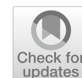
Tropical Plant Pathology (2022) 47:693–697
https://doi.org/10.1007/s40858-022-00519-8

SHORT COMMUNICATION



Protocol optimization for elimination of sugarcane bacilliform virus and rapid propagation of virus-free sugarcane using meristem tip culture

Mereme Abide[1] · Dawit Kidanemariam[2] · Teslim Yimam[1,3] · Yonas Worku[4] · Misrak Kebede[1] · Adane Abraham[1,5]

Received: 25 February 2022 / Accepted: 27 June 2022 / Published online: 25 July 2022
© The Author(s), under exclusive license to Sociedade Brasileira de Fitopatologia 2022

Abstract
Tissue culture protocol was optimized for the propagation of virus-free sugarcane from infected plants using meristem tips as an explant source for the elimination of sugarcane bacilliform virus (SCBV).Virus identification on the mother (source) plant and virus indexing to monitor elimination in the tissue culture-derived plants were done by polymerase chain reaction (PCR) using degenerate SCBV primers. Murashige and Skoog (MS) media supplemented with 0.5 mg/l BAP + 0.25 mg/l kinetin and 0.1 mg/l GA_3 + 0.5 mg/l NAA were the best hormone combination for shoot multiplication and root induction, respectively. Two explant size categories (< 1 mm and 1–2 mm) were used to assess the effect of explant size on shoot regeneration and virus elimination. The results showed that explant size affects shoot regeneration. Smaller sized (< 1 mm) explants showed higher virus elimination efficiency; however, the survival frequency of explants during initiation of shoot cultures was higher in larger (1–2 mm) meristems (64.3%) in comparison to the smaller ones (35.7%). In conclusion, *in vitro* meristem tip culture alone is not a satisfactory approach for the generation of SCBV-free plant from infected mother plant. The virus elimination efficiency could be enhanced by using the combination of meristem tip culture with other therapies.

Keywords Ethiopia · Plant growth hormones · *In vitro* · Regeneration · SCBV

Sugarcane viral diseases are responsible for declining in the production of sugarcane in different countries. Sugarcane viruses such as *sugarcane mosaic virus* (SCMV), *sugarcane streak virus* (SCSV), different sugarcane bacilliform viruses (SCBVs), and *sugarcane yellow leaf virus* (SCYLV) are among the most critical viruses reported in different parts of the world (Viswanathan and Rao 2011; Ahmad et al. 2019; Lu et al. 2021).

Ethiopia is experiencing an increasing demand for sugar. The Ethiopian Sugar Corporation is undertaking large-scale expansion and new sugar development projects (Kamski 2016; Hamza and Alebjo 2017). The expansion program requires a large amount of sugarcane clones. To satisfy the plant material requirement, different national tissue culture laboratories propagate sugarcane in large amount and disseminate the plantlets to different sugar estates (Abraham 2009). However, the risk of distributing virus-infected plants remained very high because little attempts were made to identify and/or eliminate viruses during micro-propagation procedures. Available information indicates that SCBV is a major viral pathogen widely distributed in Ethiopia sugarcane cultivation areas (Haregu et al. 2022). There is, however, no study on the elimination of viruses from Ethiopian sugarcane cultivars. Hence, this study was carried out with the objective of developing protocol for the production of SCBV-free sugarcane.

✉ Mereme Abide
mereme.abide@aastu.edu.et

✉ Adane Abraham
abrahama@biust.ac.bw

[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, Pretoria, South Africa

[3] Department of Biotechnology, School of Life Science and Technology, Center for Informational Biology, University of Electronic Science and Technology of China, Chengdu 610054, China

