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Generation of *Sugarcane streak mosaic virus*-free sugarcane (*Saccharum* spp. hybrid) from infected plants by *in vitro* meristem tip culture

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Abstract *Sugarcane streak mosaic virus* (SStMV), an unassigned member of the family *Potyviridae*, is an emerging cause of sugarcane mosaic disease in Asia. SStMV-free sugarcane was generated from infected plants by *in vitro* meristem tip culture technology. Meristem-tip explants regenerated satisfactorily when treated with a solution of anti-necrotic compounds (combination of ascorbic acid, cystine hydrochloride and silver nitrate) before culture initiation. None of the plants that generated from meristem tips showed visible mosaic symptoms even after 12 months of cultivation. When indexed by bioassay on *Sorghum bicolor* cv. Rio and direct antigen coating-ELISA (DAC-ELISA), all of the regenerated plants were negative for SStMV infection. When tested by immunocapture-reverse transcription-PCR (IC-RT-PCR), ~92% of the micropropagated plants were SStMV-free and the remaining plants found to be positive for SStMV infections. This confirms the higher sensitivity of IC-RT-PCR over bioassay and DAC-ELISA. *In vitro* meristem tip culture in combination with sensitive molecular detection tests appears to be a rapid and reliable approach for generation of SStMV-free sugarcane planting material from infected sugarcane germplasm.

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Introduction

Mosaic is one of the most widely distributed virus diseases of sugarcane and is a serious problem in many sugarcane-growing countries of the world. The mosaic disease causes devastating economic losses to the sugar industry and is involved in the decline of important commercial clones in several countries. Estimated losses from the disease vary greatly, yield losses of 30–40% are commonly reported and sometimes 60–80%. The incidence of mosaic disease in commercial sugarcane fields appears to be almost 100% in some varieties of this crop growing in States of India (reviewed in Hema et al. 2008).

Around the world, *Sugarcane mosaic virus* (SCMV), a definitive species in the genus *Potyvirus*, family *Potyviridae,* is frequently the causative agent of the mosaic disease. However in India and several other Asian countries, *Sugarcane streak mosaic virus* (SStMV), a distinct unassigned species in the family *Potyviridae,* is a major cause of mosaic disease (Hema et al. 2008). In sugarcane growing States of India, SStMV is more prevalent than SCMV (Rao et al. 2004; Viswanathan et al. 2008).

Until recently, SStMV was only reported from India and a few other Asian countries (Hema et al. 2008). Countries wishing to import sugarcane varie-

ties from SStMV prevalent regions are concerned about the accidental introduction of SStMV through infected sugarcane planting material. Quarantine procedures for sugarcane viruses involve growing plants for up to 2 years and disease screening by regular visual inspections as well as by various other techniques (Saumtally et al. 1995). If the plant material is found to be infected, it is destroyed, hence loss of valuable sugarcane planting material with superior agronomic traits. Moreover, sugarcane plants are maintained year after year for hybridization and breeding purposes (Balamuralikrishnan et al. 2003). Therefore, virus build up is very fast over the period and drastically reduces the crop stand and vigour during subsequent years. Consequently, there is a need for rapid and reliable method for the elimination of SStMV from infected sugarcane.

Various tissue culture methods have been developed for virus elimination from infected plants (Faccioli and Marani 1998). Meristem tip culture is widely used for the generation of virus-free plant material in many plant species propagated mainly or exclusively by vegetative means (Hull 2002). A virus associated with chlorotic streak disease and SCMV were eliminated from infected sugarcane using apical meristem tip culture (Hendre et al. 1975; Dean 1982). Successful eliminations of SCMV and *Fiji disease virus* (FDV), a type species of the genus *Fijivirus* of the family *Reoviridae,* were reported in sugarcane through apical meristem tip or axillary bud culture (Leu 1972; Wagih et al. 1995). The elimination of *Sugarcane yellow leaf virus* (SCYLV) using tissue culture was also reported (Chatenet et al. 2001; Fitch et al. 2001; Parmessur et al. 2002).

