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Molecular Characterization and Diagnostics for Effective Management of Phytoplasma and Viral Diseases in Sugarcane

G Vamsi Krishna, V Manoj Kumar, P Kishore Varma, B Bhavani, G Vijaya Kumar and V Prasanna Kumari

Department of Plant Pathology, Agricultural College, ANGRAU, Bapatla, Andhra Pradesh

ABSTRACT

Sugarcane is the third largest crop in terms of value next to rice and wheat in India. Vegetative propagation favours carryover of different pathogens through seed canes leads to spread of the diseases in the main field. Increasing virus and phytoplasma titre in the canes severely affects cane growth and varietal degeneration in elite varieties. This severely affect sugarcane cultivation in Andhra Pradesh and in the country. Hence, healthy seed combined with molecular diagnostics become the starting point for disease management in sugarcane. The advancements in molecular diagnosis and detection techniques have paved a way for efficiently discriminating of the virus infected seedlings benefit the sugar industry and agricultural research stations in supplying virus-free planting materials through tissue culture techniques. Now it is very well established that tissue culture derived planting materials are free from the viruses and exhibit varietal vigour. In this context, molecular characterization of viruses causing diseases like mosaic by *Sugarcane mosaic virus* (SCMV), *Sugarcane streak mosaic virus* (SCSMV) and *Maize yellow mosaic virus* (MaYMV), leaf fleck by *Sugarcane bacilliform virus* (SCBV), yellow leaf by *Sugarcane yellow leaf virus* (SCYLV) and grassy shoot by *Phytoplasma* was done based on partial genome analyses. Characterization of the viruses into new genus, species, strains and genotypes aided in developing molecular tools especially PCR, RT-PCR assays, and recombinant antisera for the viruses. Andhra Pradesh context, for the first time, all the viruses were characterized based on partial genomes.

Key Words: *Diagnosis, Molecular Characterization, Phytoplasma, Sugarcane and Viruses*

Sugarcane is a perennial grass belonging to the family Poaceae, of the genus *Saccharum*. It is an important field crop grown for sugar and bioenergy in more than 110 countries around the world under varied agroclimatic conditions. Globally, sugarcane is cultivated in about 26.34 M ha^{-1} with a production of 1859.39 million tons. Around 80 % of the world sugar requirement is met by this crop, and it has predominantly occupied Asian farmlands since prehistoric times. Globally, India, Brazil, China, Thailand, Pakistan, Mexico, Colombia, Indonesia, Philippines, and the United States are the major sugarcane producers; among them, India is the second largest producer of sugarcane in the world and accounts for about 20 % of world's cane production covering 5.15 M ha^{-1} with a production of 405.39 Mt (FAOSTAT, 2021). Moreover, the Asian continent is considered as "sugarcane hub" in the world as it has the richest genetic diversity of sugarcane, with Southeast Asia being the origin of the crop. The currently cultivated sugarcane varieties are interspecific hybrids with high sugar content. The crop gains economic importance by virtue of its industrial potential in terms of products like crystal white sugar, bagasse, ethanol, power, and press mud. This crop has been considered as energy cane or biofuel crop in the recent years (Matsuoka *et al*., 2014). Such a potential crop is being affected by several fungal, bacterial, viral, and phytoplasmal diseases (Rott *et al*., 2000; Viswanathan and Rao, 2011). Among them, viral and phytoplasma diseases are emerging as a major threat to sugarcane cultivation due to varietal degeneration, and this affects the performance of popular varieties under field conditions (Viswanathan, 2016). Mosaic, leaf fleck, yellow leaf and grassy shoot diseases have been reported in major sugarcane-

growing regions of India, and are considered as important as their incidence is increasing at an alarming rate and often resulting in a significant reduction in cane yield and juice quality (Viswanathan *et al*., 2018). In India, sugarcane is mainly grown in Uttar Pradesh, Maharashtra, Andhra Pradesh, Karnataka, Tamil Nadu, Gujarat, Bihar, Haryana and Punjab. In Andhra Pradesh, the black alluvial soils are suitable for sugarcane cultivation. The districts around Krishna and Godavari benefit from these rivers and produce sugarcane in bulk quantities, where the crop occupies an area of 0.055 lakh hectares with a production of 41.35 lakh tonnes and productivity of 75.24 tonness (Annonymous, 2021).

