Sugarcane bacilliform viruses in Ethiopia: Genetic diversity and transmission by pink sugarcane mealybug

Mereme Abide[1*] • Dawit Kidanemariam[2] • Misrak Kebede[1] and Adane Abraham[1, 3*]

[1] Department of Biotechnology, Addis Ababa Science and Technology University, Addis Ababa, Ethiopia

[2] Department of Plant and Soil Sciences, Forestry and Agricultural Biotechnology Institute, University of Pretoria, South Africa

[3] Department of Biological Sciences and Biotechnology, Botswana International University of Science and Technology, Private Bag 16, Palapye, Botswana

*Corresponding author: mereme.abide@aastu.edu.et and abrahama@biust.ac.bw

Abstract

Badnaviruses infecting sugarcane, collectively called sugarcane bacilliform viruses (SCBVs), are reported worldwide and are responsible for causing leaf fleck disease in sugarcane. SCBVs are genetically heterogeneous members of the badnavirus species complex. The objective of this study was to evaluate the genetic diversity of sugarcane bacilliform viruses (SCBVs) in four distinct sugarcane growing sites in Ethiopia. Additionally, the study aimed to examine the transmission of SCBV through vectors. A total of 270 sugarcane leaf samples, including both virus-suspected (symptomatic) and asymptomatic leaves, were collected and tested using a PCR assay with SCBV-F and SCBV-R primer pairs. Out of these samples, 67 (24.8%) were found to be SCBV-positive. Phylogenetic analysis and pairwise sequence comparisons based on the partial RT-RNase H coding region showed that the SCBVs in Ethiopia are genetically diverse. The phylogenetic analysis revealed that isolates from the

current study formed four clusters together with SCBV-G, L, Q and S isolates reported from different parts of the world. This suggests that the introduction of SCBV to Ethiopia may have occurred in multiple countries. The glasshouse experiments demonstrated the efficient transmission of SCBV from infected to healthy sugarcane plants by pink sugarcane mealybugs (*Saccharicoccus sacchari)*, which are associated with the sugarcane crop in the field. These findings contribute to the current understanding of the genetic diversity of SCBVs in Ethiopia.

Keywords: Badnaviruses, Leaf fleck disease, RT-RNase H, mealybug

Introduction

Sugarcane bacilliform virus (SCBV) is the name given to double stranded DNA viruses that infect sugarcane and belong to the genus *Badnavirus*, family *Caulimoviridae*. These viruses have non-enveloped bacilliform particles containing a 7.4–7.9 kb circular dsDNA genome. The genome is interrupted by two site-specific gaps and contains three open reading frames (ORFs I–III) (Ashraf et al. 2022). Molecular identification and characterization of SCBVs from different parts of the world have confirmed the heterogeneous and complex genetic structure of badnaviruses infecting sugarcane (Sun et al. 2016; Rao et al. 2018). Although initially reported as one virus species (Lockhart et al. 1995), the International Committee on Taxonomy of Viruses currently recognizes four sugarcane-infecting badnavirus species, namely Sugarcane bacilliform Guadeloupe A virus (SCBGAV), Sugarcane bacilliform Guadeloupe D virus (SCBGDV), Sugarcane bacilliform MO virus (SCBMOV), and Sugarcane bacilliform IM virus (SCBIMV) (ICTV 2021) which causes leaf fleck disease in sugarcane (Balan et al. 2020; Viswanathan et al. 2019). SCBV infection was first reported in Cuba in 1985 and then in Morocco in 1986 (Lockhart and Autrey 1988). Subsequently, the

disease was reported in other major sugarcane-growing countries such as Mauritius, the USA, Australia, India, South Africa, and Malawi (Rao et al. 2018). Recently, its occurrence has also been reported in Ethiopia (Abide et al. 2022; Haregu et al. 2022).

SCBVs are considered one of the most economically damaging pathogens for sugarcane production worldwide. Sugarcane infected with SCBV may often exhibit a wide range of symptoms, which include chlorotic stripes, chlorotic mottling, leaf flecks, yellowing, reddening, premature drying of leaves and stunted growth (Sun et al. 2016; Krishna et al 2023), but some of the infected plants remain asymptomatic. On the other hand, the symptoms are more prominent when there is a combined infection of SCBV with other viruses, such as the sugarcane mild mosaic virus and the sugarcane mosaic virus (Li et al. 2010). Sugarcane that has been infected with SCBV exhibits reduced juice yield, lower sucrose content, decreased purity, and reduced stalk weight, lead to significant losses in biomass production (Viswanathan et al. 2018; Ahmad et al. 2019; Bhat et al 2023). These viruses also pose significant constraints on the exchange of germplasm materials (Ahmad et al. 2019).

SCBVs are primarily transmitted to new geographical areas through infected vegetative propagative materials (Bhat et al. 2016). In addition, insect vectors play a significant role in the spread of the virus. Mealybug vectors transmit SCBVs in a semi-persistent manner from infected plants to healthy ones (Rao et al. 2018). Three species of mealybugs are reported to transmit SCBV: *Saccharicoccus sacchari* (pink sugarcane mealybug), *Dysmicoccus boninsis* (grey mealybug) (Lockhart et al. 1992), and *Planococcus citri* (citrus mealybug) (Lockhart et al. 1995). These mealybugs are considered major pests of sugarcane as they feed on the sap of the plant, cause direct damage to the plant, and also act as vectors for the transmission of SCBV, which further affects the health of the sugarcane crop. Therefore, effective

management strategies are necessary to control the populations of these mealybugs and limit the spread of SCBV (Filloux et al. 2018; Bhat et al. 2023). Alternative hosts also serve as epidemiologically significant sources of viruses for cane infection (Jayanthi et al. 2016). SCBVs can naturally infect *Saccharum officinarum*, *S. spontaneum*, *S. robustum*, *S. barberi, S. sinense, Sorghum halepense*, *Brachiaria* and *Rottboellia exaltata.* They have also been experimentally transmitted to rice, banana, and sorghum (Karuppaiah et al. 2013; Rao et al. 2014; Sharma et al. 2015; Bhat et al. 2016; Ashraf et al. 2022)**.**

A previous study has described the occurrence and prevalence of SCBVs in the Oromia region, specifically Metahara and Wonji-Shewa in Central Ethiopia (Abide et al. 2022; Haregu et al. 2022). The present investigation was conducted to study the distribution and genetic diversity of SCBV in the other major sugarcane growing sites, namely Tana Beles and Chis Abay, in Ethiopia, in addition to the previously studied sites. The study involved sequencing (RT/RNase H region) and phylogenetic analysis of SCBV isolates from four different locations. Furthermore, the transmission of SCBV by pink sugarcane mealybugs associated with sugarcane in the field was investigated. The findings will contribute to the current understanding of the genetic diversity of SCBV in Ethiopia.

Materials and methods

Leaf sample collection

Sugarcane leaf samples were collected from sugarcane fields owned by the Ethiopian Sugar Corporation in central and northern Ethiopia (Wonji-Shewa, Metahara, and Tana Beles) and from small-scale farmers around Chis Abay (Amhara region). Samples from plants showing viral disease-like symptoms such as chlorosis, leaf flecks, yellowing, and stunted growth

(Putra et al. 2023) and asymptomatic samples were randomly collected and preserved using silica gel or anhydrous calcium chloride ($CaCl_2$) (Horváth J and Besada H, 1980). A total of 270 leaf samples were collected, comprising 90 from Wonji-Shewa, 50 from Metahara, 50 from Chis Abay, and 80 from Tana Beles regions.

