The occurrence of *Sugarcane bacilliform virus* on sugarcane in Egypt

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ABSTRACT

Sugarcane bacilliform virus (SCBV), *Badnavirus*, *Caulimoviridae* was detected in the noble sugarcane (*Saccharum officinarum)* plants in Giza and Assiut governorates showing leaf symptoms of pronounced flecks or freckles and vein banding which turn later to chlorotic and necrotic strips. Polymerase chain reaction (PCR) utilizing degenerate primers for badnaviruses for the reverse trancriptase, RT/RNase genome regions of ORF III detected the virus in infected sugarcane leaves and in its vector *Saccharicoccus sacchari* and in another unidentified *Pseudococcidae*- mealybug. Amplicons for the RT/RNaseH motifs of the two virus isolates were cloned, sequenced, and submitted to the GenBanak. Pair-wise nucleotide identity indicated 86% identity between the Giza and Assiut isolates. NCBI-blastn analysis for the two isolates with four SCBV accessions retrieved from the GenBank indicated identity range of 66-91%. Phylogenetic analysis based on amino acid sequence identity indicated the clustering of the two SCBV in a separate branch from the other known SCBV; indicating the special nature of the Egyptian SCBV isolates. Close serologic relationship between SCBV and BSV from Egypt was detected. The Egyptian antiserum of BSV captured SCBV antigens in leaves and insect vectors in Immunocapture (IC) PCR analysis. The present study represents the first record of SCBV presence in Egypt.

Key words: *Badnavirus*, *Sugarcane bacilliform virus*, sugarcane (*Saccharum officinarum)*, *Saccharicoccus sacchari,* virus diversity, immunocapture PCR

INTRODUCTION

Sugarcane bacilliform virus (SCBV), a pararetrovirus of the genus *Badnavirus* within the family *Caulimoviridae* with no enveloped, bacilliform-viral particles , with 30 X 130–150 nm, containing a genome consisting of a circular, relaxed,7.5–8.0 kb dsDNA molecule (Hull *et al*., 2005). Unlike *Banana streak virus* (BSV), a badnavirus, whose genome presents as either exogenous or endogenous entities (Ndowora *et al*., 1999), SCBV does not present as an integrant in sugarcane genome Geijskes *et al*., 2004).

SCBV is a pathogen of sugarcane (*Saccharum officinarum*, *S. barberi*, *S. sinense*, S. *robustum* and *Saccharum* interspecific hybrids). SCBV was first reported in Cuba, infecting cultivar B34104, in 1985 and subsequently in Morocco 1987 by Lockhart and Autrey (1988). The occurrence of SCBV has been reported in more than 20 countries, including major sugarcane producers such as, Australia, Brazil, Cuba, India, and Madagascar (Karuppaiah *et al.*, 2013). SCBV is one of the frequently detected viruses in quarantine during germplasm exchange ((Karuppaiah *et al.*, 2013).

Earlier, SCBV caused yield reductions in some ancient varieties (Comstock and Lockhart, 1990; Lockhart and Autrey, 1988; Viswanathan *et al*., 1996). Currently, SCBV has no impact on the yield of modern sugarcane varieties.

Foliar symptoms caused by SCBV are variable and virus isolate-dependent as well as cultivar-dependent. Some sugarcane genotypes

or cultivars display severe chlorotic mottling, chlorotic strips, stunted growth, and pronounced flecks or freckles), while others remain symptomless (Viswanathan *et al.*, 1996; Muller *et al*., 2011).

SCBV is mechanically transmitted to healthy sugarcane. The major vectors transmitting SCBV are the pink mealybug, *Saccharicoccus sacchari* (Cockerelle) (Lockhart and Autrey, 1988) and the gray sugarcane mealybug Dysmicoccus *bonensis* (Kuwana) (Lockhart and Olszewski, 1994).

SCBV is closely related to another badnavirus, *Banana streak virus* (BSV), which infects banana plants. Some SCBV and BSV are serologically closely related (Lockhart and Autrey , 1988). BSV was reported to multiply in sugarcane without causing leaf symptoms. Similarly, SCBV is able to infect banana plants mechanically (Lokhart and Autrey, 1991) and by agroinoculation (Bouhida *et al*., 1993). SCBV can be experimentally transmitted by the mealybug *Saccharicoccus sacchari* from sugarcane to banana and causes streak symptoms similar to those caused by BSV. This situation has been raising major concern of the possibility of virus transmission between sugarcane and banana under natural conditions (Muller *et al*., 2011).