Sugarcane mosaic was first reported in India from Pusa in 1921 on sugarcane variety D 99 (imported from the USA) and Sathi 131, an indigenous cane of Bihar (Dastur 1923). Since then, virus isolates causing mosaic disease on sugarcane in India have continued to be a potential threat to the sugarcane industry as it is a very common disease because of the virus perpetuation through vegetative cuttings where *Sugarcane mosaic virus* (SCMV)is the pathogenic agent (Bhargava, 1975). Later, it was proved that sugarcane mosaic disease in India was due to SCMV and *Sugarcane streak mosaic virus* (SCSMV) either alone or together (Viswanathan *et al*., 2007). Mosaic disease is so destructive, causes yield losses up to 10–90% in India (Rao *et al*., 1998; Singh, 2001). Yellow leaf disease (YLD) in sugarcane characterized by a yellowing of the midrib and lamina occurred in most of the sugarcane growing regions of the country and the disease intensity was recorded up to 100 per cent in certain susceptible varieties (Rao *et al*. 2000, Viswanathan 2002). *Sugarcane yellow leaf virus* (SCYLV) has been identified as the causative virus of the disease yellow leaf, which spreads through vegetative cuttings and causes yield losses up to 100 % (Singh and Rao 2011). Leaf fleck incited by *Sugarcane bacilliform virus* (SCBV) exhibits pronounced flecks, chlorotic stripes, mottling and stunted growth in the affected genotypes and impacts the exchange of sugarcane germplasm and production globally which results in the protection of the sugarcane industry by imposing quarantine measures (Krishna *et al*., 2023). Recently *Maize yellow mosaic virus* (MaYMV) was reported in the field-grown maize crop showing mosaic and foliar yellowing symptoms in Yunnan provinces of China during 2016 by deep sequencing platform of small RNAs and Sanger sequencing of RT-PCR samples (Chen *et al*., 2016). In India, MaYMV was first reported in sugarcane in the year 2021 (Nithya *et al*., 2021). Grassy shoot, the phytoplasma disease is incited by sugarcane grassy shoot (SCGS) phytoplasma is a vector-borne and graft-transmissible bacteria mainly transmitted through vegetative propagation material and phloem-feeding leafhoppers (Srivastava *et al*. 2006); which is a major production constraint in sugarcane cultivation and reported to cause 5 % to 70 % yield reduction in plant crop and incidence increased to multifold for about 100 % in case of ratoon crops. (Gogoi *et al*., 2018). In case of severe incidence it completely affects the total number of millable cane production and the affected clumps produces numerous number of chlorotic thin tillers from the base of affected plant which gives the plant a typical grassy appearance. By this, the economically important cane stalks are lost, leading to heavy losses in cane yield under field conditions.

Standardization of diagnostic techniques especially at the molecular level is essential while monitoring the exchange of germplasm materials to contain the entry of the virus and newer viral strains into a disease free area. The international exchange of the sugarcane germplasm for breeding and commercial production has played a major role in the development of sugar industry worldwide. Reports of asymptomatic plants containing virus are of considerable concern in quarantine operations, which rely on expression of symptoms for the diagnosis of disease. All the viral pathogenic agents and phytoplasma are of concern during the movement and quarantine of sugarcane germplasm into and within the country. These viruses poses the most serious challenges in obtaining disease free seed cane and for germplasm movement. Hence, standardization of reliable and economical molecular diagnostic techniques is very much required to monitor and to prevent the movement of virus within the country and also between the countries.

MATERIAL AND METHODS

Leaf samples from 25 varieties exhibiting varying degrees of mosaic symptoms, mid rib yellowing symptoms, leaf fleck and grassy shoot disease affected samples were collected at Regional Agricultural Research station, Anakapalle. Samples

collected from each field were labelled and preserved in polythene covers, stored at -86 °C and brought to the laboratory for virus and phytoplasma diagnosis.

Serological detection

DAS-ELISA (Double antibody sandwich-enzyme linked immunosorbent assay) according to Clark and Adams (1977) was followed for the detection of SCMV and SCBV in the infected samples. The polyclonal antiserum was supplied by Nano diagnostics, USA. Reagents were prepared as per manufactures protocol. 100 µl of primary antiserum (polyclonal) was added to each well and incubated at 4 °C or at room temperature (21-24 °C) for 4 hours. SCMV and SCBV infected and healthy leaves from sugarcane were taken (preferably youngest exposed leaves) powdered with liquid nitrogen and extracted in sample extraction buffer 1:10 (pH 8.0) followed by washing the plates by filling the wells with PBST buffer then quickly emptied them again repeatedly 4 to 6 times. 100 µl of positive, negative and healthy samples were dispensed in to each well followed by incubation at room temperature for 2.5 hours. 100 µl of secondary antiserum, conjugate antibody, was added to each well and incubated at 4 °C for 4 hours at room temperature (21-24 °C) followed by washing. 100 µl of enzyme substrate was added (p-nitrophenyl phosphate) to each well and incubated for 30 min at room temperature for colour development. Colour development was arrested by adding 50 µl of 3M NaOH or KOH. Colour development (yellow) indicates the presence of target protein (virus) in the suspected sample. Absorbance at 405nm was taken using ELISA reader and the readings were compared between healthy and infected samples. Tested samples were considered as positive when its absorbance values were two times the mean value of healthy control.

PCR assay

DNA isolation from grassy shoot and leaf fleck affected samples

Total DNA was extracted from the samples collected during the survey by following the CTAB technique (Doyle and Doyle. 1987). Leaf samples (each 100 mg), kept at -86 °C, were grounded to fine powder using liquid nitrogen in a mortar and pestle. CTAB buffer of about 1 ml was added to the finely powdered samples in a 1.5 ml sterile micro centrifuge tube. The samples were then incubated in water bath at 65 °C for 45 mins and allowed them to cool to room temperature followed by centrifugation at 4 °C for 10 minutes at 12,000 rpm. The supernatant was collected in a fresh micro centrifuge tube followed by addition of chloroform and isoamyl alcohol (24:1). Following a thorough mixing process, tubes were centrifuged at 4 °C for 10 min at 12,000 rpm. Three layers were formed *i.e*., upper aqueous phase, middle protein phase and lower organic phase, among the three upper aqueous phase which included DNA, was moved in to a fresh micro centrifuge tube followed by the addition of 0.6 volume of isopropanol and mixed by gentle inversion for 4-5 times. Later DNA was allowed to precipitate at 4 °C for 30 min. After that tubes were centrifuged for 10 minutes at 12000 rpm to form pellets. The pellet was then centrifuged at 4 °C for 10 minutes at 12,000 rpm with 75% ethanol. The pellet was retained and the supernatant discarded. The pellet was then allowed to dissolved in 40 µl of sterile distilled water after being air dried.