DNA Extraction, PCR, and Sanger sequencing

DNA extraction from leaf samples was carried out using the CTAB method as described by Tamari and Hinkleyt (2016). The reverse transcriptase/ribonuclease H (RT/RNase H) genomic region was targeted with degenerate SCBV-specific primer pairs (SCBV-F 5'GTTCA TCGCHGTNTAYATTGA TGAC 3' and SCBV-R 5'GAAGGYTT RTGTTCTV CAC TCTTGTTG3') in PCR to amplify SCBV DNA (Wu et al. 2016). A PCR reaction consisting of 10 µl of 2x Master Mix (TIANGEN Biotech, China), 0.5 µl of each primer, and 1 µl of temple DNA in a final volume of 20 µl was used for PCR amplification. The PCR amplification program was as follows: 94 °C for 4 min (1 cycle), 94 °C for 45s, 55 °C for 45s, 72 °C for 45s (35 cycles), and a final extension of 72 °C for 10 minutes. The amplified products were visualized by gel electrophoresis using 1% agarose stained with GelRed. Randomly selected PCR products representing the different sampling sites were purified using the SureClean Plus purification kit and Sanger sequenced.

Mealybug collection and rearing

The mealybugs were collected from infested sugarcane stalks at the Wonji-Shewa research farm and carefully transferred into sterile Petri dishes. The rearing method described by Amarasekare et al. (2008) was used to rear the collected mealybugs. Briefly, potatoes were surface sterilised by soaking in a 1% solution of bleach for 15 minutes, followed by thorough rinsing with water and air-dried. Subsequently, the potatoes were allowed to sprout and then infested with three to five mealybugs, depending on the size of the potato. Infested potatoes

were kept in plastic containers in dark conditions at 27 °C until the transmission study was conducted. The potatoes were changed or replaced every two weeks.

DNA extraction from mealybugs and PCR assay

DNA was extracted from mealybugs using the SDS method described by Tian and Yu (2013). PCR assay was performed using two pairs of primers amplifying housekeeping genes separately, COI (C1-J-2183- 5' CAACATTTATTTTGATTTTTTGG 3' and TL2-N-3014- 5' TCCAATGCACTAATCTGC CATATTA 3') (Simon et al. 1994) and 18S (F-1183- 5'AATTTGACTCAACACGGG3' and R-1631a 5'TACAAAGGGCAGGGACG3') (Hadziav dic et al. 2014). A PCR reaction consisting of 5 µl 5x master mix (GeneMark, BioElegen, Taiwan), 0.5 µl primer, and 1 µl template DNA in a final volume of 25 µl was used for PCR assay. The PCR amplification program included 94°C for 3 minutes (1 cycle), 94°C for 45s, annealing at 50°C (for COI primer) or 52°C (for 18S primer) for 45s, 72°C for 45s (32 cycles), and a final extension of 72°C for 7 minutes. The amplified PCR products were electrophoresed and purified, and seven samples were sequenced using the Sanger method as described above.

Establishment of a virus source and healthy test plants in the greenhouse

A greenhouse experiment was conducted to determine the transmission ability of virus vectors. Sugarcane setts were prepared from sugarcane plants with and without virus-like symptoms at the research farm in Wonji-Shewa. Cuttings were planted in a sterilized soil mixture of a 1:1:1 ratio of soil, sand, and farmyard manure in plastic pots and maintained at the Addis Ababa Science and Technology University (AASTU) greenhouse. Plants were tested by PCR assay using SCBVF/R degenerate primers after two to three months of growth in the greenhouse to determine whether SCBV was present or not. Virus-free and positive

sugarcane plants were maintained in separate insect-proof cages for the disease transmission study.

Mealybug transmission study

Non-viruliferous sugarcane mealybugs were transferred from potato sprouts to filter paper and allowed to fast for 1-2 hours before placing them on SCBV-positive plants. Sugarcane variety N-7 was used as virus source plants for the insect transmission study. Healthy plants of three sugarcane varieties, CP29/1230, TSP 92-4245, and B52-298 were used for transmission test. Six plants were used from each of the three test plants and the experiment was repeated twice. The mealybugs were fed on the virus source plants for 3-5 days, and then 5-8 mealybugs were transferred to healthy test plants after a 2-hour post-acquisition fasting period. Non-viruliferous 5-8 mealybugs were directly transferred to SCBV free plants and used as negative controls. About two to three months after inoculation, the test plants and negative control sugarcane plants were screened for the presence of the virus by PCR using SCBVF/R degenerate primers as described above.

Sequence analysis

Sanger sequences from the randomly selected PCR products of SCBV samples were evaluated and trimmed using Chromas version 2.6.6 software. The trimmed 440 bp regions of SCBV sequences were then aligned online using BLASTn, which is available on the NCBI database. Multiple sequence alignment was performed using ClustalW, and the aligned sequences were used to construct a phylogenetic tree using MEGA 11 software with the maximum likelihood method and 1000 bootstrap replications, employing the Kimura-2 parameter model (Tamura et al. 2021). A phylogenetic tree was constructed using the RT/RNase H sequence of 35 SCBV isolates representing different SCBV groups retrieved from GenBank, along with sixteen isolates from the present study (seven from Chis Abay,

five from Tana Beles, two from Metahara, and two from Wonji-Shewa) and using Taro bacilliform virus as an outgroup. The quality assessment and trimming of the mealybug sequence were also performed using the described software. The trimmed mealybugs sequences were compared by search in the BLASTn (NCBI) and BOLD systems available online. A phylogenetic tree was constructed after alignment using the COI sequences of five mealybugs retrieved from GenBank along with three mealybugs from the present study, using *Maconellicoccus hirsutus* as an outgroup. Pairwise sequence comparison was performed using SDT version 1.2 software.

Results

Symptoms

A range of symptoms, such as chlorosis, leaf flecks, yellowing, and stunted growth, were observed on different sugarcane varieties at the four sampling sites, namely Wonji-Shewa, Tana Beles, Metahara, and around Chis Abay (Fig. 1). Out of the 270 samples analysed, 155 exhibited symptoms that closely resembled those typically associated with viral infections. These symptoms included leaf flecks, chlorosis, yellowing, and stunted growth. The remaining samples, on the other hand, did not display any noticeable symptoms. The severity of the disease symptoms ranged from mild to severe chlorosis. Leaf flecks were the most common symptom observed on most of the symptomatic sugarcane varieties under field conditions.