Currently SCBV is considered to be a group of separate viruses infecting sugarcane rather than isolates of one virus due to the high genomic diversity between them (Fauquet *et al*., 2005; Muller *et al.* 2011; Karuppaiah *et al*., 2013). Differences in polymerase (RT/RNase H) nt sequences of (20 %) and differences in other gene product sequences were the criteria followed by the International Committee of Taxonomy of Viruses (ICTV) to demarcate the species in the genus Badnavirus (Fauquet *et al.,* 2005; King *et al.*, 2011). For instance, considering the differences in the nucleotide sequences of RT/RNase H-region, which exceeds 20 % variation, the ICTV had described SCBV-Mor and SCBV-IM as two different species and named them as Sugar-cane bacilliform Morocco virus (SCBMV) and *Sugarcane bacilliform Ireng Maleng virus* (SCBIMV), respectively.

The present study is recording the incidence of SCBV for the first time in Egypt. The study is concerned with detecting and identifying SCBV on the molecular and serologic levels from two different governorates, viz Giza and Assiut. The Egyptian SCBV will hence forward be named according to ICTV nomination as SCBEV-Giza and SCBEV-Assiut.

MATERIALS AND METHODS

Virus isolates:

SCBEVes were obtained from infected nobel sugarcane (*S. officinarum* L.) commercial varieties. One virus isolate was collected from Giza governorate (SCBEV-Giza), while another isolate was collected from Assiut governorate (SCBEV-Assiut).

Nucleic acid extraction:

Total nucleic acid was extracted from leaves and insect vector according to the silica based method described by Echevarría-Machado *et al*. (2005). 0.1 g of tissue was homogenized with 1 ml extraction buffer (10 mM Tris-HCL, 50 mM EDTA, 500 mM NaCl, 10 mM 2-mercatoethanol, pH 7.0), transferred to a 2 ml sterilized Eppendorf tube. 100 µl of 20% SDS were added, mixed, and tube was incubated at 65^{o}C for 10 min. 500 µl of 5 M potassium acetate were added to the tube which was shaken vigorously, incubated for 20 min in ice, and spun at 16,000 g for 20

min. The supernatant was transferred to a new sterilized 1.5 ml Eppendorf tube and 300 µl of washed silica were added, and mixed manually and gently for 3-5 min. The tube was spun at 16,000 g for 30 s. The resulting pellet was washed twice with 70 % ethanol and dried at 37°C for 10 min. The pellet was resuspended in 50 µl sterilized bi-distilled water and incubated at 55°C for 5 min. The tube was spun at 16,000 g for 2 min and supernatant was transferred to a new 500 µl sterilized Eppendorf tube.

PCR analysis

Badnavirus DNA was amplified using Badna FP: 5'ATG CCI TTY GGI ITI AAR AAY GCI CC 3` and Badna RP:5' CCA YTT RCA IAC ISC ICC CCA ICC 3', designed based on the consensus sequence of the RT and RNase H coding regions to amplify a 580 bp product (Yang *et al.,* 2003). PCR reaction mixes of 25 µl contained 1 µl of each primer (10 pm), 0.5 µl of 10 mM dNTPs mix, 5 µl of 5X GoTaq DNA polymerase reaction buffer (Cat No. M8301, Promega, Madison, WI, USA), 2.5 µl of 25 mM $MgCl_2$, 0.25 µl (1.25 U) of Taq DNA polymerase, 2.5 µl of DNA template, and 12.25 µl of water. PCR cycle conditions, as described by (Yang *et al.,* 2003) were 94 °C for 4 min, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s and a final extension of 72°C for 5 min (Yang *et al*., 2003). Amplified products were stained with ethidium bromide, electrophoresed through a 1% agarose gel and visualised using a UV transilluminator.