Nested PCR for phytoplasma

Based on concentration of genomic DNA it was diluted up to 100 ng/ul for the PCR analysis. The first round of PCR was performed using the universal phytoplasma specific primers P1/P7 (Deng and Hiruki, 1991) and second set of primer pairs (R16F2 52 -GAA ACG ACT GCT AAG ACT GG-32 ; R16R2 52 -TGA CGG GCG GTG TGT ACA AAC CCC G-32) were used in the nested PCR by diluting the first round PCR product in 1:10 dilutions (Gunderson and Lee, 1996) for the amplification of both large (23S), small (16S), and 16S-23S ribosomal spacer regions. The PCR reaction was performed in a total volume of 25 ìl containing, 2.5 ìl of 10× Taq buffer with 15 mM $MgCl_2$, 2 ìl of 2.5 mM dNTP mix, 10 ìM of each forward and reverse primers, 0.33 units of Taq polymerase (Origin), 1 ìl of genomic DNA and sterile MilliQ water to make up the final volume.

The PCR cyclic conditions for the P1/P7 primer was initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min 30 s, extension at 72°C for 1 min 30 s, and a final extension at 72°C for 15 min following that nested PCR was performed using the 1:10 dilution of P1/P7 product with the same cyclic conditions except the annealing temperature at 58°C for 1 min 30s.

PCR amplification for SCBV

The most conserved RT/RNase H domains and sequences have been widely employed in *Badnavirus* taxonomy and viral detection. Primers specific to SCBV were used in the PCR reaction to target the RT/RNase H coding region of the genome, which is a region of 794 bp in size. A total PCR reaction volume of 25 µl consisting of 2 µl of DNA, 2µl of Taq buffer, 2 µl of $Mgcl_{2,}$ 2 µl of dNTPs, 10 pmol of both forward and reverse primers, 1.25 units of Taq DNA polymerase (Genei, Bangalore), and sterile milliQ water makeup to final volume. Thermocycler (Mastercycler, Eppendorf, Germany) was used to carry out the PCR reaction (15). PCR conditions included initial denaturation at 95 °C for 5 min followed by 34 cycles of 95 °C for 1 min of denaturation, 59 °C for 45 seconds for annealing, 72 °C for 45 seconds for primer extension, and a final extension of 72 °C for 10 min followed by hold at 4 °C. The PCR products were separated by electrophoresis in a 1.5 % agarose gel stained with ethidium bromide at 100 volts for approximately 45 minutes in a 1X-Tris-Acetate-EDTA (TAE) buffer with a pH of 8. Gel images were documented using a gel documentation system (Vilber E: Box, UK).

RNA isolation and RT-PCR assay for SCMV, SCSMV, SCYLV and MaYMV

All the samples were processed for total RNA extraction and RT-PCR analysis. One hundred mg of fresh sample was powdered using liquid nitrogen and transferred to a 1.5 ml DEPC treated micro centrifuge tube and to that 1 ml of TRI reagent (Sigma, USA) was added. The tubes were vigorously shaken for homogenous mixing of TRI reagent with the sample and kept at 4°C until all the samples were homogenized. The samples were allowed to complete dissociation of nucleoprotein complexes and release of RNA by incubating at room temperature for 5 min. All the insoluble materials such as cellular membranes, high molecular weight DNA and polysaccharides were precipitated at the bottom of the tubes by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant containing the RNA was transferred to a fresh centrifuge tube and to that 200 ìl of the chloroform was added. The tubes were shaken vigorously for 15 sec and again incubated at room temperature for 5–10 min and later centrifuged at 12,000 rpm for 15 min at 4°C. After centrifugation, the mixture separated into three phases: a red organic phenolic phase contained protein, an inter-phase of DNA and a colourless upper aqueous phase which contained the RNA. The RNA containing upper aqueous phase was transferred into a fresh tube and added 500 ìl of isopropanol to precipitate the RNA. After incubation for 5 min at room temperature, the tubes were centrifuged at 12,000 rpm for 15 min at 4°C for pelletizing the RNA. The pellet was air dried for 10 min and dissolved the RNA with 40 ìl of molecular grade water and stored at "86°C (Chomczynski P and Sacchi 1987). The quality of the RNA was checked in 1.5% agarose gel.

cDNA synthesis (RT reaction)

All the reagents are placed on crushed ice, RT mixture was prepared by adding the following components in a sterile 0.2 ml PCR tubes.