[4] Ethiopia Sugar Corporation, research center, Wonji, East Shoa, Ethiopia

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

Sugarcane seed (variety cp29/1230) samples were collected from sugarcane plants at the research farms of Wonji Sugarcane Research Center, Ethiopia. Seeds were planted on plastic pot and maintained at Addis Ababa Science and Technology University (AASTU) greenhouse. After growing in the greenhouse for about 2 months, leaf samples were collected and tested for SCBV by PCR as described below. Furthermore, the collected sugarcane plants were also tested for the major sugarcane viruses other than SCBV, namely SCMV, SCYLV, and sugarcane streak virus using PCR and RT-PCR. The SCBV positive plants were used as source of explants for the virus cleaning study. Actively growing shoot tips were harvested followed by surface sterilization by spraying with 70% ethanol and leaves surrounding meristem were carefully removed. The apical portions (1 cm) were washed under running tap water with liquid detergents for 15 min followed by shaking in a beaker with five drops of liquid detergent for 10 min and again washed under running tap water. Then, the plant materials were sterilized in 70% alcohol for 1 min followed by sodium hypochlorite solution (5%) containing one drop of tween 20 for 10 min with continuous shaking and rinsed thoroughly in sterile distilled water in an aseptic condition within a laminar airflow cabinet. Apical meristems measuring < 1 and 1–2 mm in size were aseptically excised out and immediately placed on MS medium (Murashige and Skoog 1962) containing 3% sucrose, 0.1% activated charcoal, and 0.8% agar supplemented with different concentration of BAP and Kin (0.25 mg/l BAP + 0.1 mg/l Kin, 0.5 mg/l BAP + 0.25 Kin, and 0.5 mg/l BAP alone) and the pH adjusted to 5.8. The cultured meristems were kept in the incubation chamber at 26 °C with 16-h photoperiod. Cultures were transferred to fresh medium every 2 weeks. Fourteen samples per treatments were used and the experiment was repeated twice. Regeneration rate was evaluated 4 weeks after meristem was cultured. Explants were considered regenerated if new leaves developed and persisted on growing following inoculation of meristems to the regeneration media. Regeneration rate was calculated as:

$$\text{Shoot regeneration rate}\,(\%) = \frac{\textit{Number of regenerated explants}}{\textit{Total number of explant scultured}} \times 100$$

Different combinations of cytokinins were tested for shoot multiplication. After 6 weeks, the regenerated shoots were transferred onto shoot multiplication MS media supplemented with different concentration of BAP (0, 0.1, 0.25, 0.5, 1, 1.5, and 2 mg/l) and Kin (0, 0.1, 0.25, 0.5 mg/l) and kept in the growth chamber at 26 °C with 16-h photoperiod. Cultures were transferred to fresh medium every 2 weeks and multiple shoot development was evaluated. Shoot number per explant were recorded for each hormone combination. Shoots greater than 5 cm in length were transferred in to growth regulator-free MS media with 0.1% activated charcoal for about 21 days and then cultured in to root induction MS media supplemented with 0.1 mg/l GA_3 with different concentrations of NAA, IBA, and IAA (0, 0.1, 0.25, 0.5 mg/l). The cultures were transferred to fresh medium every 2 weeks and root induction was compared after 4 weeks. The rooted plants were removed from the medium and washed thoroughly in running tap water to remove residues of medium. Then, the shoots were transferred to pots filled with autoclaved red soil, sand, and manure mix in 2:1:1 ratio. The experiments were repeated three times and 8 samples per treatment were used. The plants were maintained in the greenhouse and survival rate of the shoot was observed and recorded.

DNA was extracted using the CTAB method as described by Tamari and Hinkley (2016). Green GoTaq® master mix (Promega) was used for PCR amplification. The amplified products were detected by gel electrophoresis using 1% agarose stained with gel-red.

Data were analyzed using SPSS software and statistical analysis was carried out using ANOVA and a comparison of means using Tukey's test (P 0.05).

Shoot initiation of the apical meristem tip was observed after 5 days of culturing. Multiple shoot development was observed within 4 weeks. Among the three hormone combinations (0.25 mg/l BAP + 0.1 mg/l Kin, 0.5 mg/l BAP + 0.25 Kin, and 0.5 mg/l BAP alone) used for shoot initiation, the MS media supplemented with 0.5 mg/l BAP + 0.25 mg/l Kin showed higher propensity for shoot regeneration (Fig. 1).