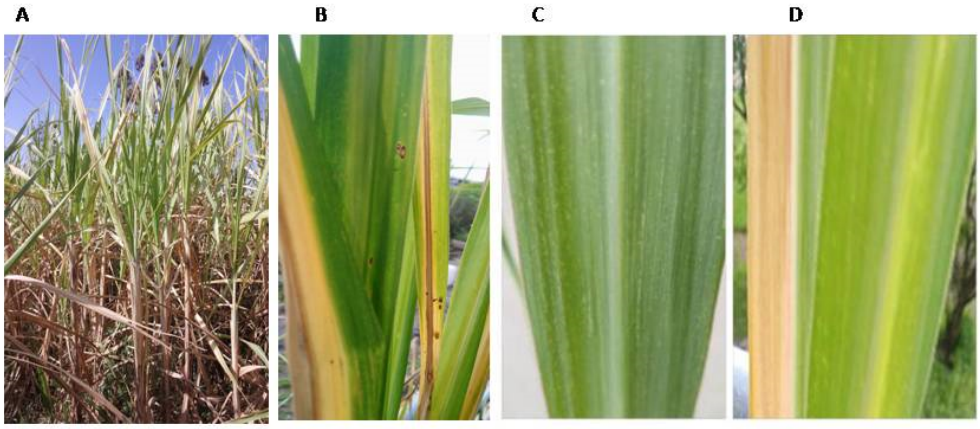

Fig. 1. Different virus-like symptoms observed in the field: (A) dwarf and yellowing; (B) leaf yellowing; (C) leaf fleck; (D) yellowing and chlorosis

Detection of SCBV

A total of 270 samples were subjected to PCR analysis using the SCBVF/R primers. In this analysis, an amplicon of the expected size (720 bp) corresponding to the RT/RNase H region was detected in 40 symptomatic and 27 asymptomatic samples (Table 1) accounting for 24.8% of the total samples collected. Based on the observations made at the four sampling sites, it was found that the prevalence of SCBV was higher in Chis Abay. At this specific location, 19 out of the 50 samples (38%) tested positive for SCBV. Following that, the Wonji-Shewa region demonstrated an incidence rate of 23 out of 90 samples (25.5%). The prevalence of SCBV in the Tana Beles was found to be 22% (11 out of 50 samples), while in the Metahara region it was 17.5% (14 out of 80 samples). Out of the 67 PCR positive samples, 16 samples (seven samples from Chis Abay, five samples from Tana Beles, two samples from Metahara, and two samples from Wonji-Shewa) were randomly selected for Sanger sequencing and further genetic analysis.

Table 1. Number of leaf samples collected from different sampling sites and summary of PCR screening for SCBV

Sampling site	Total number of samples	Symptomatic samples	Asymptomatic samples	SCBV positive symptomatic samples	SCBV positive asymptomatic samples	Total number of positive samples
Chis Abay	50	31	19	11	8	19 (38%)
Metahara	50	23	27	7	4	11 (22%)
Wonji-Shewa	90	44	46	13	10	23 (25.5%)
TanaBeles	80	57	23	9	5	14 (17.5%)
Total	270	155 (57.4%)	115 (42.6%)	40(25.8%)	27(23.5%)	67 (24.8%)

Pairwise sequence comparisons

Pairwise sequence analysis was used to compare the nucleotide sequence identity of the identified SCBVs among themselves and with reference sequences. This analysis showed that the SCBV isolates used in this study shared 77–98.9% nucleotide sequence identity among themselves. The seven isolates from Chis Abay (SCBV ET1, SCBV ET2, SCBV ET8, SCBV ET9, SCBV ET14, SCBV ET15, and SCBV ET16) showed 86.8%–98.9% nucleotide sequence identity among themselves. Additionally, these isolates showed sequence identities of 78%–87%, 79%–86.4%, and 79.1%–85.6% with SCBV isolates from Tana Beles, Metahara, and Wonji-Shewa, respectively. The other five SCBV isolates (SCBV ET3, SCBV ET4, SCBV ET5, SCBV ET12, and SCBV ET13) from Tana Beles showed nucleotide sequence identities of 81.7%–98.2% and 77.2%–96.4% with SCBV isolates identified from Metahara and Wonji-Shewa, respectively. SCBV ET6 and SCBV ET11 isolates from the Metahara site exhibited nucleotide sequence identity ranging from 84.9% to 95.8% with two SCBV isolates (ET7 and ET10) from Wonji-Shewa. Three of the four previously reported SCBV isolates from Ethiopia (MT157333.1, MT157334.1, and MT157336.1) shard a nucleotide sequence identity of 77.6%–93% with the SCBV isolate of the current study. Interestingly, one of the SCBV isolate from the previous report (MT157335.1) exhibited a relatively low level of similarity (<77%) when compared to the isolates analysed in the present study.

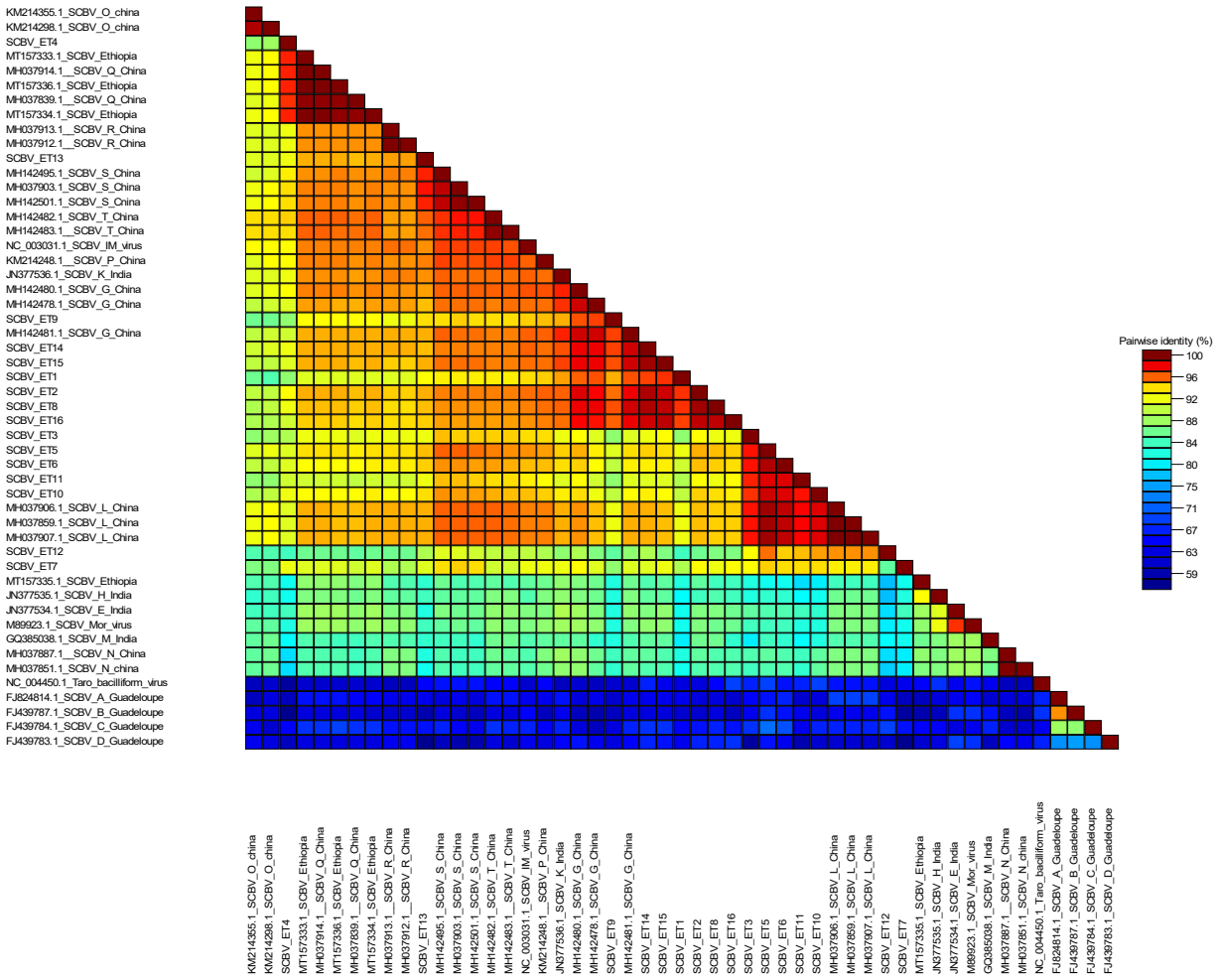

Fig. 2. Pairwise nucleotide identity matrix based on the partial RT/RNase H regions of SCBV isolates from Ethiopia together with isolates from NCBI.