Several methods are available to index *in vitro* generated plant material to ascertain the elimination of virus. Enzyme-linked immunosorbent assay (ELISA) is currently the main diagnostic tool for large-scale testing of plant materials for viruses (Martin 1998). However, virus detection with *in vitro* generated planting material requires more reliable and sensitive methods. Reverse transcriptase-polymerase chain reaction (RT-PCR) is suitable for detecting RNA viruses in very small tissue samples with extremely low virus titre, as found in meristem-tip culture (Dovas et al. 2001). RT-PCR, using immuno-captured viral particles (IC-RT-PCR), eliminates the need for isolation of RNA and is more sensitive than conventional RT-PCR in the detection of SStMV (Hema et al. 2008; Viswanathan et al. 2010).

The importance of the SStMV elimination procedure is that it would provide virus-free plants in order to investigate yield loss and virus transmission, eliminate the virus from the existing infected germplasm, to avoid the introduction of new virus/strains from imported varieties. A literature survey revealed that no promising method was reported for elimination of SStMV and indexation of virus-free sugarcane planting material using sensitive detection tests. The objective of the present study was to generate SStMV-free sugarcane by meristem tip culture and to index generated plants by bioassay, DAC-ELISA and IC-RT-PCR.

Materials and methods

Plant material

Mosaic disease affected sugarcane (*Saccharum* spp. hybrid) var CoV 94102 was collected from local commercial fields, and tested by DAC-ELISA and IC-RT-PCR to detect the presence of SStMV. Apical stem portions of about 5 cm in length containing apical meristems were collected from SStMV positive plants. The tops were rinsed for 5 min with 0.1% Tween-20 followed by 70% ethanol for 10 min. The explants were finally soaked in 5 g l^{-1} mercuric chloride solution for 10 min and rinsed thrice with sterile distilled water. The outer whorls of leaves were removed from apical stem portions until the meristem tip with one or two leaf primordia was reached (Fig. 1a). Meristem tips (~1 mm) were excised with a sterile blade and soaked in a sterile solution of anti-necrotic compounds: ascorbic acid (Sigma), 15 mg l^{-1}; cystine hydrochloride (Sigma), 40 mg l^{-1} and silver nitrate (Sigma), 2 mg l^{-1}. Finally, the explants were washed with sterile distilled water under aseptic conditions and used for culture initiation.

Media, culture conditions and meristem tip culture

All the culture media were based on Murashige and Skoog (1962) with appropriate modifications. All the media components were prepared in autoclaved double distilled water and stored in aliquots at −20°C.