The growth hormones used for this study markedly affected the development of multiple shoots; these observations are reported in Table 1.

Out of the eight hormone combinations used for multiplication, T5 resulted in a higher number of shoots (28.5 ± 4.3) per explant. T4 was the second-best medium for multiple shoot development and resulted in 19.3 ± 4.7 shoots per explant. On the other hand, a lower propagation rate was recorded on hormone-free, T2 and T3 media. The results showed that increasing the concentration of BAP from 0.1 to 0.5 mg/l increased multiplication capacity of the explant and resulted in a higher number of shoots per explant, whereas an increase in BAP concentration beyond 0.5 mg/l (1, 1.5, and 2 mg/l BAP) while withholding kinetin at 0.25 mg/l showed a considerable decline in the number of shoots per explant (from 28.5 ± 4.3 in T5 to 6.7 ± 1.1 in T9). Similarly, keeping the concentration of BAP at 0.5 mg/l while increasing the concentration of kinetin from 0.25 to 0.5 mg/l considerably reduced the number of shoots obtained per explant from 28.5 ± 4.3 in T5 to 5.9 ± 0.9 in T6.

Nine hormone combinations with one hormone-free control were tested for root induction; the result is presented in Table 2. Out of these rooting media tested, best rooting was observed on R10 which resulted higher number of roots (9.3 ± 2.3). At this hormone concentration, 75% of the shoots formed root and root length per plant was also higher (5.5 ± 1.1).

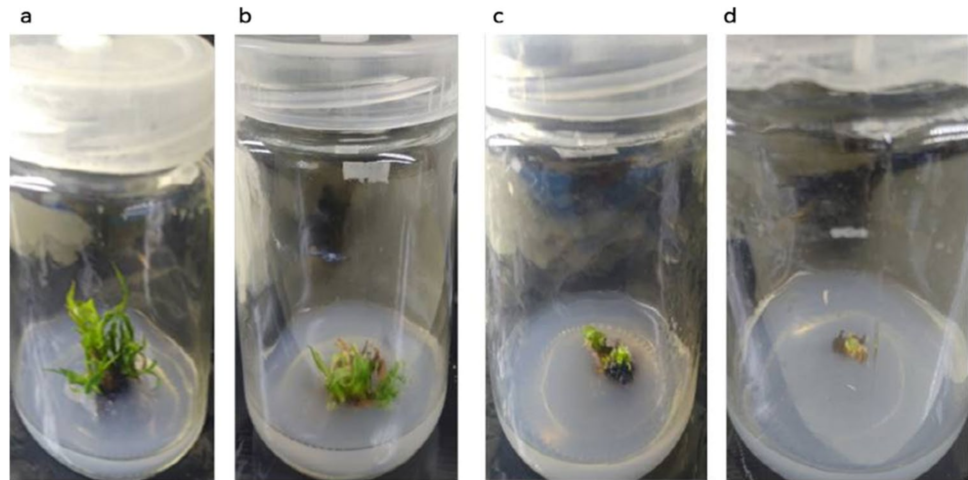


Fig. 1 Shoot regeneration of sugarcane from meristem tip after 4 weeks. **a** Culture on MS media supplemented with 0.5 mg/l BAP + 0.25 mg/l Kin, **b** culture on MS media supplemented with 0.5 mg/l BAP alone, **c** culture on MS media supplemented with 0.25 mg/l BAP + 0.1 mg/l Kin, **d** culture on hormone-free MS media