The analysis of pairwise sequence comparison also revealed that the SCBV isolates obtained in this study exhibited nucleotide sequence identities ranging from 56% to 98.4% with other SCBV isolates from China, India, Guadeloupe, Australia, and Morocco (Fig. 2). Out of the sixteen isolates, SCBV ET2, SCBV ET8, SCBV ET14, SCBV ET15, and SCBV ET16 showed the highest nucleotide identity (96.9–98.9%) among themselves. These isolates also showed 93–96.7% nucleotide identity with group G SCBV isolates identified from China (MH142478.1, MH142481.1, and MH142480.1) and 89.8–91.6% identity with group K SCBV isolates from

India (JN377536.1). On the other hand, these isolates shared a limited identity (<64.5%) with group A, B, C, and D isolates from Guadeloupe (FJ824814.1, FJ439787.1, FJ439784.1, and FJ439783.1). The other seven isolates from this study, SCBV ET3, SCBV ET5, SCBV ET6, SCBV ET7, SCBV ET10, SCBV ET11, and SCBV ET12, had 84.4–98.4% nucleotide identity with group L SCBV isolates identified from China (MH037859.1, MH037907.1, and MH037906.1). Two isolates, SCBV ET1 and SCBV ET9, showed 87–91.9% nucleotide identity with group G and K SCBV isolates identified from China and India, respectively (MH142478.1, MH142481.1, MH142480.1, and JN377536.1). Isolate SCBV ET4 had 93% nucleotide identity with group Q SCBV isolates identified from China (MH037839.1 and MH037914.1). SCBV ET13 isolate showed 93–94% nucleotide identity with group S SCBV isolates identified from China (MH142495.1, MH037903.1, and MH142501.1).

Phylogenetic analysis

A maximum likelihood phylogenetic tree was constructed to analyse the evolutionary relationships among the SCBV isolates. The present study has identified the presence of four genotypes, namely SCBV-G, L, Q, and S, in Ethiopia. These genotypes were identified form the different sampling sites and form four distinct clusters along with other SCBV isolates. The first cluster within the phylogenetic tree consisted of seven isolates from the current study, namely SCBV ET1, SCBV ET2, SCBV ET8, SCBV ET9, SCBV ET14, SCBV ET15, and SCBV ET16, as well as SCBV isolates from China and India that were previously classified as groups G and K (Fig 3). SCBV ET13 isolate, together with groups S, T, and P isolates of SCBV from China and SCBV IM isolate from Australia, constituted the second sequence cluster. The third cluster in the phylogenetic tree was formed by the SCBV ET4 isolate, along with previously described isolates from Ethiopia and a group Q SCBV isolate from China (MH142495.1). The last sequence cluster

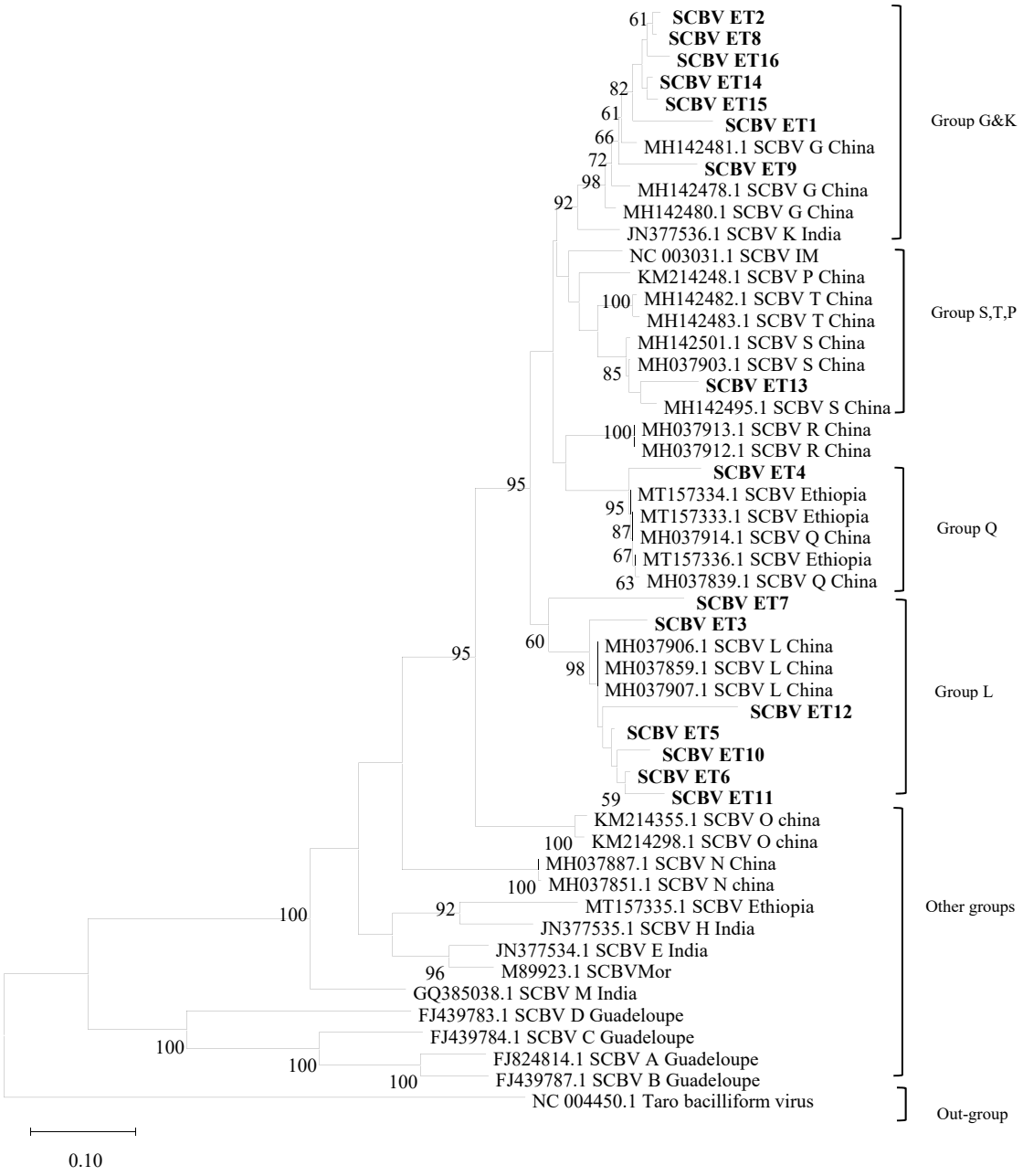

Fig. 3. The phylogenetic tree of SCBV generated by the Maximum Likelihood method of MEGA 11 based on partial RT/RNase H sequences with selected SCBV sequences from the GenBank. Taro bacilliform virus was used as an out-group. Isolates from this study are highlighted in bold.

includes seven isolates from this study (SCBV ET3, SCBV ET5, SCBV ET6, SCBV ET7, SCBV ET10, SCBV ET11, and SCBV ET12) as well as group L SCBV isolates reported from China (Fig.3).