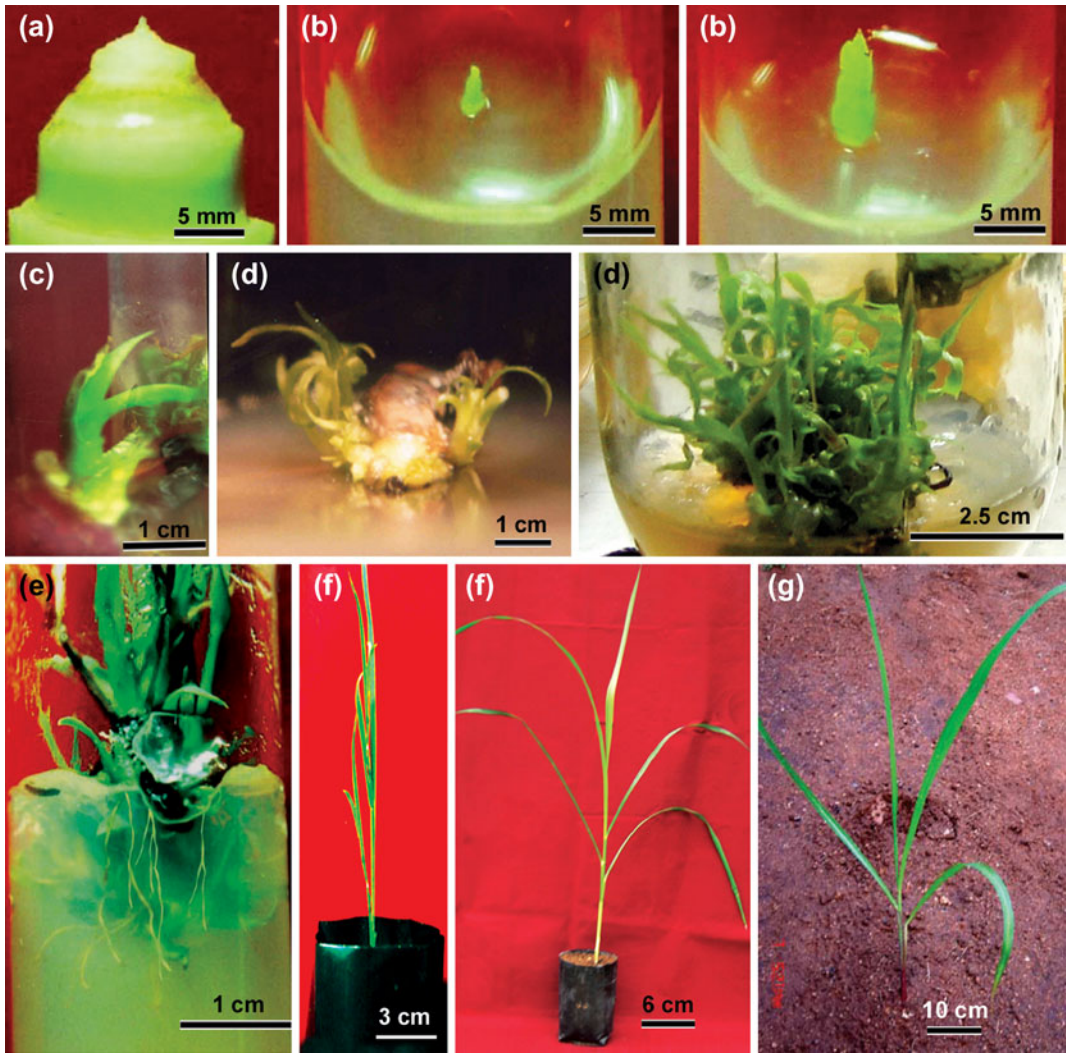

Fig. 1 Stages of *in vitro* meristem tip culture for generation of SStMV-free sugarcane. **a** apical meristem; **b** shoot emerged from excised meristem surrounded by leaf primordia; **c** shoot grown from meristem; **d** growth of multiple shoots; **e** rooted individual shoots; **f** rooted plantlets transplanted into soil in polythene bags; **g** rooted plantlets transplanted into experimental plot

Explants were maintained in a growth chamber (MB Instruments, Mumbai, India) at 25±1°C with a 16 h photoperiod provided by Philips 20 W cool white fluorescent lamps.

Meristem tips were initially cultured on semi-solid shoot regeneration medium (SRM) which consisted of Murashige and Skoog (MS) salts (Murashige and Skoog 1962) supplemented with myoinositol 100 mg l^{-1}, thiamine-HCl 0.5 mg l^{-1}, benzyl amino purine (BAP) 3 mg l^{-1}, sucrose 2% (w/v) and 0.8% (w/v) agar. The pH was adjusted to 5.8 before autoclaving at 121°C for 15 min. Meristem tip explants were aseptically placed on SRM and incubated under the above tissue culture conditions.

The regenerated shoot was transferred onto semi-solid shoot multiplication medium (SMM) [MS salts supplemented with myoinositol 100 mg l^{-1}, thiamine-HCl 0.5 mg l^{-1}, BAP 2 mg l^{-1}, kinetin (KIN) 1 mg l^{-1}, naphthalene acetic acid (NAA) 0.5 mg l^{-1}, sucrose 2% (w/v) and 0.8% (w/v) agar] for formation of multiple shoots. Subculturing was done at 15-day intervals.