Table 1 Summary of *in vitro* shoot multiplication from meristem of sugarcane

Treatment	Media combination	Number of samples	Number of shoots per plant(mean ± SD)
T1	MS (hormone free)	8	0.5 ± 0.5
T2	MS + 0.1 mg/lBAP + 0.1 mg/l Kin	8	1.0 ± 0.7
T3	MS + 0.25 mg/l BAP + 0.1 mg/l Kin	8	1.9 ± 0.6
T4	MS + 0.5 mg/l BAP	8	19.3 ± 4.7
T5	MS + 0.5 mg/l BAP + 0.25 mg/l Kin	8	28.5 ± 4.3
T6	MS + 0.5 mg/l BAP + 0.5 mg/l Kin	8	5.9 ± 0.9
T7	MS + 1 mg/l BAP + 0.25 mg/l Kin	8	12.2 ± 2.2
T8	MS + 1.5 mg/l BAP + 0.25 mg/l Kin	8	9.0 ± 2
T9	MS + 2 mg/l BAP + 0.25 mg/l Kin	8	6.7 ± 1.1

Table 2 The effect of growth hormones (auxins) on root development

Treatment	Media combination	Number of samples	Number of root per explant (mean ± SD)	Root length (mean ± SD)
R1	MS (hormone free)	8	1.6 ± 0.7	2.7 ± 0.2
R2	MS + 0.1 mg/l GA_3 + 0.1 mg/l IAA	8	3.9 ± 0.7	3.3 ± 0.5
R3	MS + 0.1 mg/l GA_3 + 0.25 mg/l IAA	8	4.8 ± 0.9	2.9 ± 0.5
R4	MS + 0.1 mg/l GA_3 + 0.5 mg/l IAA	8	4.0 ± 1.1	3.1 ± 0.5
R5	MS + 0.1 mg/l GA_3 + 0.1 mg/l IBA	8	5.6 ± 2	3.3 ± 0.8
R6	MS + 0.1 mg/l GA_3 + 0.25 mg/l IBA	8	7.8 ± 2.4	3.6 ± 1.6
R7	MS + 0.1 mg/l GA_3 + 0.5 mg/l IBA	8	5.6 ± 1.9	3.4 ± 0.8
R8	MS + 0.1 mg/l GA_3 + 0.1 mg/l NAA	8	5.8 ± 2.1	4.3 ± 2.1
R9	MS + 0.1 mg/l GA_3 + 0.25 mg/l NAA	8	6.1 ± 1	3.8 ± 1.5
R10	MS + 0.1 mg/l GA_3 + 0.5 mg/l NAA	8	9.3 ± 2.3	5.5 ± 1.1

The current study also revealed that MS media supplemented with 0.1 mg/l GA_3 and 0.25 mg/l IBA were the second best rooting media. *In vitro* rooted plantlets (Fig. 2a) were transferred to pots in the green house, and the survival rate was recorded after 1 month. The survival percentage of rooted shoots was 85% and hardened as shown in Fig. 2b.

The successes of shoot regeneration and virus elimination were affected by the size of the explants used for meristem tip culture. From plants with size category of < 1 mm, only 35.7% (10/28) of the explants develops into shoots, while 64.3% (18/28) of larger explants (1–2 mm) showed shoot regeneration. PCR amplification was performed before and after tissue culture treatment. All of the PCR amplifications