Mealybug identification and phylogenetic analysis

PCR assays on mealybugs using COI and 18S primer pairs yielded an amplicon of approximately 800 bp and 500bp respectively. Seven randomly selected PCR products (four from 18S and three from COI) were purified and Sanger-sequenced for further analysis. Pairwise comparison was carried out using these sequences together with four reference mealybug (*Phenacoccus solenopsis*, *Pseudococcus baliteus, Planococcus citri*, and *Saccharicoccus sacchari*) sequences retrieved from GenBank. Pairwise sequence comparison analysis showed that mealybugs sequences obtained from COI region showed highest nucleotide sequence identity (99.8%-100%) with pink sugarcane mealybug (*Saccharicoccus sacchari)*. On the other hand the pairwise sequence comparison of 18S ribosomal gene with the reference sequences showed less than 94% sequence identity with other mealybug species (*Planococcus citri* and *Phenacoccus solenopsis*). Sequences from the COI primers revealed a very high sequence identity and hence enabled us to identify the mealybugs to species level. These sequences were used for further phylogenetic analysis (Fig. 4). On the other hand sequences amplified by the 18S primers were had low identity being unsuitable for species level identification of mealybugs and hence disregarded from subsequent analysis. A maximum likelihood phylogenetic analysis using sequences generated by amplification with COX primers indicated that mealybugs used for SCBV transmission in the current study are the pink sugarcane mealybug, *Saccharicoccus sacchari*, which forms a distinct clade within the phylogeny (Fig. 4).

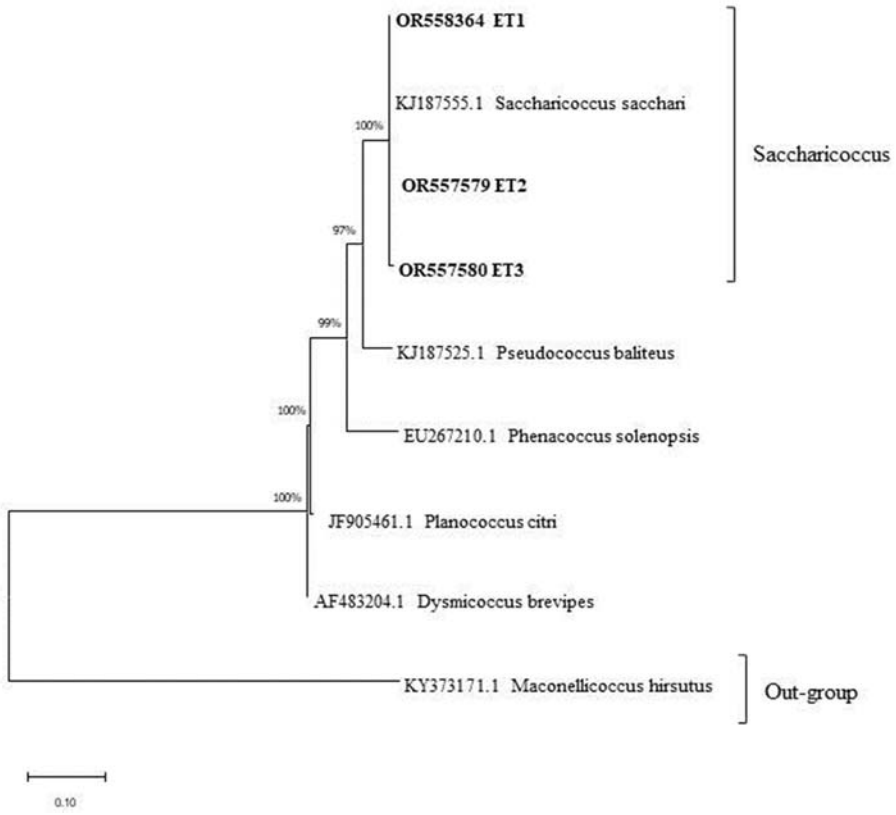

Fig. 4. The phylogenetic tree of sugarcane mealybug generated by the Maximum Likelihood method of MEGA 11 based on COI sequences with selected mealybug sequences from the GenBank. *Maconellicoccus hirsutus* was used as an out-group. Mealybugs from this study are highlighted in bold.

SCBV disease transmission by mealybugs

The rearing technique using sprouted potato yielded an ample number of mealybugs for the virus transmission study (Fig 5A and 5B). In the transmission study, nine of the 36 inoculated test plants showed leaf fleck symptoms 3-6 months after inoculation feeding (Fig. 5C and 5D). The remaining test plants did not exhibit any visible signs of infection. All the six negative control plants also remained healthy throughout the study period.

.

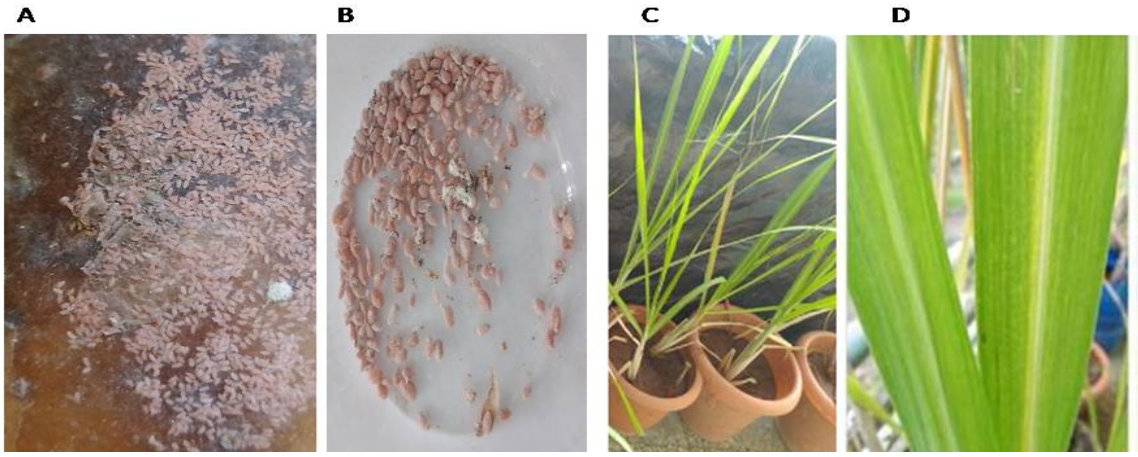

Fig. 5. Different stages of the disease transmission study A) Reared mealybugs on potatoes; B) Starvation of mealybugs on filter paper; C) Test plants before inoculation D) Symptoms developed 3-6 months post inoculation feeding.

All the plants from the virus transmission study were screened with PCR to assess the transmission of SCBV by mealybugs. Twenty two test plants (13 asymptomatic and nine symptomatic sugarcane plants) were found to be PCR positive for SCBV out of the 36 test plants (Fig. 6), indicating successful transmission of the virus by the mealybug.

