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Detection of Sugarcane Mosaic Virus in Diseased Sugarcane using ELISA and RT-PCR Technique

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Sugarcane mosaic virus (ScMV) causes yield losses in almost all the genotypes/ clones of sugarcane. So the detection of ScMV is very important for disease management. In present study serological and molecular methods for rapid virus detections were used. Serological study including DAS-ELISA (Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay) was optimized to identify the virus by using a monoclonal antibody. Leaf samples of sugarcane were collected from symptomatic and non-symptomatic plants. ELISA was done by sap extraction method. Total 80 cultivars of sugarcane were selected on the basis of morphological symptoms for ELISA test 70 % cultivars represented the presence of ScMV infection through ELISA. Two stepsRT-PCR (Reverse Transcriptase-Polymerase Chain Reaction) was performed for detecting ScMV using a pair of primers design to amplify a fragment in the coding region of ScMV coat protein. 93.75% cultivars showed the presence of ScMV infection through twostep RT-PCR. Further it was found that two step RT-PCR method is more stringent and will be used for the detection of low titer sample which were not detected by DAS-ELISA. In conclusion our findings suggest that ELISA and RT-PCR can be routinely used for ScMV detection with high efficiency of which RT-PCR is most reliable technique.

Key words: Sugarcane genotypes, ScMV, DAS-ELISA, RT-PCR, serology.

Sugarcane is one of the major commercial crops cultivated widely in tropical and subtropical regions of the world. The cane production in these regions is limited by various etiological agents like viruses, bacteria, fungi and nematodes[1]. Unlike fungi and bacteria there is no chemical or physical treatment to eradicate effectively viruses from infected plants. Virus infected plants either deteriorate quality or reduce the yield to a significant level. ScMV is one of the most widely distributed and important pathogens of sugarcane worldwide, causing the disease known simply as mosaic[2]. In South Africa, under favorable conditions of severe mosaic infection in a susceptible variety, reduction in sucrose yield may be as high as 42%[3]. ScMV is widely spread and almost all the cultivars grown in the continent are infected with the virus. A significant part of yield (39-46%) is lost every year due to ScMV harboring. The crop yield is significantly reduced (10-22%) when incidence of infection level reaches to 50%[4]. ScMV has been reported to be prevalent in more than 70 countries[5].

In India, sugarcane is cultivated under a wide range of agro-climatic conditions. In tropical climate, the cultivation of sugarcane is more successful in terms of cane yield and sugar recovery throughout the year. Sugarcane occupies

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a distinct position as an agro-industrial crop of India, covering around 4.94 million hectares area (over 3% of the total cultivated area) with an annual cane production of 339.17 million tons (2010-2011), contributing about 9.5% to the gross value of the agricultural production in the country (www.faostat.fao.org/). India ranks second in world sugar production, next to Brazil. Sugar Industry is the second largest agro-industry next to textile industry in India.

ScMV is member of the large and economically important plant virus family, the Potyviridae. The virus transmission occurs in a persistent and non persistent manner. Detection of ScMV is very important for disease management. Mosaic is identified primarily by its leaf symptoms. As with most sugarcane diseases, the symptoms may vary in intensity with the cane variety, growing conditions and the strain of the virus involved. The most distinctive symptom is a pattern of contrasting shades of green, often islands of normal green on a background of pale green or yellowish chlorotic areas on the leaf blade. As a vegetatively propagated crop, sugarcane is prone to viral infection via seed cane. Viral screening plays an important role for controlling the disease during germplasm exchange and seed production. Therefore, it is valuable to establish a rapid, convenient and reliable method of viral detection. Serological and molecular methods including the enzyme-linked Immunosorbent assay (ELISA) and polymerase chain reaction (PCR) based techniques have been developed for the detection of sugarcane viruses over the past two decades. Tosic[6] demonstrated that serological detection method such as ELISA can exactly distinguish ScMV from other mixed infecting ScMV subgroup viruses. Also Balamuralikrishnan[7]revealed that antibody based techniques can be employed for screening ScMV. A Double Antibody Sandwich (DAS) ELISA based method has already been reported for the detection of ScMV infecting black pepper. DAS-ELISA was reported to be sensitive and suitable for specific detection of many plant viruses[8]. Reverse transcription (RT)-PCR was developed for ScMV detection in 1994 by Smith[9] and later improved by Alegria[10] and Xu[11]. For more sensitive and specific detection we had implemented the detection of ScMV through morphological, ELISA and RT-PCR technique.

MATERIALS AND METHODS

Collection and maintenance of ScMV-infected samples

Surveys were conducted in Sugarcane cultivation plots of R & D section, Vasantdada Sugar Institute, Manjari (Bk), Pune, Maharashtra, India, to account the occurrence of sugarcane mosaic disease during the years 2012–2013. Naturally infected sugarcane plantlets exhibiting disease symptoms were collected and immediately dipped in liquid nitrogen. Leaf samples were stored at -80p C deepfreezer for further use.

