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

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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 was done by polymerase chain reaction (PCR) using degenerate SCBV primers. Murashige and Skoog(MS) media supplemented with 0.5mg/l BAP + 0.25mg/l kinetin and 0.1mg/l GA_3 + 0.5mg/l NAA were the best hormone combination for shoot multiplication and root induction, respectively. Two

explant size categories (<1mm and 1-2mm) were used to assess the effect of explant size on shoot regeneration and virus elimination. The results showed that explant size significantly affects shoot regeneration. Smaller sized (<1mm) explants showed higher virus elimination efficiency, however the survival frequency of explants during initiation of shoot cultures was higher in larger (1-2mm) 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.

Key words: 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.

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 (1cm) 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 1-2mm 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 (0.25mg/l BAP + 0.1mg/l Kin, 0.5mg/l BAP + 0.25 Kin and 0.5mg/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 were evaluated four 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:

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

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 2mg/l) and Kin (0, 0.1, 0.25, 0.5mg/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 0.1mg/l GA_3 with different concentrations of NAA, IBA and IAA (0, 0.1, 0.25, 0.5mg/l). The cultures were transferred to fresh medium every two weeks and root induction was compared after four 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 five days of culturing. Multiple shoot development was observed within four weeks. Among the three hormone combinations (0.25mg/l BAP + 0.1mg/l Kin, 0.5mg/l BAP + 0.25 Kin and 0.5mg/l BAP alone) used for shoot initiation the MS media supplemented with 0.5mg/l BAP + 0.25mg/l Kin showed higher propensity for shoot regeneration (Fig.1).

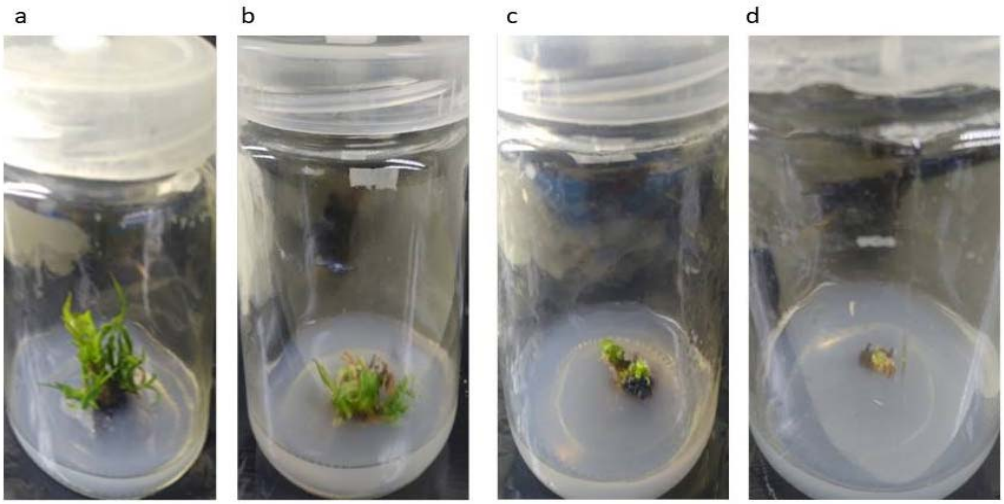

Fig. 1: Shoot regeneration of sugarcane from meristem tip after 4 weeks. a) culture on MS media supplemented with 0.5mg/l BAP + 0.25mg/l Kin b) culture on MS media supplemented with 0.5mg/l BAP alone c) culture on MS media supplemented with 0.25mg/l BAP + 0.1mg/l Kin d) culture on hormone free MS media

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

Table1. 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+0.1mg/lBAP + 0.1mg/l Kin	8	1.0±0.7
T3	MS+0.25mg/l BAP + 0.1mg/l Kin	8	1.9±0.6
T4	MS+0.5mg/l BAP	8	19.3±4.7
T5	MS+0.5mg/l BAP + 0.25mg/l Kin	8	28.5±4.3
T6	MS+0.5mg/l BAP + 0.5mg/l Kin	8	5.9±0.9
T7	MS+1 mg/l BAP + 0.25mg/l Kin	8	12.2±2.2
T8	MS+1.5mg/l BAP + 0.25mg/l Kin	8	9.0±2
T9	MS+2mg/l BAP + 0.25mg/l Kin	8	6.7±1.1

Out of the eight hormone combinations used for multiplication, T5 resulted in a higher number of shoots (28.5±4.27) per explant. T4 was the second-best medium for multiple shoot development and resulted in 19.33±4.67shoots 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 0.1mg/l to 0.5mg/l increased multiplication capacity of the explant and resulted in a higher number of shoots per explant. Whereas, an increase in BAP concentration beyond 0.5mg/l (1, 1.5 and 2mg/l BAP) while withholding kinetin at 0.25mg/l showed a considerable decline in the number of shoots per explant (from 28.5±4.27 in T5 to 6.7±1.1 in T9). Similarly, keeping the concentration of BAP at 0.5 mg/l while increase the concentration of kinetin from 0.25 to 0.5mg/l considerably reduced the number of shoots obtained per explant from 28.5±4.27 in T5 to 5.87±0.9 in T6.