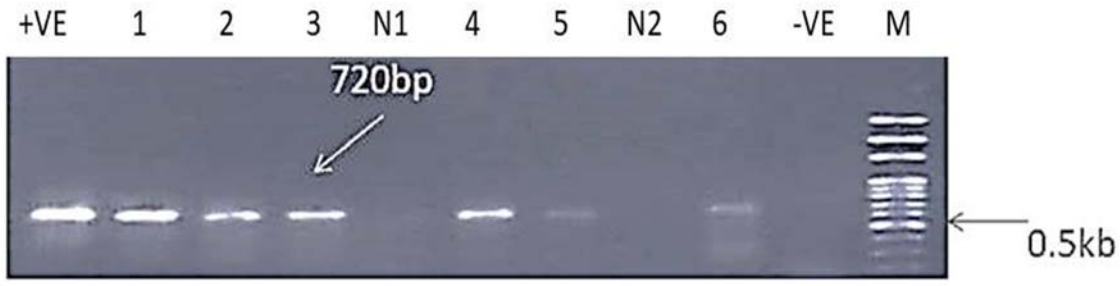

Fig. 6. PCR assay result of the disease transmission study: (+VE) positive control, (1-6) infected test plants, (N1 and N2) negative test plants, (-VE) negative control, (M) 1 Kb plus marker.

Leaf fleck symptoms were observed on four samples of CP29/1230, three samples of TSP 92-4245, and two samples of B52-298. The results are summarized in Table 2. None of the negative control plants tested positive for SCBV.

Table 2. The transmission of SCBV by mealybugs from infected sugarcane to healthy test sugarcanes plant

Sugarcane varieties	Total number of Inoculated plants	Symptomatic plants	PCR-Positive plants
cp29/1230	12	4	9 (75%)
TSP 92-4245	12	3	7 (58.3%)
B52- 298	12	2	6 (50%)
Total	36	9	22 (61.1%)

Discussion

Sugarcane is an extensively cultivated crop globally, and the global exchange of germplasm has occurred frequently. This exchange allows for the development of improved varieties that are adapted to different climates and environmental conditions (Perera et al. 2023). However, the exchange of sugarcane germplasm can also lead to the spread of diseases, including viruses, which can have negative impacts on local ecosystems and agricultural productivity (Bhat et al. 2023). Knowledge of virus prevalence and their genetic diversity is critical to the management of viral diseases through sanitary and exclusionary measures. To date, there has been only one study conducted to examine the prevalence of SCBV in limited sugarcane-growing regions of Ethiopia (Haregu et al. 2022). The results obtained from this study expand knowledge on the presence and prevalence of SCBV in other sugarcane-growing regions of Ethiopia.

The current study involved the collection and screening of 270 sugarcane samples (155 symptomatic and 115 asymptomatic) from four distinct locations in Ethiopia for SCBV using a PCR assay. Out of the total samples, 67 tested positive for SCBV. The study showed that SCBV is present in all the sampled regions, with varying levels of infection and in both symptomatic and asymptomatic sugarcane plants. The higher number (40) of positive samples was identified from the symptomatic group. SCBV infection was found to be more prevalent (38%) in the Chis Abay region where majority of the farmers are smallholder. Sugarcane production under smallholder farming systems in Ethiopia is constrained by biotic, abiotic, and socioeconomic factors. The lack of improved varieties is identified as a significant limitation to the production of sugarcane in small scale farm (Tena et al. 2016). As the Chis Abay site is a small-scale farm and not under the ownership of the Sugar Corporation of Ethiopia, the possibility of obtaining improved (disease-resistant and disease-free) varieties is limited. This might be a possible reason for the high prevalence of the virus on this site. The prevalence of SCBV in these regions highlights the importance of continued surveillance and management efforts to control the spread of this virus in sugarcane crops. In order to effectively combat SCBV, it is crucial to implement preventive measures such as regular monitoring of crops, early detection of infected plants, and prompt removal of diseased material. Additionally, implementing strict quarantine measures and promoting the use of disease-resistant sugarcane varieties can significantly contribute to reducing the spread of SCBV. Furthermore, educating agricultural workers about the symptoms and transmission of SCBV can help them identify and report potential cases, further aiding in containment efforts.

The RT/RNase H genomic region is generally used for genetic variability analysis of badnaviruses as it is the most conserved site considered for badnavirus species demarcation by

the ICTV. Extensive genetic diversity is reported in SCBV worldwide based on the sequence differences in RT/RNase H region (Muller et al. 2011; Karuppaiah et al. 2013; Ahmad et al. 2019; Janiga et al. 2023). To date, 25 SCBV genotypes (SCBV A-Y) assigned to different phylogenetic groups have been reported worldwide (Ahmad et al. 2019; Janiga et al. 2023). Five genotypes, SCBVA-D and SCBV-G, were identified in Guadeloupe (French West Indies) (Muller et al. 2011), whereas seven genotypes, SCBV E and SCBV H-M, have been reported from India (Karuppaiah et al. 2013; Rao et al. 2014). Furthermore, five SCBV genotypes, SCBV-A, SCBV-C, SCBV-F, SCBV-M, and SCBV-H, have been identified in Brazil (de Silva et al. 2015), and ten genotypes of SCBV (SCBV-G, SCBV-H, SCBV-L, and from SCBV-N to SCBV-T) were reported from China (Wu et al. 2016). Recently five novel subgroups, SCBV-U, SCBV-V, SCBV-W, SCBV-X, and SCBV-Y were identified from India (Janiga et al. 2023). In the current study, the existence of four genotypes, namely SCBV-G, L, Q, and S, in Ethiopia has been identified. This finding, together with our recent report on the occurrence of SCBV-Q and -H genotypes in central Ethiopia (Haregu et al. 2022), showed that at least five SCBV genotypes exist in Ethiopia. The most prevalent of these are SCBV-G, resembling those previously reported from Guadeloupe and China, and SCBV-L, similar to those reported from the USA and India (Wu et al. 2016; Rao et al. 2014). The SCBV-Q genotype was previously reported from China and Australia (Wu et al. 2016). The isolate ET13 sequence has been observed to cluster with the SCBV-S genotype, which has been recently identified as a novel genotype in China. The isolate exhibits a nucleotide variation of only 9.4–11.8% from SCBIMV, which is genetically the closest to it at the RT-H region (Ahmad et al. 2019). This indicates that the isolate belongs to the same species. Finally, the SCBV-H genotype reported in our previous study (Haregu et al. 2022) is reported in Brazil, India, and China (Ahmad et al. 2019). Before this study, among the 25

genotypes described so far globally, only one genotype, SCBV-E (representing SCBMOV), was reported from Morocco (Ahmad et al. 2019).