IC-PCR

IC-PCR was basically similar to the one described by Gambley (2008) with minor modifications. A rabbit polyclonal antiserum prepared for BSV-Egypt, BSEV (Abdel-Salam *et al*., 2005) was used to entrap virus antigens on sterile polypropylene thin-walled 0.2 ml microfuge. Tubes were coated overnight at 4°C or for 2 hr at 37°C with 25 µl of BSV polyclonal antiserum diluted 10^{-2} in sodium carbonate coating buffer (15 mM sodium carbonate, 34 mM sodium bicarbonate, pH 9.6), then washed three times with 100 µl of PBST washing buffer (136 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 8 mM Na_2HPO_4, 0.05% Tween-20, pH 7.4,) and incubated overnight at 4°C with 50 µl of sap extract. Plant and insect extracts were prepared by macerating 0.1 g of leaf tissue and 10 insects/tube in 1 ml of citrate extraction buffer (0.05 M citrate pH 7.0, 0.5 mM EDTA, 1% skim milk powder, 0.5% glycerol, 0.05% Tween-20, and 0.05% of 2-mercaptoethanol) and clarified by centrifugation at 14,000 x g for 5 min. The tubes were washed as before then incubated at 37°C for 30 min with 3 U of DNAse I (Invitrogen) prepared in 1 × DNAse I buffer (Invitrogen) in a total reaction volume of 50 µl. The DNAse I was removed by washing tubes with 100 µl washing buffer. The tubes were then rinsed with 100 µl sterile water, and the complete 25 µl PCR mix was added directly to the tube using the degenerate BadnaF/P primers. PCR reactions were made in 25 µl as described in PCR analysis above except no DNA template was added and water was adjusted to 14.75 µl. The PCR cycle conditions were as above except no DNA template was added and water was adjusted to 14.75 µl. The PCR cycle conditions were as described in PCR analysis above. Amplified products were examined as described before.

Cloning, sequencing and analysis:

The PCR products were gel-purified using QIAEX II gel extraction kit (Qiagen) and then cloned into pTZ57R/T vector using InsT/Aclone

PCR product cloning kit #1214 (Fermentas). Plasmids were transformed in *E. coli* JM 109 cells using standard molecular techniques. Purified plasmids were sent for DNA sequencing (Macrogen Korean Biotechnology Company). DNA sequences were submitted to GenBank and were assigned accession numbers of KM591919 and KM591920 for SCBEV-Giza and SCBEV-Assiut, respectively.

The two sequences were compared for matching sequences (see Table 1) in the Genbank databases, at http://www.ncbi.nlm.nih.gov**.,** after removing primer positions. Percentage nucleotide identities were determined using the Blastn of GenBank. The encoded amino acid sequences were translated from the nucleotide sequences, with Standard genetic code, using the EditSeq program in DNASTAR. Translated amino acids of the two SCBEVes were aligned with amino acid sequences of four other members of the *Caulimoviridae* using ClustalW method (Thompson *et al*., 1994). Phylogenetic relationships of SCBV tested isolates were inferred from multiple sequence alignment using the Neighbor-Joining method (Saitou and Nei, 1987) and a consensus tree was generated following 500 rounds of bootstrapping (Rzhetsky and Nei, 1992; Dopazo, 1994) using MEGA 6 program (Tamura *et al*., 2013). The evolutionary distances were computed using the p-distance method (Nei *et al*., 2000) and are in the units of the number of amino acid differences per site.

RESULTS

Symptomatology

Symptoms observed in SCBEV-infected sugarcane fields in Giza and Assiut were variable and difficult to observe. Symptoms start as small green vein- banding (VB) that develop around small veins and may extend to the midrib (Fig. 1-A, B). The previous symptoms are followed later on by chlorotic flecks or strips (Fig. 1-B). Upon disease progress, chlortotic stripes become necrotic (Fig. 1-B). In some sugarcane plants only vein banding and general chlorosis can be observed (Fig. 1-C).