Individual shoots from these subcultures (>5 cm in length) were transferred to semi-solid root induction medium (RIM) [1/2 strength MS salts supplemented with myoinositol 100 mg l^{-1}, thiamine-HCl 0.25 mg l^{-1}, NAA 5 mg l^{-1}, sucrose 2% (w/v) and 0.8% (w/v) agar] for rooting, and the rooted plantlets were transferred into liquid root elongation medium (REM) [1/2 strength MS salts supplemented with myoinositol 100 mg l^{-1}, thiamine-HCl 0.25 mg l^{-1}, NAA 0.5 mg l^{-1} and sucrose 2% (w/v)] for further root elongation.

Plantlets with roots >4 cm long were removed from the medium and the roots were thoroughly washed in running tap water for several minutes to remove residues of medium and planted in autoclaved potting mixture (2 parts of red soil, 1 part of fine sand and 1 part of farm-yard manure) in black polythene bags. The plantlets were placed for 2 weeks in the plant growth chamber for acclimatization and the potting mix was regularly wetted with sterile ¼ strength MS salts. They were then transferred to a wire-mesh house and grown for 6 weeks. Finally, the plantlets were transplanted into the field soil drenched with a commercial fungicide (Carbendizim) and watered regularly.

Virus detection in sugarcane

The acclimatized sugarcane plants (SC-1 to SC-64) were indexed by performing bioassay, DAC-ELISA and IC-RT-PCR. The plants were tested after 1 month of transplantation into plots and again after 6 months. Young fully expanded leaves were sampled for virus detection, as these leaves displayed more severe symptoms than older leaves.

Bioassay Leaf samples from meristem tip culture generated sugarcane plants were separately macerated at 1:10 (w/v) dilution in chilled 10 mmol potassium phosphate buffer, pH 7.0 containing 0.2% 2-mercaptoethanol and used for mechanical inoculation of *Sorghum bicolor* (cv Rio) seedlings at 3 to 4 leaf stage (~14 days). Each plant inoculum was separately inoculated onto not less than 20 seedlings and appropriate positive and healthy (var. Co6907) controls were also included.

DAC-ELISA DAC-ELISA was performed as described by Clark and Bar-Joseph (1984). Sugarcane leaf extracts were prepared by grinding in carbonate buffer, pH 9.6 at 1:10 (w/v) dilution and used as an antigen source. SStMV polyclonal antiserum raised against recombinant coat protein (rCP) (Hema et al. 2003) was used at 1:10,000 dilution (v/v). Goat ant rabbit antibodies-ALP (alkaline phosphatase) conjugate at 1:2000 dilution (v/v) (Bangalore Genei, Bangalore, India) was used as a secondary antibody, and 0.05% paranitrophenyl phosphate (pNPP; Sigma, USA) in diethanolamine buffer (pH 9.8) was used as a substrate. Appropriate positive, healthy (variety Co6907 collected from a local Agricultural research station and confirmed both by DAC-ELISA and IC-RT-PCR as SStMV-free) and buffer controls were also included. Absorbance values were recorded at 405 nm in microplate reader (Model 680, Bio-Rad, USA). Test samples with absorbance values greater than two times the mean of healthy control were considered as positive.