Total RNA	2 µl
10 mM dNTPs	1µl
Oligonucleotide primer (SCMV & SCSMV) Reverse primer (MaYMV and SCYLV)	0.25µl
RT buffer	2 µl
RT enzyme	0.125µl
RNase inhibitor	0.25µl
Molecular grade water	4.375µl

The reaction mixture was incubated at 70 °C for 5 mins followed by inactivation of the enzyme at 37 °C for 90 mins, followed by termination of reaction at 72 °C for 10 mins. The partial sequence of the SCMV, SCSMV, SCYLV and MaYMV was amplified by RT-PCR with the below mentioned primers (Table 1).

RESULTS AND DISCUSSION

Detection of viruses in sugarcane

The globalisation of the sugar business has been significantly aided by the exchange of germplasm of sugarcane for commercial production and breeding and on a global scale. The early and sensitive detection of such pathogens is vital and necessary to decrease the danger of introducing new diseases or pathogen strains into sugarcane growing regions. Both serological and molecular methods were used in this investigation to identify the virus at the protein and nucleic acid levels (Fig 5).

Table.1 Synthetic oligonucleotide primers used for PCR amplification

Virus	Region	Synthetic oligonucleotide primers (5'---3')	Reference
SCMV	Coat protein	CCCGAAGCTTGCTGGAACAGTCGATGCAGG	Viswanathan *et al*. (2010)
		ATCGCGGCCGCTTAGCCAGCTGTGTGTCTCT	
SCSMV	Coat protein	GGATCCGGACAAGGAACGCAGCCAC	
		AGATCTCGCACGTCGATTTCTGCTGGTG	
SCYLV	Coat protein	ATGAATACGGGCGCTAACCGYYCAC	Viswanathan *et al*. (2014)
		GTGTTGGGGRAGCGTCGCYTCC	
MaYMV	Coat protein and Movement protein	CGCGCTCGCAATAATAACCG	Nithya *et al*. (2021)
		TTCTGATGAGTCGCGCCAAA	

Serological detection

The leaf samples exhibiting mosaic and leaf fleck symptoms were collected from RARS, Anakapalle and subjected to protein based detection *i.e.*, ELISA. Infected samples from different varieties *viz.*, 2009A 107, 87A 298, 2003V 46, Co 86032, 81V 48, 2017A 553, 2015A 311 and 2006A 223 were collected. Out of 25 samples tested, 7 samples were tested positive for protein based detection of SCMV with absorbance (405 nm) values ranging from 0.99 to 2.22, proving that serological test is successful at finding the virus in plant samples. Titre values (Absorbance values A_{405}) for healthy, positive and negative control were recorded as 0.30, 1.89 and 0.25 respectively. Samples tested for SCMV are also tested for SCBV, 13 samples were tested positive with absorbance values (405 nm) ranging from 0.62 to 1.95 Titre values (Absorbance values A_{405}) for healthy control was recorded as 0.25, where as for positive and negative control titre values were recorded as 2.78 and 0.16 respectively (Fig 1). Balamuralikrishnan and Viswanathan. (2005), reported that eight susceptible clones have shown positive for SCBV out of 11 clones. Similarly, Rao *et al*., 2003 reported that 81 mosaic infected samples have shown positive for SCMV, out of 101 samples tested. Among the varieties/genotypes of sugarcane, which were found to have positive reaction in DAS-ELISA for SCMV, ratoon crops of 2009A 107 and VCF 517 are having highest viral titre values of 2.20 and 1.21 respectively, while genotype 2017A 553 showed negative in reaction. In case of SCBV, Co 86032 and 2003V 46 are having highest titre values 1.98 and 1.67 respectively, while genotype 2017A 553 showed negative in reaction. The intensity of colour as well as titre values were more in case of infected samples collected from ratoon crop indicating the susceptive nature of crop as well as persistence of sett borne inoculum in the stubbles.

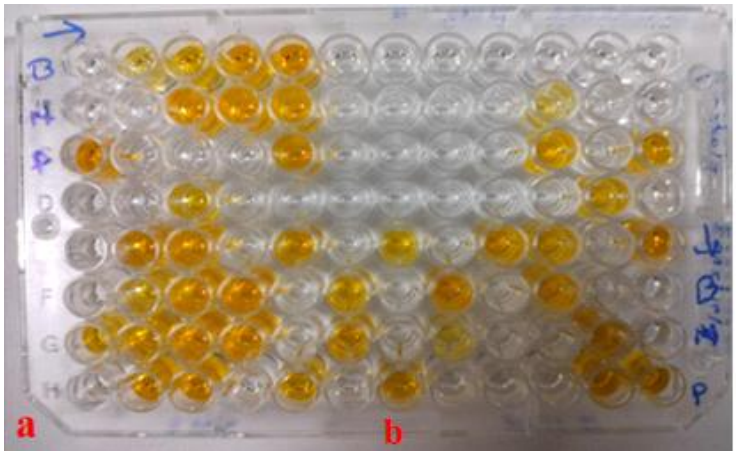

Fig.1. Micro titer plate showing yellow coloured wells indicating positive (405 nm) for a) SCBV b) SCMV infection from sugarcane samples (B: blank, N: Negative control; P: positive control) (Wells 1 to 48 = SCBV; 49 to 96 = SCMV).