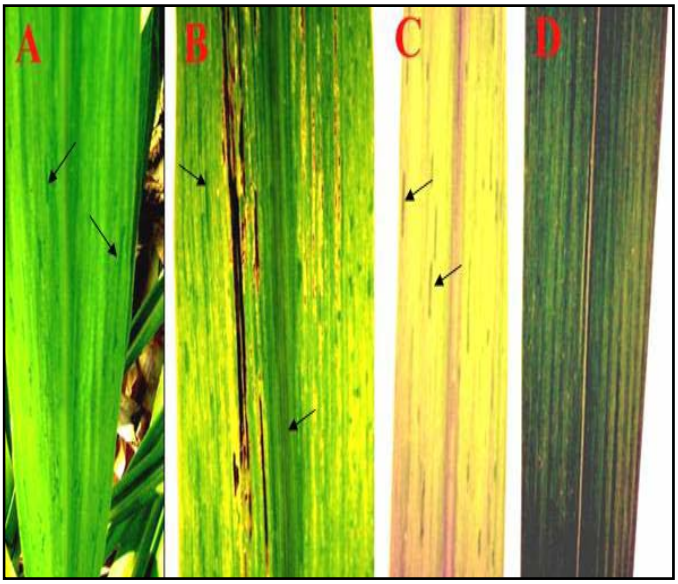

Fig. (1): Symptoms observed on commercial sugarcane plants in the field upon infection with SCBV. A, vein-banding (VB); B, VB and chlorotic and necrotic strips; C, VB and general chlorosis; D, healthy leaf. Arrows depict VB on small veins and midrib.

PCR analysis

The results shown in Figure 2 indicated the success of the Badna F/P primers in amplifying 580 bp from the RT/RNase motifs from both sugarcane samples from Giza, Assiut, and banana infected with BSV. No PCR amplicons were detected in healthy sugarcane sap.

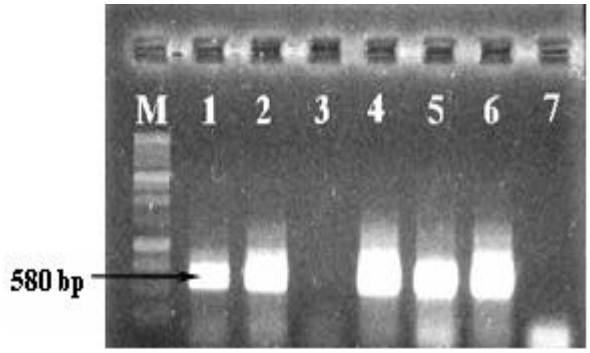

Fig. (2): PCR analysis showing the 580 bp products amplified from the RT/RNase H region for total DNAs extracted from infected sugarcane from Giza (1&2), Assiut (4&5), and from BSV-infected banana (6). Lanes 3 and 7 contained healthy sugarcane. M, 1kb DNA ladder

IC-PCR

Results in Figure (3) indicated the detection of IC-PCR to amplicons of 580 bp in infected sugarcane from Giza, Assiut governorates and in BSV-infected banana with no virus captured in healthy sugarcane control. The obtained results also indicated the presence of SCBEV in both mealybug insects collected from sugarcane plants including *S. sacchari* (Fig.4-A) and another unknown species of mealybug (Fig. 4-B).

IC-PCR detected SCBEV in symptomless sugarcane samples from Giza and Minya governorates (data not shown).

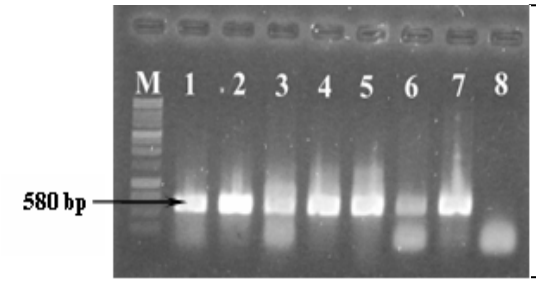

Fig. (3). Results of IC-PCR performed on leaf extracts from: infected sugarcane from Giza (1&2), Assiut (3&4), BSV-infected banana (5); *Saccharicoccus sacchari* insects (6), unidentified mealybug species (7), and healthy sugarcane (8). M, 1kb DNA ladder