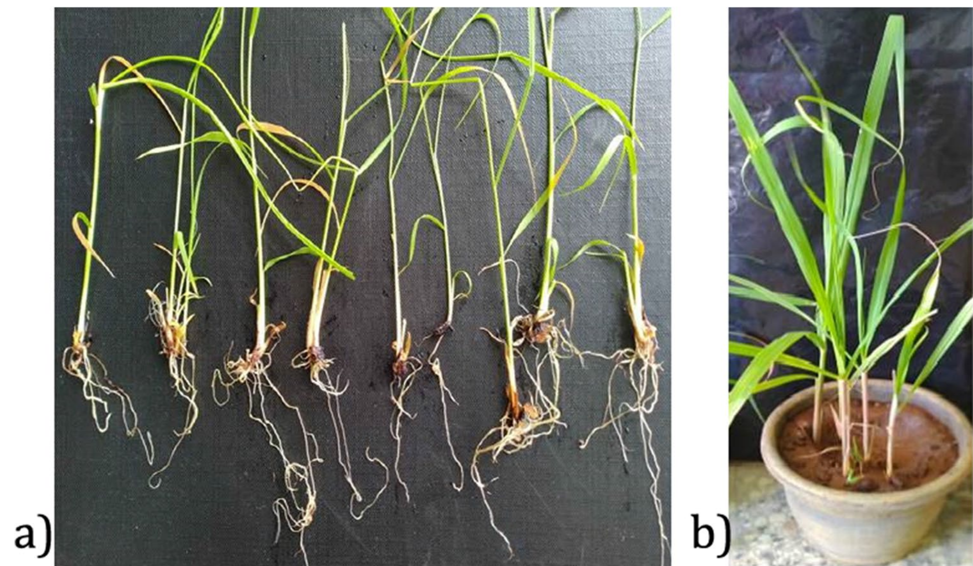


Fig. 2 Rooting and hardening of tissue culture-derived plantlets of sugarcane: **a** *in vitro* rooted shoots, **b** survived shoots in the greenhouse after 2 month

done on samples before tissue culture treatment yielded the expected amplicon size for SCBV and representative samples were Sanger sequenced and confirmed. Even though all the meristem tips used for tissue culture were derived from SCBV positive plants, some of the regenerated plantlets proved to be virus free. The PCR analysis on tissue culture-derived plants after 6 months of growing in the glasshouse revealed 16.7% and 30% virus-free plants from larger and smaller explant sizes, respectively.

Tissue culture technology has been used as the best way to ensure rapid multiplication of disease-free planting material. The present study shows the use of shoot apical meristem culture for simultaneous mass propagation and virus elimination in sugarcane. The results of this experiment showed that both higher and lower levels of growth regulator hormones negatively affect shoot multiplication. This might be associated with the fact that higher levels of cytokinin hinder cell division and a lower level of hormone might not be enough to induce shoot multiplication. This result agrees with Salokhe (2021) finding, which reported noticeable growth regulator (BAP and Kin) effect on reduction of shoot multiplication when it exceeds the optimum concentration. On the other hand, our result contrasts with the earlier report by Mekonnen et al. (2014) in which they claimed the optimum shoot multiplication on MS medium supplemented with high concentration of growth regulator (3 mg/l BAP alone and 3 mg/l BAP + 1.5 mg/l Kin). In contrast, Ramgareeb et al. (2010) reported maximum shoot multiplication on MS medium with a low concentration of hormones (0.1 mg/l BAP + 0.015 mg/l Kin). Optimum hormone concentration likely depends on the sugarcane varieties and therefore the use of balanced cytokinin is important for shoot multiplication. On the other hand, the current study revealed that MS media supplemented with 0.1 mg/l GA_3 + 0.5 mg/l NAA and 0.1 mg/l GA_3 + 0.25 mg/l IBA were the best rooting media. NAA and IBA have been reported as the best rooting hormone for an *in vitro* root initiation in sugarcane (Khan et al. 2008; Pathak 2009; Mekonnene 2014; Nawaz et al. 2013; Salokhe 2021).

Attempts have previously been made by different researchers to eliminate sugarcane viruses from infected plant using meristem tip culture and successful elimination of the SCMV, SCYLV, SCSMV, and FDV was reported (Ramgareeb et al. 2010; Subba-Reddy and Sreenivasulu 2011; Cheong et al. 2012). Even though SCBV is one of major threats in causing yield decline and affecting germplasm exchange globally (Balan et al. 2020), little attempts have been made to study SCBV elimination using tissue culture. In the present study, meristem tip culture was used for SCBV elimination and lower rate of elimination (16% and 30% from 1–2 mm and < 1 mm meristem, respectively) was found. The efficiency of virus elimination from infected sugarcane could be affected by various factors. Difference in elimination efficiency of sugarcane viruses might be attributed to differences in infecting virus and cultivar response to the virus. The elimination of phloem restricted viruses via meristem tip culture perhaps is effective than other viruses. Lower elimination efficiency found in the present study could be due to the virus characteristic, genotype, and physiological condition of the cultivar. Therefore, the combination of meristem tip culture with thermotherapy, chemotherapy, and cryotherapy should be considered in the future to enhance the eradication of SCBV from infected plant.