Molecular Detection of Viruses by PCR and RT-PCR

Collected samples were subjected to RT-PCR analysis with SCMV and SCSMV specific primers targeting coat protein. Out of 25 samples tested, 11 samples were positive for SCMV with an amplicon size of 891 bp and no amplification was obtained in healthy sample and negative control (Fig. 2a). Whereas for SCSMV 15 samples were tested positive with an amplicon size of 690 bp and amplification was observed in healthy sample and negative control (Fig. 2b). 13 samples have showed positive for SCYLV with an amplicon size of 615 bp and no amplification was obtained in healthy sample and negative control (Fig. 2c). RT-PCR analysis with MaYMV specific primers targeting coat protein and movement protein revealed that 4 samples were tested positive for MaYMV revealing its distribution in Andhra Pradesh (Fig. 2d). Further all the collected samples were assayed with PCR using SCBV specific primers, targeting conserved RT/RNase H coding region which is a taxonomic marker for delineation of species in Badnaviruses, amplifying 794 bp. Out of 25 samples collected, 14 samples gave positive results and no amplification was observed in healthy sample and negative control (Fig 3). Viruses were also detected in some of the symptom less plants which revealing masked infections in the host. SCMV, SCSMV, SCYLV, MaYMV and SCBV infection in the widely-cultivated popular cultivars was validated by the amplification of 891 bp, 690 bp, 615bp, 453 bp and 794 bp amplicons, respectively in different samples collected from sugarcane varieties.

Grassy shoot disease infected samples were also subjected to nested PCR which reveals all the symptomatic samples were positive with first round of PCR with an amplicon size of 1800 bp followed by nested PCR with an amplicon size of 1200 bp (Fig. 4a and 4b).

Molecular characterization

The synthetic oligonucleotide primers amplified a specific product of 891 bp, 690 bp, 615 bp, 453 bp, 794 bp and 1200 bp corresponding to the coat protein region of SCMV, SCSMV and SCYLV, coat protein and movement protein of MaYMV and RT-RNase H region of SCBV, respectively. This was further confirmed by direct sequencing of the amplified products by Sanger method at both ends. After ensuring the quality of the sequences (q = > 30), they were aligned and subjected to Blastn analysis. All the SCMV, SCSMV, SCYLV, MaYMV and SCBV sequences were submitted to GenBank and NCBI accession numbers given are listed in Table 2.

Table 2. List of accession numbers for nucleotide sequences of SCMV, SCSMV, SCYLV MaYMV, SCBV and phytoplasma submitted to GenBank

S.No	Virus	Accession number
1	SCMV	OR099818
2	SCSMV	OR099811
3	MaYMV	OR130184
4	SCBV	OR060699
5	SCYLV	OR099876
6	Phytoplasma	OR09995

Vegetative propagation in sugarcane favours accumulation of pathogens inside the canes and carryover of pathogenic inoculum through planting material. Continuous accumulation of pathogens systemically leads to loss of vigour in sugarcane varieties referred to as 'varietal degeneration'. This results in loss of yield potential in elite commercial varieties. Advancements made in diagnosis of the viruses through serological and molecular techniques led to precise diagnosis of the virus(es) and established the losses with more assurance and authority. Knowledge on virus presence and its prevalence is very much essential for management of viral and phytoplasma diseases through exclusionary and sanitary measures. There is an increasing need for the improvement of current methods for rapid and specific detection of plant viruses of agriculturally important crops. Ideally these procedures should allow the assay of large number of samples at a low cost with high sensitivity to reliably detect low levels of causative disease as well as its various strains that may occur in the field. In an infected field all plants may not be affected and certain plants may avoid infection completely. An efficient method should be able to detect virus infection in those sub-optimally infected plants, which may not show any apparent symptom typically of the virus. Such symptomless infected plants are often mistakenly considered as healthy and farmers

tend to use them as the propagule for the next generation of crop. Considering these facts of viral diseases, it is very urgent tactics to develop the techniques for detection of viruses and phytoplasma.