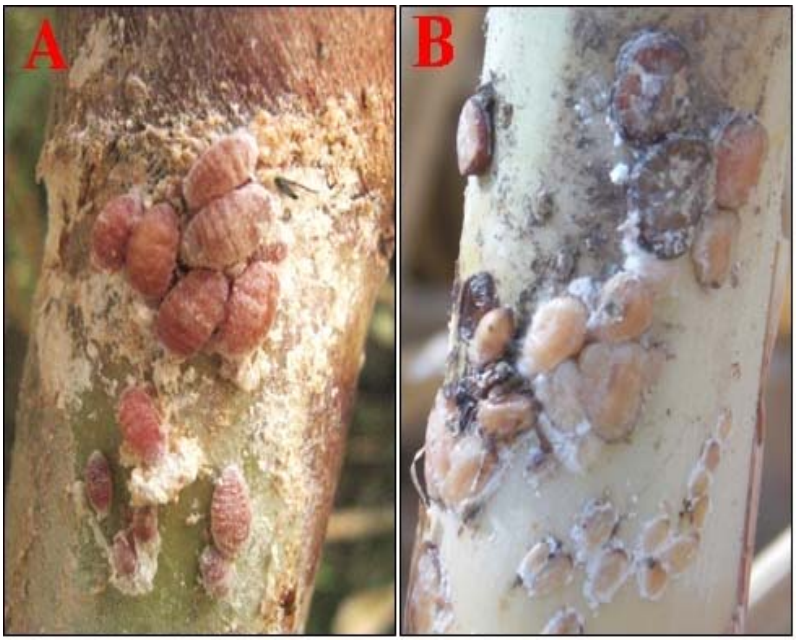

Fig. (4): Mealybug vectors of SCBEV detected on sugarcane in Giza governorate. A & B, respectively represent adults and nymphs of *Saccharicoccus sacchari* and an unidentified species of mealybug observed on sugarcane.

Sequence analysis and phylogeny

Both SCBEV-Giza and SCBEV-Assiut shared 86% nucleotide sequence identity in the RT/RNase. Results in Table (1) indicated that the SCBEV-Giza and –Assiut isolates had 81% and 91% nucleotide sequence similarity with isolates from Morocco and Guadaloup, respectively. Whereas, the two isolates were less similar to those from Australia and India.

Table (1): Nucleotide (NT) sequence identity between RT/RNase H sequences of two tested Egyptian SCBV (SCBEV) isolates against four corresponding isolates retrieved from the GenBank

SCBV.\ Country. Acc. No	**SCBEV Giza** ***Acc No.*** **KM591919**	**SCBEV Assiut** ***Acc No.*** **KM591920**
SCBMV, Morocco, M89923	81	90
SCBIMV, Australia, AJ277091	72	66
SCBGV-R570-78, Guadaloupe,FJ439815	89	91
SCBIV-B091, India, JN377533	74	Not available

As shown in Figure (5), phylogenetic analysis based on amino acid similarity between SCBEVes and other SCBVes indicated that SCBEV-Giza and –Assiut clustered together in one clade apart from the tested SCBVes.

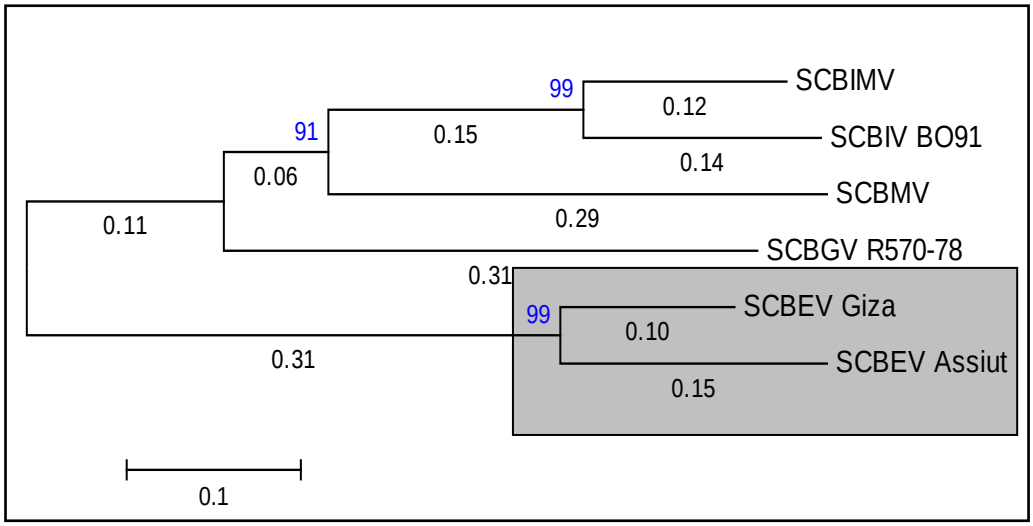

Fig. (5). Phylogenetic tree depicting the relationships of SCBEV tested isolates with other SCBVes based on amino acid sequence similarities of RT/RNase H using the Neighbor-Joining method. The dendrogram was bootstrapped 500 times (score are shown on nodes). The shaded box