Overall, the phylogenetic analysis shows that the isolates from Ethiopia show relatedness to isolates originating from a wider geographic area, including China, India, Brazil, the USA, and Guadeloupe (the Caribbean). This suggests that SCBV isolates may have had multiple introductions to Ethiopia from these countries at different times. In Ethiopia, sugarcane was first commercially cultivated in 1951 by a Dutch company, and the sugar industry of Ethiopia is so far dependent on the introduction of exotic varieties. Such germplasm imports from diverse geographical areas are likely to be the reason for the occurrence of genetically diverse isolates similar to those in these countries. On the other hand, SCBV isolates from Morocco, which is geographically closer to Ethiopia, branch distantly from the Ethiopian isolates (Fig.3). This suggests that geographical proximity may not necessarily be the determining factor for genetic similarity among SCBV isolates. Further research is needed to understand the underlying mechanisms driving the genetic variation of SCBV and its implications for disease management strategies in different regions. Abraham et al. (2018) reported a new badnavirus tentatively named the enset streak virus infecting enset, a widely cultivated crop in the country, that is phylogenetically most closely related to the sugarcane bacilliform Guadeloupe D virus reported from sugarcane in the French West Indies (Muller et al. 2011). However, we did not observe any close relationship with the badnaviruses reported in enset and sugarcane in Ethiopia, although these crops are grown in overlapping geographical areas in some parts of the country.

Mealybug infestations cause not only direct plant damage but also spread badnaviruses within a sugarcane plantation. Mealybugs and infected vegetative cuttings are known to be the major means of natural transmission for SCBVs (Bhat and Rao 2020). Three species of mealybug are

so far reported to transmit SCBV: the pink mealybug (*Saccharicoccus sacchari*), the grey mealybug (*Dysmicoccus boninsis*) (Lockhart et al. 1992), and the citrus mealybug, *Planococcus citri* (Lockhart et al. 1995).

The ribosomal DNA (subunits 28S , 18S, and ITS) and mitochondrial DNA (cytochrome oxidase I (COI) and II (COII) are the common genes used in identifying insects (Simon et al., 1994; Rokas et al., 2002; Li et al., 2005). The cytochrome oxidase I (COI) mitochondrial genes and the 18S ribosomal gene were used for molecular identification of mealybuges in the current study. COI sequences amplified by C1-J-2183 and TL2-N-301 primer pairs provided an unambiguous identification of the mealybugs and confirmed their identity (99.8-100%) as *Saccharicoccus sacchari*. Preliminary morphological observations suggested that the mealybugs have a whitish-pink color. The coloration is consistent across all life stages, from nymphs to adults. These observations align with the molecular identification of mealybugs, confirming the species identification as pink sugarcane mealybug. Our attempts to identify mealybug species using 18S primer pairs (F-1183 and R-1631a) yielded unsatisfactory result as the sequences obtained by these primer pair exhibited a relatively low identity percentage of 90–94% which is too low for species-level identification of mealybugs. We therefore recommend the use of Cox primer pairs for molecular identification of mealybugs. This information could be valuable for developing effective control strategies to manage the virus and its vectors in sugarcane crops. Therefore, it is important to carefully inspect and quarantine vegetative cuttings and monitor for mealybug infestations to prevent the spread of SCBV.

During the course of our research, we encountered challenges in assigning the genotypes of the SCBV genotypes we studied to established species. This difficulty arises from the fact that the

identified genotypes do not align with any of the four SCBV species (SCBGAV, SCBGDV, SCBMOV, and SCBIMV) officially recognized by the International Committee on Taxonomy of Viruses (ICTV). Apart from these four species, other possible SCBV species have been proposed by different authors in Guadeloupe (Muller et al. 2011), India (Karuppaiah et al. 2013), and China (Ahmad et al. 2019); however, ICTV has not approved these proposals. Using the available species demarcation criteria, it is likely that some of the genotypes from the different countries, including some from Ethiopia, may be classified into species other than the four recognized. Hence, we recommended that the ICTV reassess and clarify the classification of SCBV genotypes at the species level through its *Caulimoviridae* working group, as the current state of knowledge in this regard is insufficient and might lead to confusion. This would help provide a more accurate understanding of the diversity and distribution of SCBV genotypes.

In conclusion, the present study provided insight into the occurrence, genetic diversity, and evolutionary relationships of SCBV isolates from Ethiopia. The existence of four genotypes, namely SCBV-G, L, Q, and S, in Ethiopia has been identified. This genetic diversity suggests that the virus may have multiple origins. The study reveals that pink sugarcane mealybugs are potential vectors for the transmission of the viruses, highlighting the need for continuous surveillance and management of these pests in sugarcane production systems. The study provides valuable insights for understanding the dynamics of SCBV transmission and developing effective control strategies to prevent its further spread.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Mereme Abide, Adane Abraham, Dawit Kidanemariam, and

Misrak Kebede. The first draft of the manuscript was written by Mereme Abide, and all the authors commented on the previous version of the manuscript. All authors read and approved the final manuscript.

Data availability

Sequences described in this paper are available under GenBank accession numbers OQ401597–OQ401612 (SCBV) and OR557579, OR557580 and OR558364 (mealybug). The other datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Acknowledgment

The authors would like to thank Addis Ababa Science and Technology University for financial support and the Molecular Biology Laboratory of the Biotechnology Department for lab facilities.

Funding: This study was financially supported by the Addis Ababa Science and Technology University.

Statements and declarations

Conflict of interest: The authors declare that they have no conflict of interest.

Reference

Abide M, Kidanemariam D, Yimam T, Worku Y, Kebede M, Abraham A (2022) Protocol optimization for elimination of sugarcane bacilliform virus and rapid propagation of virus-free sugarcane using meristem tip culture. Trop Plant Pathol 47:693-697

Abraham A, Winter S, Richert-Pöggeler K, Menzel W (2018) Molecular characterization of a new badnavirus associated with streak symptoms on enset (*Enseteventricosum*, *Musaceae*). J Phytopathol 166:565-571

Ahmad K, Sun SR, Chen JL, Huang MT, Fu HY, Gao SJ (2019) Presence of diverse sugarcane bacilliform viruses infecting sugarcane in China revealed by pairwise sequence comparisons and phylogenetic analysis. J Plant Pathol 35:41-50

Amarasekare KG, Mannion CM, Osborne LS, Epsky ND (2008) Life history of *Paracoccus marginatus* (Hemiptera: Pseudococcidae) on four host plant species under laboratory conditions. Environ Entomol 37:630- 635

Ashraf MA, Feng X, Hu X, Ashraf F, Shen L, Iqbal MS, Zhang S (2022) Insilico identification of sugarcane (*Saccharum officinarum* L.) genome encoded microRNAs targeting sugarcane bacilliform virus. PLoS One 20:17

Balan S, Viswanathan R, Cherian K (2020) Status of leaf feck caused by sugarcane bacilliform virus incidence and severity in different sugarcane growing areas of Kerala and Tamil Nadu. J Sugarcane Res 10:74-86

Bhat AI, Hohn T, Selvarajan R (2016) badnaviruses: the current global scenario. Viruses 8:177. https://doi.org/10.3390/v8060177

Bhat AI, Rao GP (2020) Transmission of viruses through mealybugs. In: Characterization of plant viruses. Springer Protocols Handbooks. Humana, New York, NY. Https. 1007/978-1 - 0716-0334-5_15

Bhat AI, Selvarajan R, Balasubramanian (2023) Emerging and re-emerging diseases caused by badnaviruses. Pathogens 12:245: https://doi.org/10.3390/pathogens12020245

da Silva JM, Jobim LJ, Ramos-Sobrinho R, Lima S, Assunção P, Cruz M, Lim A (2015) Incidence and species diversity of badnaviruses infecting sugarcane from a germplasm collection in Brazil. Trop. Plant Pathol 40:212-217