DAS-ELISA

Double antibody sandwich-ELISA (DAS-ELISA) is used for diagnosis of viral diseases. This test was carried out using a monoclonal antibody (Agdia Company, USA). ELISA microplates were coated by incubating for 4 hours at 37º C with the monoclonal antibody diluted (1:200) in carbonate coating buffer (1.59 g Na_2CO_3, 2.93 g $NaHCO_3$, 0.2 g NaN_3 in 1 L water pH 9.6). The plates were washed with washing buffer (8.0 g NaCl, 1.15 g Na_2PO_4, 0.2 g KPO_4, 0.2 g KCl, 0.5 g Tween-20 in 1 L water pH 7.4) and incubated with extracts from healthy and infected plants, overnight at 4ºC. Extracts were prepared by grinding the leaf samples at a ratio of 1:10 (w/v) in extraction buffer (1.3 g Na_2SO_3, 20.0g Polyvinyl pyrrolidone (PVP), 0.2 g NaN_3 , 2.0 g Powdered egg albumin, Grade II, 20.0 g Tween 20 in 1 L of washing buffer pH 7.4) the plates were washed and incubated for 2 hours at 37ºC with conjugate antibody diluted (1:200) in conjugate buffer (2.0 g BSA-Bovine serum albumin), 20.0 g PVP,0.2 g NaN_3 in 1 L wash buffer pH 7.4) after washing the plates were incubated for 1 hour at room temperature 1 mg/ml of p-nitrophenyl phosphate (pNPP) in substrate buffer (97 ml diethanolamine, 0.2 g NaN_3, 0.1 g $MgCl_2$ $6H_2O$ in 800 ml of water pH 9.8). Results were measured by an ELISA-reader (Labsystem Multiscan Company) at 405 nm.

Total RNA extraction from plants

Alwash and Nulit[12] Trizol RNA extraction method was used to extract total RNA from healthy and infected leaves with few modifications as. About 0.2 gm frozen leaves of sugarcane were collected and ground with mortar and pestle to fine powder by mixing with liquid nitrogen. 2ml Trizol reagent was added (carefully under fume

hood). Vortexed for few seconds and 100µl chloroform was added followed by Centrifugation at 12000rpm (room temperature). The supernatant was transferred to new tube. To supernatant 200µl isopropanol was added followed by incubation in ice for few minutes and then Centrifuge 10 sec at 4°C. the supernatant was discarded carefully and given the ethanol wash by Centrifugation for 5min at 7500rpm (room temperature). The pellet was dried by fixing it horizontally, not upside down to prevent the pellet from going out. The resulting pellet was dissolved in 50µl of DEPC/treated water.

RT-PCR (reverse transcription polymerase chain reaction)

A pair of primers a forward (ScMV- F3; 5'-GATGCAGGCGCTCAAGGAGGAGGTGG-3') and a reverse primer (ScMV- R3; 5'-GGTGAGATT TCGCTGAAGTCCATATCGT-3') was designed for amplification of a conserved region (a fragment of 718-bp) in the coding region of ScMV coat protein gene[13]. Reverse transcription of transcripts and cDNA synthesis from 1 µg total RNAs was carried out using SMART™ PCR cDNA Synthesis Kit (Clontech, USA) and quantified by measuring OD at 260 nm. The sugarcane constitutive gene specific primers ²- actins were used for checking the quality of cDNA. The cDNA were amplified by using above said pair of coat protein specific primers as; 50ng of cDNA, 1X taq buffer, 250µM dNTP's, 1.5mM $MgCl_2$, 0.25µM each primer and 1 U of taq DNA polymerase were used for PCR amplification in 20 ¼l reaction mixture. The thermal cycling conditions were: Initial denaturation 94°C for 4 minutes, 30 cycles of 94°C for 45 seconds, 58°C for 45 seconds, 72ºC for 45 seconds and a final extension at 72°C 10 minutes. PCR amplified products were analyzed by electrophoresis on 1% agarose gel[14].

Cloning and sequencing of ScMV coat protein gene

The PCR product was eluted from the gel using QIAEX II gel extraction Kit (QIAGEN) and cloned into pGEMT vector using pGMET T/A PCR Product Cloning Kit as described by the manufacturers. White transformants were examined for the presence of inserts by colony-pick PCR. Plasmid was isolated from the transformants appeared positive in colony-PCR, using Qiagen plasmid isolation kit. Purified plasmids were sequenced using T7 promoter single pass sequencing through, Eurofins Genomics India Pvt Ltd.