Nine hormone combinations with one hormone free control were tested for root induction; the result presented in Table 2. Out of these rooting media tested, best rooting was observed on R10 resulted higher number of roots (9.25±2.26). At this hormone concentration, 75% of the shoots formed root and root length per plant was also higher (5.5±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+ 0.1mg/l GA_3 + 0.1mg/l IAA	8	3.9±0.7	3.3±0.5
R3	MS+0.1mg/l GA_3 + 0.25mg/l IAA	8	4.8±0.9	2.9±0.5
R4	MS+0.1mg/l GA_3 + 0.5mg/l IAA	8	4.0±1.1	3.1±0.5
R5	MS+0.1mg/l GA_3 + 0.1mg/l IBA	8	5.6±2	3.3±0.8
R6	MS+0.1mg/l GA_3 + 0.25mg/l IBA	8	7.8±2.4	3.6±1.6
R7	MS+0.1mg/l GA_3 + 0.5mg/l IBA	8	5.6±1.9	3.4±0.8
R8	MS+0.1mg/l GA_3 + 0.1 mg/l NAA	8	5.8±2.1	4.3±2.1
R9	MS+0.1mg/l GA_3 + 0.25mg/l NAA	8	6.1±1	3.8±1.5
R10	MS+0.1mg/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 0.1mg/l GA_3 and 0.25 mg/l IBA was 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 one month. The survival percentage of rooted shoots was 85% and hardened as shown in Fig. 2b.

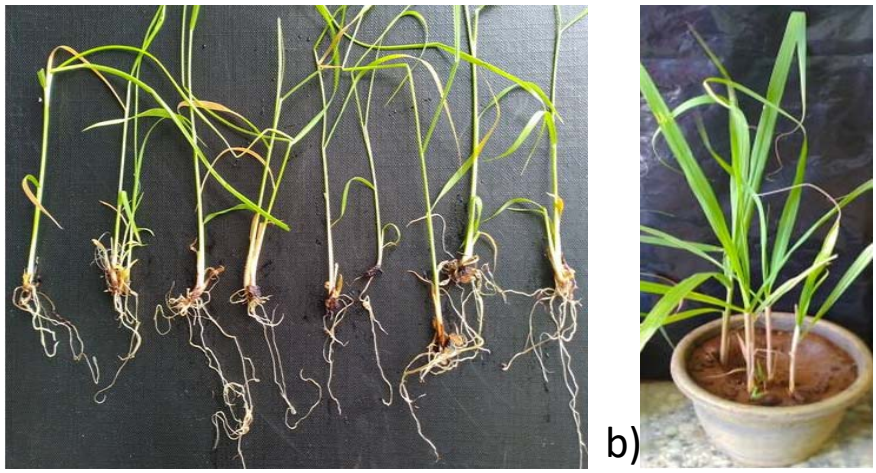

Fig. 2. Rooting and hardening of tissue cultured-derived plantlets of sugarcane: a) *in vitro* rooted shoots b) survived shoots in the greenhouse after two month

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 <1mm only 35.7% (10/28) of the explants develops into shoots; while 64.3% (18/28) of larger explants (1-2mm) showed shoot regeneration. PCR amplification was performed before and after

tissue culture treatment. All of the PCR amplifications done on samples before tissue culture treatment yielded the expected amplicon size of 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 six months of growing in the glasshouse revealed 16.7% and 30% virus free plants from large 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. These results 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 (3mg/l BAP alone and 3mg/l BAP + 1.5mg/l Kin). In contrast, Ramgareeb et al (2010) reported maximum shoot multiplication on MS medium with a low concentration of hormones (0.1mg/l BAP + 0.015mg/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 0.1mg/l GA_3 + 0.5mg/l NAA and 0.1mg/l GA_3 + 0.25mg/l IBA was 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 were 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 1-2mm and <1mm 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.

Author contribution

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.

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Data Availability Statement

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

Conflict of interest

The authors declare no conflict of interests

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