IC-RT-PCR SStMV-AP rCP polyclonal antiserum (Hema et al. 2003), diluted in carbonate buffer, pH 9.6 (1:500) was added (50 µl) to nuclease-free 0.2 ml polypropylene tubes and incubated at 37°C for 2 h. The tubes were washed thrice with PBS-T (phosphate buffered saline containing 0.05% tween-20) and finally once with PBS. *In vitro* generated sugarcane leaf samples, healthy (var Co6907) and positive samples were separately macerated in extraction buffer [500 mM Tris-HCl, pH 8.3, containing 2% polyvinyl pyrollidone-40 (PVP-40), 1% polyethylene glycol-6000, 140 mM NaCl and 0.05% Tween-20] at 1:10 (w/v) dilution (Hema et al. 2003) and the extracts were used as a template. An oligo 5′-d $(T)_{18}$(AGC)-3′ was used as reverse primer, and a forward primer (5′-AAGTGGTTAAACGCCTGTGG-3′) amplifying ~1,400 bp fragment of 3′ terminus of SStMV genome was designed using Primer3 online tool (http://frodo.wi.mit.edu/primer3/) based on SStMV sequences deposited in the GenBank. To the tubes with immunocaptured virions, 20 pmol of reverse primer and RNase-free water were added to a 25 µl final volume. The tubes were heated at 80°C for 10 min and chilled on ice. To each tube, 8 µl of 5 X M-MuLV reverse transcriptase buffer, 2 µl of 25 mM $MgCl_2$, 2 µl of 10 mM dNTPs mixture, 10 U of RiboLock™ RNase inhibitor (Fermentas, Burlington, Ontario, Canada) and 200 U of M-MuLV reverse transcriptase (Fermentas) were added and the final volume was made up to 40 µl with RNase-free water. Reverse transcription was carried at 42°C for 60 min. The PCR mix was prepared by adding 2.5 µl

of 10 X PCR buffer II (Applied Biosystems, Carlsbad, CA, USA), 2.5 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTPs mixture, each 10 pmol of reverse and forward primers, 1 U of AmpliTaqGold DNA polymerase (Applied Biosystems) and final volume was made up to 20 µl with nuclease-free water. To each tube, 5 µl of reverse transcription product was added and PCR was performed: 95°C pre-PCR incubation for 5 min; 35 cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 90 s and post-PCR extension at 72°C for 10 min. The amplified products were analysed by 1% agarose gel electrophoresis (Sambrook and Russel 2001).

Results

Meristem tip culture

Microbial contamination of explants in culture ranged from 10 to 15%. The highest number of explants (>80%) were regenerated after treatment with a solution of anti-necrotic compounds immediately after excision.

Of several media tested, SRM showed highest propensity for shoot regeneration. After about 7 days, the shoot started to emerge from the apical meristem tip explant (Fig. 1b) and the meristem tip grew 1–2 cm in 3 weeks (Fig. 1c). The expanded shoots produced from meristem-tip explants formed 6–14 multiple shoots on SMM within 8 weeks (Fig. 1d). Individual shoots rooted readily on RIM medium at a rate of nearly 80% within 2 weeks (Fig. 1e) and the length of the roots was enhanced by growing in REM for 4 more weeks. About 72% of the rooted plantlets readily acclimatized after transplantation to soil (Fig. 1f and g).

Virus detection

The established sugarcane plantlets were regularly inspected and none of them showed visible mosaic symptoms even after 12 months of cultivation. In bioassay on sorghum performed after 1 month of planting into the plots, none of the total 64 plantlets was positive for SStMV infection, except those plants inoculated with the positive control (Fig. 2). None of the 64 regenerated plants was positive for SStMV in DAC-ELISA test also. Absorbance values obtained were: test samples 0.14–0.41, healthy control 0.28–0.31, buffer control 0.13–0.17 and positive control 1.78–1.96. When tested by IC-RT-PCR, no amplification was observed in 59 of the regenerated sugarcane plants (~92%), the expected size fragment (~1,400 bp) was observed in the remaining five plants (SC-12, SC-25, SC-29, SC-43 and SC-52; ~8%) and also in the positive control, indicating the presence of SStMV (Fig. 3). When indexed after 6 months of planting using the same three tests, similar results were observed and there were no new infections.