DISCUSSION

Symptomatology

Generally, developed symptoms were similar to those described on sugarcane upon SCBV infection (Viswanathan *et al.*, 1996; Muller *et al*., 2011). Variation in symptoms may be attributed to the presence of different virus genotype or due to different cultivar response (Viswanathan *et al.*, 1996). The VB symptoms appear unique for the SCBEV infestation in sugarcane. It is not known whether that the VB symptom is due to SCBEV or BSV infection. Similar VB symptoms, however, were described upon infection of banana with BSV (Harper *et a*l 2002); Abdel-Salam *et al*, In Press). BSV has been shown to be transmitted by *Planococcus citri* and *Saccharicoccus sacchari*, both of which colonize banana (Lockhart *et al.* 1992). Therefore Cross-transmission of BSV from banana to sugarcane through *S. sacchari* is expected.

PCR analysis

Badna F/P primers were successful in amplifying 580 bp from the RT/RNase motifs from sugarcane and banana infected with SCBV and BSV respectively; confirming the presence of the two viruses in their respective hosts. Several investigator have used these primers in detection of badnaviruses including SCBV and BSV (Yang *et al.,* 2003; Gayral and Iskra-Caruana, 2009; Muller *et al*., 2011).

IC-PCR was used for detection of SCBV in sugarcane in Australia (Geijskes, 2003) and in Guadalupe (Muller *et al.,* 2011). Both BSV and SCBV are serologically close related badnaviruses (Lockhart and Autrey, 1988). Therefore, an Egyptian antiserum for BSV (Abdel-Salam *et al*., 2005) was used successfully in entrapment of SCBEV and BSV virions from infected tissues. Furthermore, SCBEV virions were detected in two mealybugs species including *S. sacchari* and in another species of mealybug. *S. sacchari* is a main vector for SCBV (Lockhart and Autrey, 1988). No information at presence is available so far on the other species of mealy bug vector. IC-PCR also showed the presence of SCBEV in symptomless sugarcane plants collected from Giza and Minia governorates; indicating the ability of SCBV to cause no symptoms upon infection in some sugarcane varieties (Viswanathan *et al.*, 1996; Muller *et al*., 2011).

Sequence analysis and phylogeny

In the present study, the 80% nucleotide identity threshold in the RT/RNase H domain, conserved in badnaviruses, was used to discriminate between badnavirus species as stated by the ICTV (Fauquet *et al*., 2005; King *et al.*, 2011). Accordingly, both SCBEV-Giza and SCBEV-Assiut with 86% nucleotide sequence identity in the RT/RNase H regions are closely related isolates. Likewise, some SBCV isolates from Australia (Geijskes *et al*., 2002) and Guadeloupe (Muller *et al.,* , 2011) had *ca* 85% identity in the RT/RNase H region and grouped together; while some Indian isolates of SCBV were diverged with sequence similarity for the same region between 69 to 85% (Karuppaiah *et al*. ,2013). Other results indicated close nucleotide sequence identity between the Egyptian isolates with isolates from Morocco and Guadeloupe, and distant relatedness to those from Australia and India. Such results may indicate the high diversity in SCBV members as found by Karuppaiah *et al*. (2013) and Rao *et al.* (2014).

Phylogeny analysis indicated the clustering of the two SCBEV isolates apart from other foreign SCBV isolates. Such results reveal the distinct nature of the Egyptian SCBV. This

was similar to phylogenetic results on SCBV from Indian (Karuppaiah *et al*., 2013) and Australia (Geijskes *et al*., 2002) which indicated that SCBV from both countries clustered apart from other SCBVes and/or badnaviruses.

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