (0.61 at A₄₀₅ nm) was found to be positive when tested by PCR. This result recommended the use of PCR as a molecular technique for plant virus detection particularly with DNA viruses.

Shamloul *et al.* (2001) developed a sensitive detection of the Egyptian isolate of sugarcane streak virus by PCR-probe capture hybridization (PCR-ELISA). In South Africa, Rybicki and Hughes (1990) detected and typed MSV

and other distantly related geminiviruses of grasses by PCR amplification of a conserved viral sequence. He also discussed the use of the PCR for the amplification of gemini- and other virus genomes or genomic fragments for typing, mapping, phylogenetic analysis and taxonomy.

Over an extended period, detection of SSV was based on visual inspection of its symptoms. This was not satisfactory because (i) not all inspectors were familiar with the symptoms of SSV, (ii) the symptoms of SSV are often mild or non-existent under low light conditions and (iii) they always exists the possibility of strains of SSV that are mild or symptomless in individual cultivars. Therefore, it is important to use sensitive and rapid detection methods to evaluate *in vitro* materials as well as seeds prior cultivation in the field. Results of this investigation pointed to the successful use of ELISA and PCR as serological and molecular tools, respectively, for detection of SSV.

Table (2): Molecular detection of SSV using PCR technique.

SS	PCR	SS	PCR	SS	PCR
1	+	13	-	25	+
2	-	14	-	26	-
3	-	15	+	27	-
4	-	16	-	28	+
5	+	17	-	29	+
6	-	18	-	30	-
7	-	19	+	31	+
8	+	20	-	32	-
9	+	21	-	33	+
10	+	22	+	34	+
11	-	23	-	35	-
12	-	24	-	36	-

SS: Sugarcane samples. -: Negative reaction. +: Positive reaction.

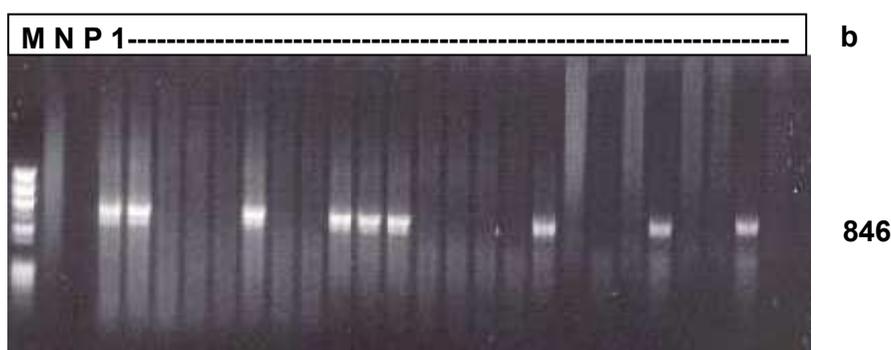


Figure (2a): Agarose gel (1%) in TAE buffer stained with ethidium bromide shows PCR detection of sugarcane samples (Lanes 1-23 M: ØX174/*Hae* III (IX) DNA standard marker (1353, 1078, 872, 603, 310, 281, 271, 234 bp). P: SSV-infected sample as a positive control. N: PCR mixture with no DNA as a negative control.

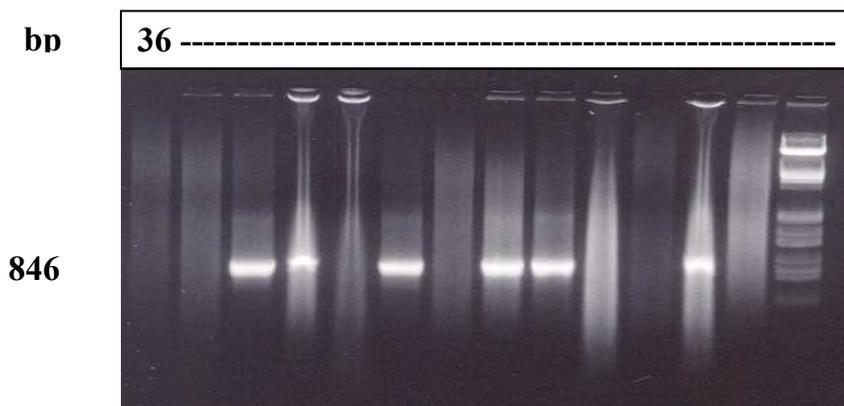


Figure (2b): Agarose gel (1%) in TAE buffer stained with ethidium bromide shows PCR detection of sugarcane samples (Lanes 24-36). M: λ DNA/*Hind* III and $\text{OX174}/\text{Hae}$ III DNA standard marker (23130, 9416, 6557, 4361, 2322, 2027, 1353, 1078, 872, 603, 564, 310, 281).

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الكشف السيرولوجي والجزيئي عن فيروس التخطيط في القصب التابع لمجموعة فيروسات الجيميبي

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يعتبر مرض التخطيط في القصب واحدا من أهم الأمراض الفيروسية التي تصيب اصناف قصب السكر المنزرعة في محافظات الوجه القبلي في مصر. وقد ثبت أن فيروس التخطيط في القصب التابع لمجموعة فيروسات الجيميبي والمعروف باسم الـ SSV هو المسبب لهذا المرض والذي عادة ما يتواجد بتركيزات منخفضة في منطقة اللحاء، مما يتطلب استخدام طرق حساسة ودقيقة للكشف عن وجوده. وبناء على هذا تم في هذه الدراسة استخدام طرق سيرولوجية وجزيئية للكشف عن الفيروس سواء كان علي مستوي البروتين أو الحامض النووي الفيروسي. ولهذا تم استخدام اجسام مضادة من النوع عديد النسل ومتخصصة لفيروس الـ SSV و طريقة ELISA الغير مباشرة في الكشف عن الفيروس في عدد 36 عينة قصب من الصنف G85-37 بها أعراض ظاهرية مشابهة لمرض التخطيط في القصب. وقد أظهرت النتائج وجود الفيروس في 13 عينة من 36 عينة بنسبة 36,11% حيث تراوحت متوسطات قراءات الـ ELISA لهذه العينات ما بين 1,02 و 1,88 في الوقت الذي اعطت العينة السليمة قراءة 0,390 عند طول موجي 405 نانومتر nm. وعند الكشف عن الفيروس باستخدام تقنية الـ PCR و اثنين من البادئات المتخصصة لفيروس الـ SSV ظهرت حزمة ذو وزن جزيئي 846 زوج من النيوكليوتيدات في عدد 14 عينة. وقد لوحظ أن العينة رقم 34 والتي أعطت نتيجة سلبية مع اختبار ELISA (0,61 عند 405 nm) قد أعطت نتيجة موجبة عند استخدام الـ PCR مما يدل على دقة وحساسية تقنية الـ PCR عن الـ ELISA في الكشف عن الفيروس. ولهذا فإننا نوصي بأهمية استخدام تقنية الـ PCR في الكشف السريع والدقيق عن الفيروسات مما يعتبر اولي خطوات المقاومة الناجحة.