Serological detection techniques are thought to be the most quick, easy, sensitive, and high throughput of the existing virus detection approaches. Hence, serological based detection was employed for rapid detection of SCMV and SCBV in samples collected during the survey. In the family *Caulimoviridae*, the RT/RNase H coding region is frequently employed as a taxonomic marker for species delineation. Moreover, this RT/RNase H region contains highly conserved motifs which can be used for PCR detection and phylogenetic analysis of Badnaviruses. Coat protein is the most conserved region in case of Potyviruses. The DAS-ELISA and PCR test results are equivalent, suggesting a higher degree of coincidence, which suggests that both diagnostic methods have more or less similar sensitivities. Results made it very evident that PCR was more sensitive than DAS-ELISA. PCR bands were extremely faint in some of the samples, which were found negative when tested with DAS-ELISA, indicating low virus concentration that is below the sensitivity limit of DAS-ELISA. Less prominent symptomatic leaves as well as symptom free leaves of cultivars 2009A 107 and 2003V 46 were failed to detect SCMV and SCBV in the DAS-ELISA, but RT-PCR assays were positive in reaction. Similar kind of results were reported by Balamuralikrishnan *et al*. (2004) who reported that RT-PCR assay method was more sensitive than DAS-ELISA in detecting the virus from plants of less predominant and symptom free plants. Low virus concentration or variation in strains may be the reason for the reduced detection rate in DAS-ELISA as compared to PCR. PCR's greater sensitivity in detecting sugarcane viral infections like SCMV (Balamuralikrishnan *et al*., 2004); SCBV (Balamuralikrishnan and Viswanathan. 2005) and SCYLV (Madugula and Gali. 2017; 2018), Scagliusi *et al*. (2009) as compared to serological methods were reported earlier. Different detection methods have been reported in India in diagnosing various viruses and phytoplasma studied *i.e.*, based on visual symptoms, protein-based detection such as DAC-ELISA, TIBA and ISEM (Thorat *et al*., 2015; Although the isolates had demonstrated cross-reactivity and less sensitive in detection. Later, it was discovered that PCR-based diagnosis was quicker, more sensitive, and more effective than serological approaches (Krishna et al., 2023; Madugula and Gali. 2017; 2018). PCR based detection was employed to screen large number of samples in quarantine, germplasm exchange and under filed conditions. Even more sophisticated techniques like q PCR, LAMP, RPA can be used for the detection of virus at quarantine level. Tissue culture derived planting materials free from the viruses, exhibit varietal vigour and by this approach the viral diseases can be efficiently managed in sugarcane in combination with molecular diagnostics. In the present study, it was found that combined or separate infections of viruses causing mosaic and yellow leaf (YL), phytoplasmas causing grassy shoot and leaf yellows are associated with varietal degeneration. The degeneration and impact was rapid and severe when all these pathogens infect sugarcane together as compared to their separate infections. The impact increased to epidemic levels in combined infections and is a serious concern for sugar industry and due to that longevity of the affected varieties in the field is threatened. Many investigators suggested the use of meristem culture combined with molecular diagnosis was found effective to eliminate the viruses like SCMV, SCSMV and SCYLV efficiently from the cane and to reduce the disease incidence (Wang *et al*., 2009). Developing disease-free nurseries is imperative to sustain productivity of sugarcane and to realize yield potential of popular sugarcane varieties in India.

In spite of the advancement of molecular diagnosis technologies for all the disease-causing pathogens, recommendation of disease-free healthy seed material serves as a viable option till this time to manage or minimize the disease incidences throughout the world. That too vegetatively propagated crop like sugarcane; this is considered as the most feasible option to manage the diseases especially viral diseases. The disease-causing virus genomes keep on changing due to the selection pressure thrust upon it when it switches from host to insect vector environment and incipient nature of the virus according to the resistant/ susceptible host environment in different geographic regions, through which different genotypes, strains, and variants of the same virus have been evolving in different countries and are being characterized and identified based on its complete genome sequences. Characterization of the viruses led to developing

precise diagnostics for the viruses in sugarcane. Both serological and molecular techniques are being applied to diagnose sugarcane seedlings derived through tissue culture. Such application led to production of virus-free planting materials in sugarcane; thereby, varietal degeneration can be addressed and cane productivity sustained in India. Virus indexing in planting material prior to distribution of seed setts to the farmers by the sugar industries, Agricultural research stations which will ensure healthy planting material to the growers leading to higher sugarcane productivity and cane quality.