Filloux D, Fernandez E, Comstock JC, Mollov D, Roumagnac P, Rott P (2018) Viral Metagenomic-Based Screening of Sugarcane from Florida Reveals Occurrence of Six Sugarcane-Infecting Viruses and High Prevalence of Sugarcane yellow leaf virus. Plant Dis 102:2317-2323. doi: 10.1094/PDIS-04-18-0581-RE

Hadziavdic K, Lekang K, Lanzen A, Jonassen I, Thompson EM, Troedsson C (2014) Characterization of the 18S rRNA Gene for Designing Universal Eukaryote Specific Primers. PLOS ONE 9:e87624. https://doi.org/10.1371/journal.pone.0087624

Haregu S, Kidanemariam D, Abraham A (2022) Molecular identification and characterization of badnaviruses infecting sugarcane in Ethiopia. ActaVirologica 66:3-10

Horváth J, Besada H (1980) Preservation of some plant viruses by dehydration over anhydrous calcium chloride ($CaCl_2$) J Plant Dis Prot 87:463–472. http://www.jstor.org/stable/43214717

International Committee on Taxonomy of Viruses ICTV (2021) https://ictv.global/ taxonomy

Janiga, K, Nithya, K, Viswanathan R (2023) Dynamics of genetic diversity among Indian sugarcane bacilliform virus species and implications of associated recombination events in the virus. Sugar Tech 25:705–716. https://doi.org/10.1007/s12355-022-01224-1

Jayanthi R, Srikanth J, Sushil SN (2016) Sugarcane. In: Mani M, Shivaraju C (ed) mealybugs and their management in agricultural and horticultural crops. Springer, New Delhi, pp 287–296. https://doi.org/10.1007/978- 81-322-2677-2_28

Karuppaiah R, Viswanathan R, Kumar G (2013) Genetic diversity of sugarcane bacilliform virus isolates infecting *saccharum* spp. in India. Virus Genes 46:505-516

Krishna GV, Kumar VM, Varma PK, Bhavani B, Kumar GV (2023) Detection of Sugarcane bacilliform virus in diseased sugarcane plants in Andhra Pradesh, India by serological and molecular methods. Braz J Microbiol 54:1399-1409. 10.1007/s42770-023-01094-z

Li F, Huang K, Jiang M, Zhang X, Zhang L, Li F (2010) Detection of sugarcane bacilliform virus isolate and its influence on yield and quality of cane in Yunnan. Acta Phytopathol Sin 6: 651-654

Li M, Deng Q, Wang W, Chen Y, Jia L, Wang Z (2005) A preliminary phylogeny of the Pentatomomorpha (Hemiptera: Heteroptera) based on nuclear 18S rDNA and mitochondrial DNA sequences. Mol Phylogenet Evol 37: 313-326

Lockhart B, Autrey L, Comstock J (1992) Partial purification and serology of sugarcane mild mosaic virus, a mealybug-transmitted clostero like virus. Phytopathology 82: 691-695

Lockhart B, Irey J, Comstock J (1995) Sugarcane bacilliform virus, *Sugarcane mild mosaic* virus, and *sugarcane yellow leaf* syndrome. In: Croft BJ, Pig CT, Wallis ES, Hogarth DM (eds) sugarcane germplasm conservation and exchange. Queensland, Australia, pp 108-112

Lockhart BE, Autrey L (1988) Occurence in sugarcane of a bacilliform virus related serologically to Banana streak virus. Plant Dis 72: 230-233

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Floors P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Ann. Entomol. Soc. Am 87: 651-701

Muller E, Dupuy V, Blondin L, Bauffe F, Daugrois JH, Nathalie L, Iskra-Caruana ML (2011) High molecular kvariability of sugarcane bacilliform viruses in Guadeloupe implying the existence of at least three new species. Virus Res 160:414–419. doi:10.1016/ j.virusres.2011.06.0

Perera MF, Ostengo S, Malavera ANP, Balsalobre TWA, Onorato GD, Noguera AS, Hoffmann HP, Carneiro MS (2023) Genetic diversity and population structure of Saccharum hybrids. PLoS One. 18:e0289504. doi: 10.1371/journal.pone.0289504

Putra LK, Kristini A, Jati WW (2023) viral diseases of sugarcane in Indonesia: Occurrence notes, pathogenic characteristics, and management strategies. Conf. Agric. & Life Sci. AIP Conf. Proc. 2583, 020038

Rao GP, Sharma SK, and Kumar PV (2018) Sugarcane Bacilliform Viruses Present Status. In: Gaur RK, Khurana SMP, Dorokhov YL (eds) Plant viruses: diversity, interaction and management. CRC Press, Boca Raton, pp 136-149

Rao GP, Sharma SK, Singh D, Arya M, Singh P, Baranwal VK (2014) Genetically diverse variants of sugarcane bacilliform virus infecting sugarcane in India and evidence of a novel recombinant Badnavirus variant. J Phytopathol 162: 779-787

Rokas A, Nylander J, Ronquist F and Stone G (2002) A maximum-likelihood analysis of eight phylogenetic markers in gallwasps (Hymenoptera: Cynipidae): implications for insect phylogenetic studies. Mol Phylogenet Evol 22:206-219

Sharma SK, Vignesh Kumar P, Geetanjali AS, Pun KB, Baranwal VK (2015) Subpopulation level variation of banana streak viruses in India and common evolution of banana and sugarcane badnaviruses. Virus Genes 50: 450-465

Sun R, Damaj B, Alabi J, Wu B, Mirkov E, Fu Y, Chen K, Rao J (2016) Molecular characterization of two divergent variants of sugarcane bacilliform viruses infecting sugarcane in China. European J Plant Pathol 145: 375

Tamari F, Hinkley CS (2016) Extraction of DNA from plant tissue: review and protocols. In: Micic M (ed) Sample Preparation Techniques for Soil, Plant, and Animal Samples. Humana Press, pp 245–263

Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol 38:3022-3027. doi:10.1093/molbev/msab120

Tena E, Mekbib F, Shimelis H, Mwadzingeni L (2016) Sugarcane production under smallholder farming systems: Farmers preferred traits, constraints and genetic resources. Cogent food agric. 2:1. DOI: 10.1080/23311932.2016.1191323

Tian W, Yu H (2013) A Simple and Rapid DNA Extraction Protocol of Small Insects for PCR Amplification. Entomol. News 123:303–310. doi:10.3157/021.123.0403

Viswanathan R, Karuppaiah K, Bagyalakshmi, Balan S, and Kaverinathan K (2019) Emergence of leaf fleck caused by sugarcane bacilliform virus in sugarcane as a serious disease under field conditions in India. Int. Sugar J 121: 146-153

Viswanathan R, Parameswari B, Nithya K (2018) Molecular characterization of sugarcane viruses and their diagnostics. In: Prasad R, Gill SS, Tuteja N (Eds) Crop Improvement through Microbial Biotechnology. The Netherlands, pp 175-193

Wu B, Alabi J, Damaj B, Sun R, Mirkov E, Fu Y, Chen K, Gao J (2016) Prevalence and RT/RNase H genealogy of sugarcane bacilliform virus isolates from China. J Phytopathol 164:595-607