Acknowledgements The authors would like to thank the Addis Ababa Science and Technology University for financial support and Plant Tissue Culture Laboratory of Biotechnology Department for lab facilities. Also the Authors acknowledge Dr. Solomon Benor and AlazarYeshitila for their kind cooperation during tissue culture experiment.

Author contribution MA: Conceived, designed, and performed the experiments; analyzed data; and prepared draft manuscript. DK: designed of the experiments; contributed reagents/materials and critically reviewed the data and manuscript. TY: carried out the experiment; critically reviewed the data and manuscript. MK: contributed reagents/materials, critically reviewed the data and manuscript. YW: prepared samples and review the manuscript. AA: conceptualized and designed of the experiments, contributed reagents/materials, and critically reviewed the data and manuscript. All authors read and approved the final manuscript.

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

Data availability The datasets generated and/or analyzed during the current study are available from the corresponding author up on request.

Declarations

Conflict of interest The authors declare no competing interests.

References

Abraham A (2009) Agricultural biotechnology research and development in Ethiopia. African Journal of Biotechnology 8:7196–7204

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. Plant Pathology Journal 35:41–50

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

Cheong EJ, Mock R, Li R (2012) Elimination of five viruses from sugarcane using in vitro culture of axillary buds and apical meristems. Plant Cell, Tissue and Organ Culture 109:439–445

Hamza TA, Alebjo AL (2017) Sugarcane Saccharum officinarum L tissue culture in Ethiopia opportunities For Ethiopias sugar industries. International Journal of Scientific & Technology Research 6:398–406

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

Kamski B (2016) The Kuraz Sugar Development Project (KSDP) in Ethiopia : between sweet visions and mounting challenges. Journal of Eastern African Studies 10:568–580

Khan SA, Rashid H, Chaudhary MF, Chaudhry Z, Afroz A (2008) Rapid micropropagation of three elite Sugarcane (Saccharum officinarum L.) varieties by shoot tip culture. African Journal of Biotechnology 7:2174–2180

Lu G, Wang Z, Xu F, Pan YB, Grisham MP, Xu L (2021) Sugarcane mosaic disease: characteristics, identification and control. Microorganisms 9:1–19

Mekonnen T, Diro M, Sharma M, Negi T (2014) Protocol optimization for in vitro mass propagation of two sugarcane (Saccharum officinarum L) clones grown in Ethiopia. African Journal of Biotechnology 13:1358–1368

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15:473–497

Nawaz M, Ullah I, Iqbal N, Iqbal MZ, Javed MA (2013) Improving in vitro leaf disk regeneration system of sugarcane (Saccharum officinarum) with concurrent shoot/root induction from somatic embryos. Turkish Journal of Biology 37:726–732

Pathak S, Lal M, Tiwari AK, Sharma ML (2009) Effect of growth regulators on in vitro multiplication and rooting of shoot cultures in sugarcane. Sugar Technologies 11:86–88

Ramgareeb S, Snyman SJ, van Antwerpen T, Rutherford RS (2010) Elimination of virus and rapid propagation of disease-free sugarcane (Saccharum spp. cultivar NCo376) using apical meristem culture. Plant Cell, Tissue and Organ Culture 100:175–181

Salokhe S (2021) Development of an efficient protocol for production of healthy sugarcane seed cane through Meristem culture. Journal of Agriculture and Food Research 4:100–126

Subba-Reddy CV, Sreenivasulu P (2011) Generation of Sugarcane streak mosaic virus-free sugarcane (Saccharum spp. hybrid) from infected plants by in vitro meristem tip culture. European Journal of Plant Pathology 130:597–604

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

Viswanathan R, Rao GP (2011) Disease scenario and management of major sugarcane diseases in India. Sugar Technologies 13:336–353

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.