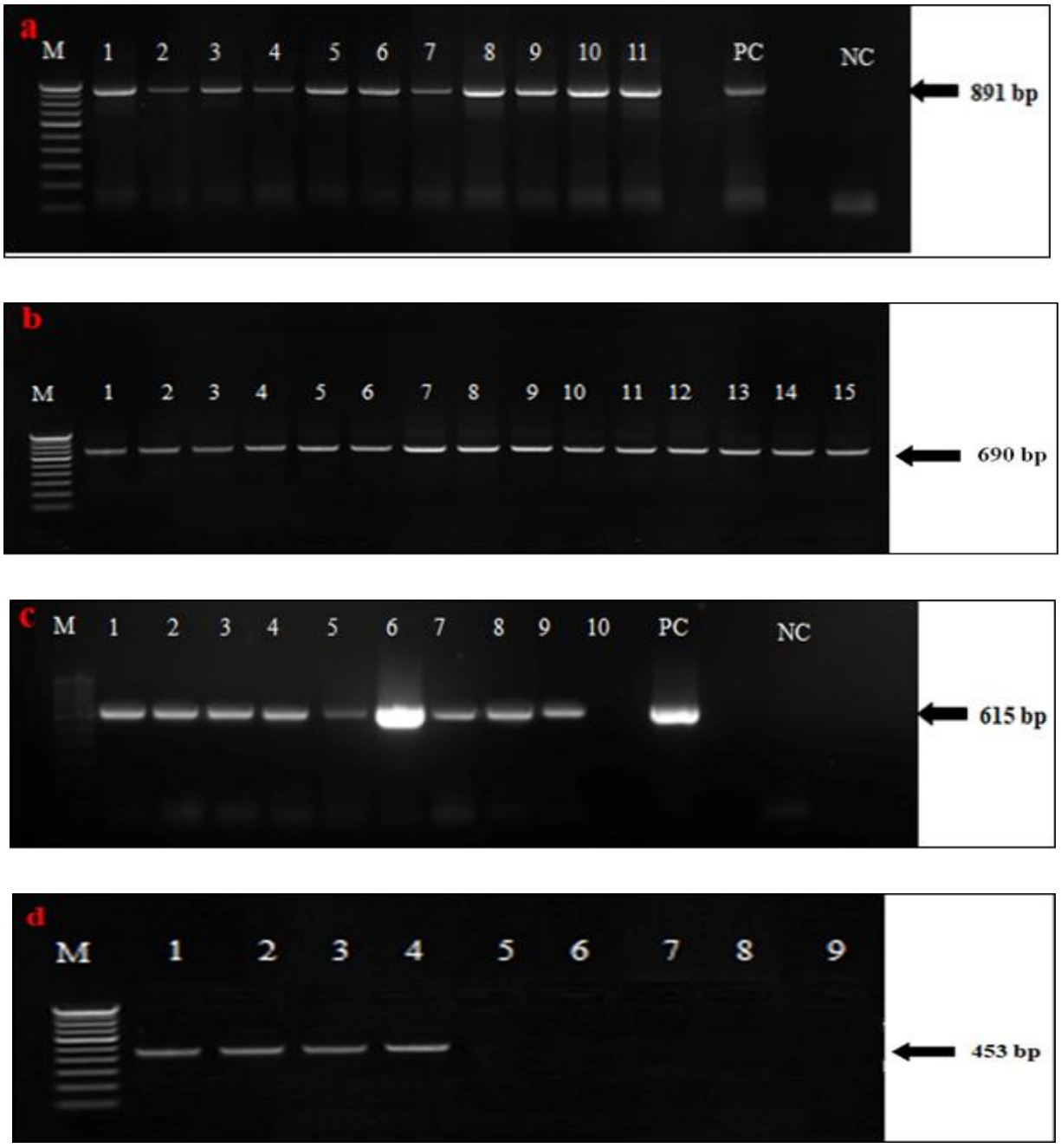

Fig 2. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of coat protein gene of a) SCMV (891 bp), b) SCSMV (690 bp), c) SCYLV (615 bp) and d) MaYMV (415 bp) of sugarcane samples collected from different sugarcane varieties. {lane M: 100 bp ladder, lane 1,2,3… indicates SCMV, SCSMV, SCYLV and MaYMV positive amplification, PC: Positive control, NC: Negative control}.

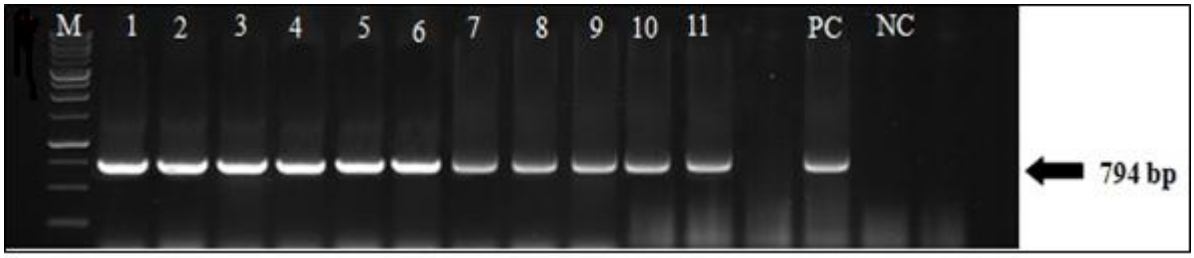

Fig 3. Polymerase chain reaction (PCR) amplification of RT-RNase H region, SCBV of sugarcane samples collected from different sugarcane varieties {lane M: 1 kb ladder, lane 1 to 11: SCBVpositive amplification from different sugarcane varieties. PC: Positive control; NC: Negative control}.