Sequence analysis

Sequences were assembled and analysed using Bioedit software. Sequences obtained were converted in FASTA format and were subjected to BLAST by using the nucleotide BLAST programme available on NCBI web portal. BLAST was carried out against the non redundant (nr) nucleotide database. Highly similar sequences with lowest expect value were considered for assigning the putative class to the new sequence.

RESULTS

Disease symptoms

The main symptoms recorded on sugarcane cultivars which exhibited pattern of contrasting shades of green, often islands of normal green on a background of pale green or yellowish Chlorotic areas on the leaf blade (Fig. 1). Chlorotic areas are most evident at the base of the leaf. Chlorotic areas may also be present on the leaf sheath, but rarely on the stalk. Infected plants appear paler and more yellow than healthy plants. The symptoms are most easily seen in young rapidly growing leaves and the symptoms tend to fade as the leaves age. In some varieties, particularly varieties with dark red to purple stalks, the mosaic pattern can be seen on the stalks. Thus such symptomatic leaves were collected from 50 genotypes and 30 promising clones which were further utilized for detection through DAS-ELISA and RT-PCR.

ELISA based detection of ScMV

Results of DAS-ELISA after incubation for one Hour with substrate (pNPP) solution were a change of yellow color in the wells that contained ScMV or the positive control, which were not observed in the wells containing negative control. The results were measured by ELISA-reader (Labsystem Multiscan) at 405 nm. A total of fifty genotypes and 30 promising clones of sugarcane, included ScMV positive and negative control were used in the tests. Among these, 44 genotypes of sugarcane showed positive reaction in DAS-ELISA (Table 1) of which Co08020 is having highest reaction titer while 6 genotypes Co07009, Co05132, Co09009, Co09006, CoM06084 and CoN08071were negative in reaction. Out of 30 clones there were 12 promising clones which showed positive reaction whereas, 18 clones were negative in DAS-

Table 1. Detection of ScMV by Double Antibody Sandwich (DAS) ELISA in different sugarcane genotypes

S. No.	Varieties/ genotypes	Reaction Screening	PCR
1.	Co09007	-	+
2.	Co07009	-	+
3.	Co05132	-	+
4.	Co86032	+	+
5.	Co09009	-	+
6.	Co99004	+	+
7.	CoN09071	+	+
8.	Co09014	+	+
9.	CoM06082	+	+
10.	PI07131	+	+
11.	CoJn08101	+	+
12.	CoN09072	+	+
13.	CoSnk08101	+	+
14.	Co94008	+	+
15.	Co08009	+	+
16.	Co06002	+	+
17.	Co07012	+	+
18.	Co08008	+	+
19.	Co06015	+	+
20.	Co09013	+	+
21.	Co08016	+	+
22.	Co06013	+	+
23.	Co85004	+	+
24.	CoC671	+	+
25.	Co06012	+	+
	ScMV + ve control	+	NA
26.	CoVC08061	+	+
27.	Co09006	-	+
28.	CoVC08062	+	+
29.	CoSnk05102	+	+
30.	Co07006	+	+
31.	CoM08081	+	+
32.	Co07003	+	+
33.	Co06002	+	+
34.	PI06132	+	+
35.	Co05002	+	+
36.	PI07131	+	+
37.	CoSnk6101	+	+
38.	Co07010	+	+
39.	CoM06084	-	-
40.	Co0720	+	+
41.	Co08020	+	+
42.	CoSnk07103	+	+
43.	Co09010	+	+
44.	Co06024	+	+
45.	Co09003	+	+
46.	Co07007	+	+
47.	CoN07071	+	+
48.	Co07008	+	+
49.	CoN08071	-	-
50.	Co09004	+	
	ScMV - ve control	-	NA

ELISA (Table 2). Hence more sensitive and specific detection technique like PCR may be necessary for samples that were negative in DAS-ELISA. However due to its cost effectiveness and adaptability in testing large number of samples, ELISA can be used for primary screening of samples.

Detection of sugarcane mosaic virus through RT PCR

RNA was isolated from ScMV infected sugarcane samples of which VSI435 uninfected leaf sample was used as negative control. For detection of ScMV at molecular level two step RT PCR method was used. The cDNA were synthesized and

Table 2. Detection of ScMV by Double Antibody Sandwich (DAS) ELISA in different sugarcane promising clones

S. No.	Clones/ genotypes	Reaction Screening	PCR
51	TC2543	-	+
52	CoVSI05058	-	+
53	153-1	-	+
54	CoVSI08004	-	-
55	40-2	+	+
56	70-6	-	+
57	1028	+	+
58	CoVSI08033	-	+
59	CoVSI09121	-	+
60	CoVSI08017	-	+
61	152-8	-	+
62	14-4	+	+
63	VSI434	-	+
64	VSI07004	+	+
65	CoVSI07012	-	+
	ScMV +ve control	+	NA
66	08-20	-	+
67	12-25	-	+
68	61-10	-	-
69	VSI08121	-	+
70	CoVSI9805	+	+
71	CoVSI08122	-	+
72	VSI08005	+	+
73	TC4160	+	+
74	169-7	-	+
75	10-17	+	+
76	92-4	+	+
77	CoVSI05132	+	+
78	CoVSI03102	-	-
79	CoVSI08058	+	+
80	139-6	+	+
	ScMV -ve control	-	NA