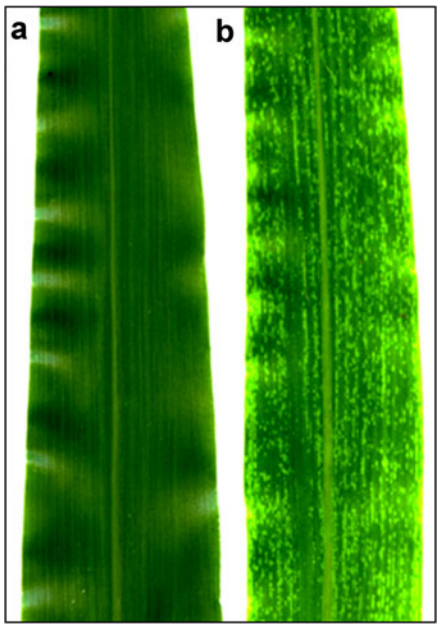

Fig. 2 Bioassay on sorghum for detection of SStMV in tissue culture generated sugarcane. **a** healthy leaf; **b** virus infected leaf

Discussion

Virus-free planting material can be generated by using various tissue culture methods. Tissue culture of sugarcane can be performed using either meristem tip culture or by somatic embryogenesis from callus (Lee 1987). Meristem tip culture is a viable, rapid and reliable method of virus elimination in plants. This technique takes an advantage of the fact that some viruses are unable to invade this region because of inhibition of replication and/or inability of the virus to keep up with the pace of rapidly dividing cells at the meristem tip (Faccioli and Marani 1998). Hence, the chance of virus elimination is greater and produces plants phenotypically very similar to the original plant.

The concentration of virus in the explant increases with increasing distance from meristem tip. The chance

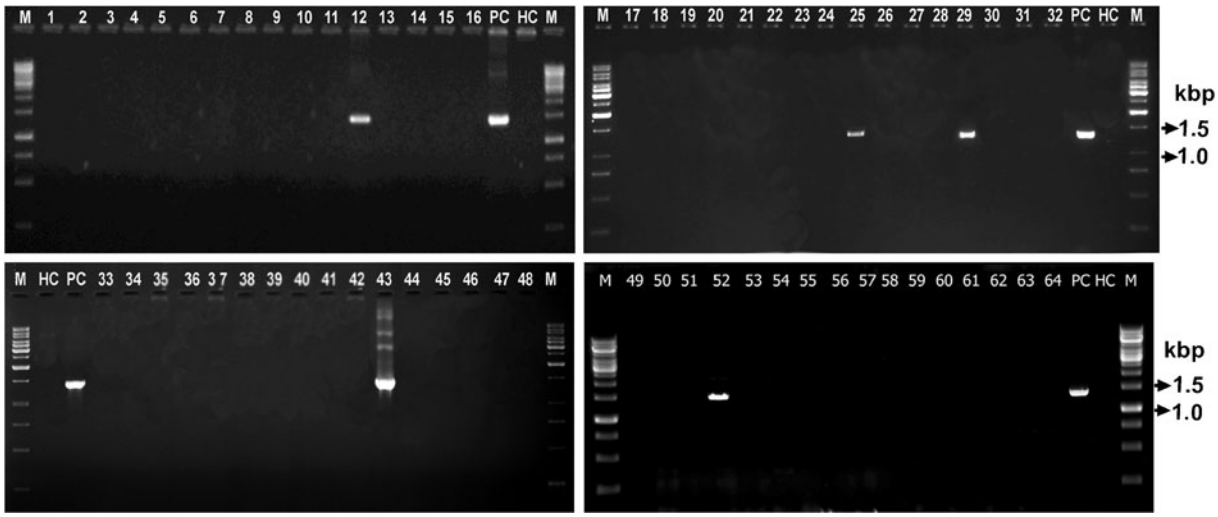

Fig. 3 IC-RT-PCR screening of sugarcane plants generated by meristem tip culture (1–64). M, 1 kb DNA ladder (Fermentas); HC, Healthy control (var. Co 6907) and PC, SStMV positive control

of virus elimination increases with decreasing meristem size and, at the same time, the regeneration capacity decreases with the decreasing size of the meristems (Faccioli and Marani 1998). Large meristem tips (>2 mm) are likely to be infected whereas smaller ones (<0.5 mm) are unlikely to regenerate. Leu (1972) studied the elimination of SCMV by cultivation of up to 3–4 mm shoot tips of sugarcane. In the present study, meristem tips of ~1 mm size regenerated satisfactorily and produced SStMV-free sugarcane plants.