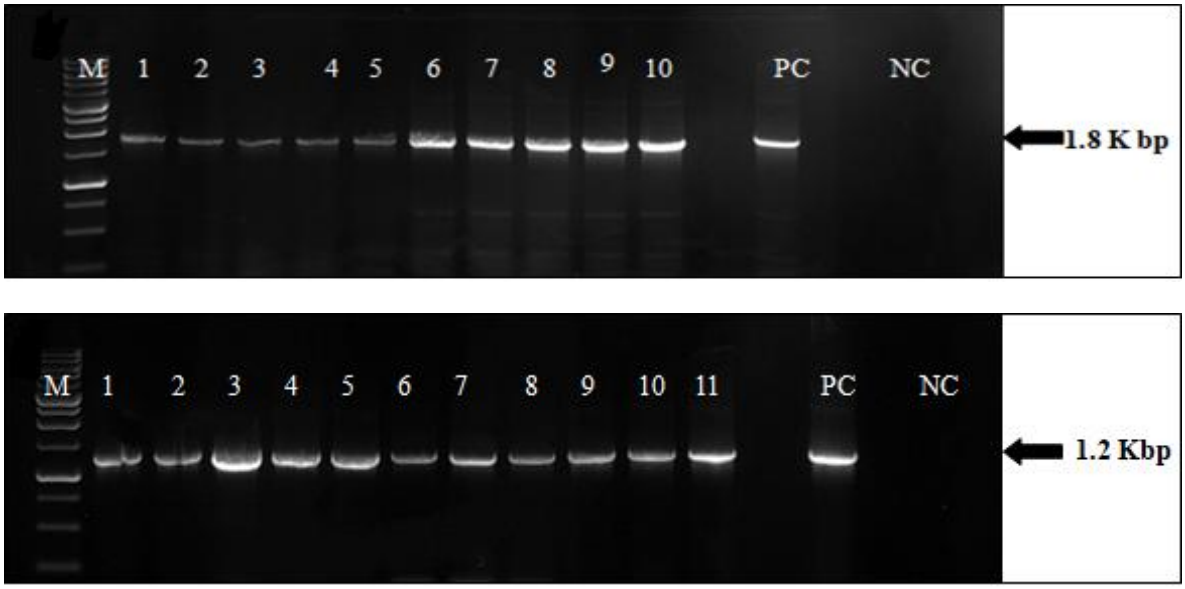

Fig 4. a) Detection of phytoplasma in grassy shoot affected sugarcane cultivars by first round of PCR using P1 and P7 primers. b) Detection of phytoplasma in grassy shoot affected sugarcane cultivars by nested PCR using R16F2n/R16R2. Molecular characterization.

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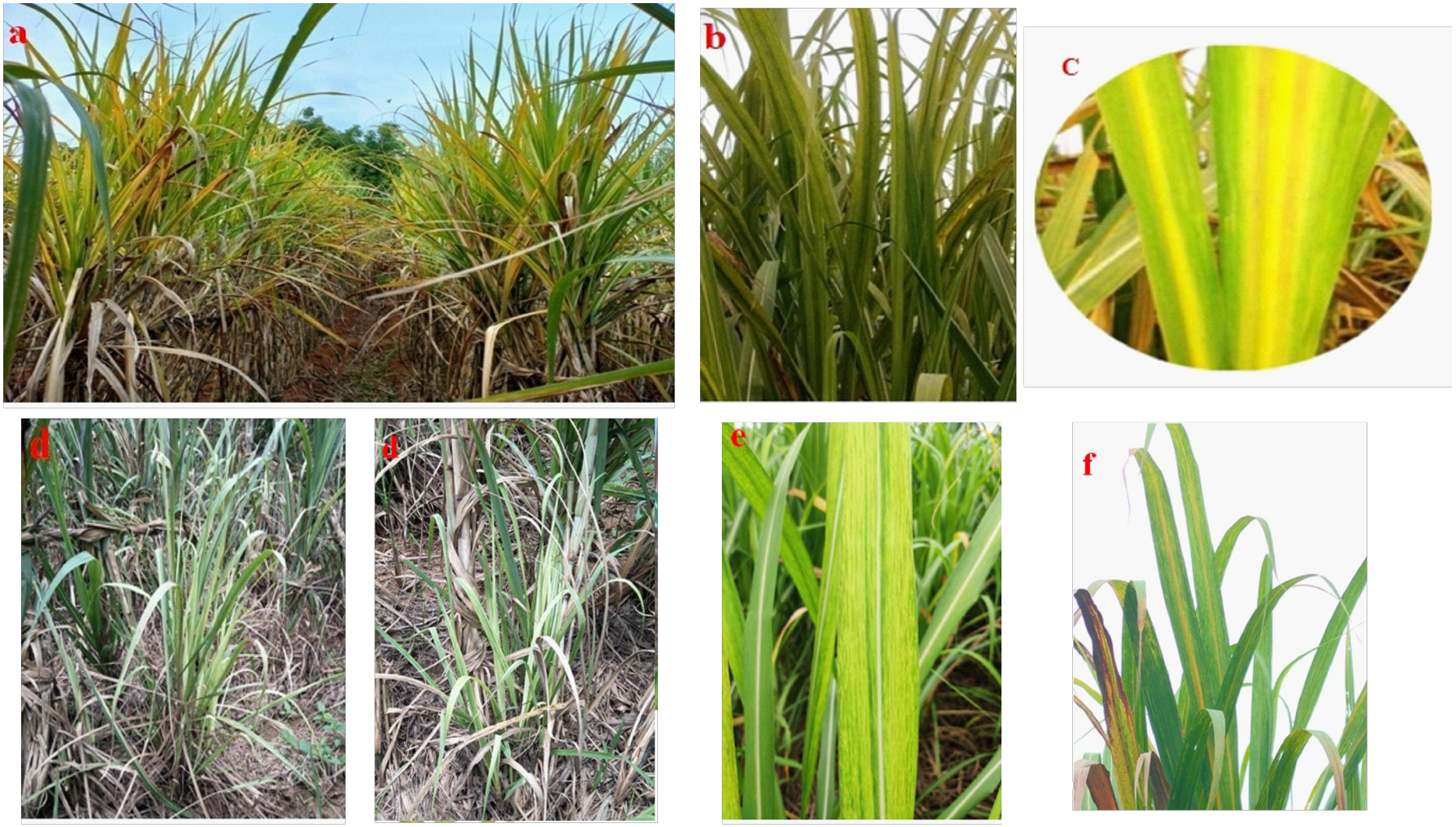

Fig 5. a) Field view of sugarcane severely infected with mosaic in the variety 2009A 107; b) Extensive discolouration of the crop canopy in leaf fleck affected plants (2017A 205); c) Typical mid rib yellowing of leaves in the whorl in YLD affected cane (2003V 46). d) Typical Grassy shoot symptom showing production of numerous chlorotic thin tillers from the base of affected plant (2009A 107); e) Chlorotic streaks/ stripes that alternate with parallel veins throughout the lamina; f) Mixed infections of mosaic and yellow leaf.

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