Initially, the majority of explants (>70%) turned brown and failed to grow, subsequently leading to death. To enhance the regeneration of sugarcane meristematic explants, the effect of combinations of anti-necrotic compounds was assessed. The best results were obtained when using a combination of ascorbic acid, cystine hydrochloride and silver nitrate. After excision, meristem tips of sugarcane excrete high levels of polyphenols from cut ends into the medium, which when oxidized are detrimental to the explants and hamper regeneration due to necrosis of tissues. The use of anti-necrotic compounds with anti-oxidative activity reduced the activity of polyphenols (Enriquez-Obregon et al. 1997; Garcia et al. 2007).

Attempts have previously been made to eliminate viruses from infected sugarcane tissues. For example, apical meristem tip culture eliminated chlorotic streak disease from infected sugarcane (Dean 1982). In India, an earlier attempt was made to generate chlorotic streak disease-free sugarcane from apical meristems, but virus infection and elimination were assessed merely based on presence or absence of visual symptoms and no sensitive detection tool was used to index the regenerated plants (Hendre et al. 1975). Sometimes symptomless diseased plants carry virus. Despite its widespread occurrence in India and several other Asian countries, no reliable methods for elimination and indexation of SStMV have yet been developed. In the present study, sugarcane apical meristem tips regenerated more efficiently and rapidly as compared to axillary bud meristem tips (data not shown). Hence, for generation of virus-free plants, apical meristem tips are more suitable explants than axillary bud meristem tips.

It is necessary to use highly specific, sensitive and rapid detection methods to determine whether virus is completely or partially eliminated from sugarcane plants generated through *in vitro* meristem tip culture. In the present study, none of the meristem tip culture-generated sugarcane plants was shown to be positive for virus infection by bioassay. The sensitivity of bioassay is less and not suitable to simultaneously screen large number of samples (Hema et al. 2008). The bioassay takes >30 days for assessment of results and several abiotic and biotic factors like different climatic conditions, and host cultivars used in the assay can profoundly influence the results (Shukla and Ward 1989).

DAC-ELISA is a widely used diagnostic test for large-scale screening and routine detection of plant

viruses (Martin 1998). All the regenerated sugarcane plants tested by DAC-ELISA were also found to be SStMV-free. The sensitivity of DAC-ELISA is more or less similar to bioassay and lacks the sensitivity required for detection of viruses that occur in very low concentration in tissues.

In the present study, IC-RT-PCR detected SStMV in ~8% of the total plants that were initially negative for virus when tested by bioassay and DAC-ELISA. This confirmed the higher sensitivity of IC-RT-PCR over bioassay and DAC-ELISA. RT-PCR has been shown to be very useful for detecting and characterizing RNA viruses and is more sensitive than serological techniques (Candresse et al. 1998; Martin 1998). RT-PCR using immunocaptured viral particles (IC-RT-PCR) can detect very low titres of virus and is suitable for detection of viruses in very small plant tissue samples with extremely high sensitivity (Dovas et al. 2001). It was reported that IC-RT-PCR is more sensitive than conventional RT-PCR in the detection of SStMV (Hema et al. 2008). IC-RT-PCR allows detection of viruses in oxidizing plant extracts as interfering plant constituents are washed off from immobilized virions and it eliminates the need for isolation of RNA, which is inconvenient for processing of large numbers of samples. Recently multiplex PCR was developed for simultaneous detection of three viruses including SStMV in sugarcane (Viswanathan et al. 2010), and which could be useful to index the tissue culture generated sugarcane.

In vitro meristem tip culture, in combination with sensitive molecular detection techniques, was successfully applied as a rapid and reliable method to generate SStMV-free planting material, and which can be applied both commercially and to germplasm.

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