Table 3. NCBI BLAST results of ScMV coat protein gene sequence

Discription	Identity	Accession
Sugarcane mosaic virus isolate S-9 coat protein mRNA, partial cds	98%	EU650180.1
Sugarcane mosaic virus polyprotein gene, partial cds	98%	EF443055.1
Sugarcane mosaic virus isolate LK-8102 coat protein gene, partial cds	98%	DQ866747.1
Sugarcane mosaic virus isolate HR-Co62399 coat protein gene, partial cds	98%	DQ866745.1
Sugarcane mosaic virus isolate PB-CoJ85 coat protein gene, partial cds	97%	DQ866746.1
Sugarcane mosaic virus isolate KL-Co86032 coat protein gene, partial cds	97%	DQ866744.1
Sugarcane mosaic virus isolate ARG-345 polyprotein gene, partial cds	96%	JX237865.1
Sugarcane mosaic virus coat protein gene, partial cds	96%	AF006735.1
Sugarcane mosaic virus genomic RNA for polyprotein (vp gene), strain A, isolate Brisbane	96%	AJ278405.1
Sugarcane mosaic virus isolate SAL-1N polyprotein gene, partial cds	96%	EU196434.1

quantified using spectrophotometer. The quality of cDNA was checked through amplification of sugarcane ^{2}-actin constitutive gene specific primers. The PCR amplification conditions and ingredients for obtaining single product (718bp) were standardized as described in material and methods. The expected size (~718 bp) fragment was successfully amplified with Path2F and Path2R primers. Out of 50 genotypes 48 samples were positive for ScMV infection and out of 30 clones 27 clones were positive for ScMV infection and there was no amplification in a negative control healthy VSI435. Thus, infection of ScMV was confirmed in 48 genotypes and 27 clones of sugarcane collected from VSI fields. The CoM06084 and CoN08071 genotypes and CoVSI08004, 61-10, CoVSI03102 clones were found negative for ScMV detection. Typical results of PCR following first strand synthesis of DNA from total RNA extracted from sugarcane leaf material are shown in Fig 2. Results show that primer pair amplifies an approximately 718 bp fragment from virally infected leaf material from sugarcane but not from negative control (symptomless) leaf material (VSI435). The results were reproducible through two steps RT-PCR. There were four genotypes Co07009, Co05132, Co09009, Co09006 and fifteen clones (TC2543, CoVSI05058, 153-1, 70-6, CoVSI08033, CoVSI09121, CoVSI08017, 152-8, VSI434, CoVSI07012, 08-20, 12-25, VSI08121, CoVSI08122 and 169-7) which were negative through DAS-ELISA but later found positive through RT-PCR.

Sequence analysis and identification of ScMV isolates

In addition to RT-PCR confirmation of ScMV the promising samples were sequenced form Co-08020 and VSI-07004 infected samples. The ScMV amplicons of ~718 bp with prominent intensity were successfully isolated and cloned in

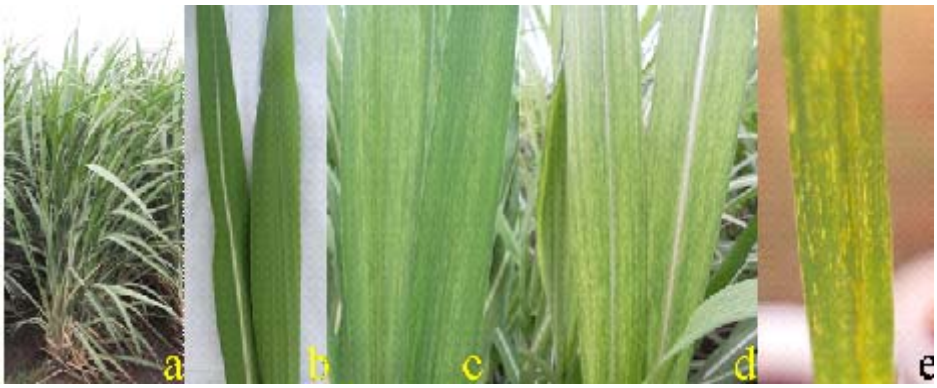

Fig. 1. Symptoms of sugarcane mosaic virus on leaf of sugarcane. a. field grown healthy plantlet, b. healthy non infected sugarcane leaves, c. infection with contrasting green shade of leaves, d. Infected leaves appear paler and more yellow than healthy plants and e. dark yellow stalks mosaic pattern.

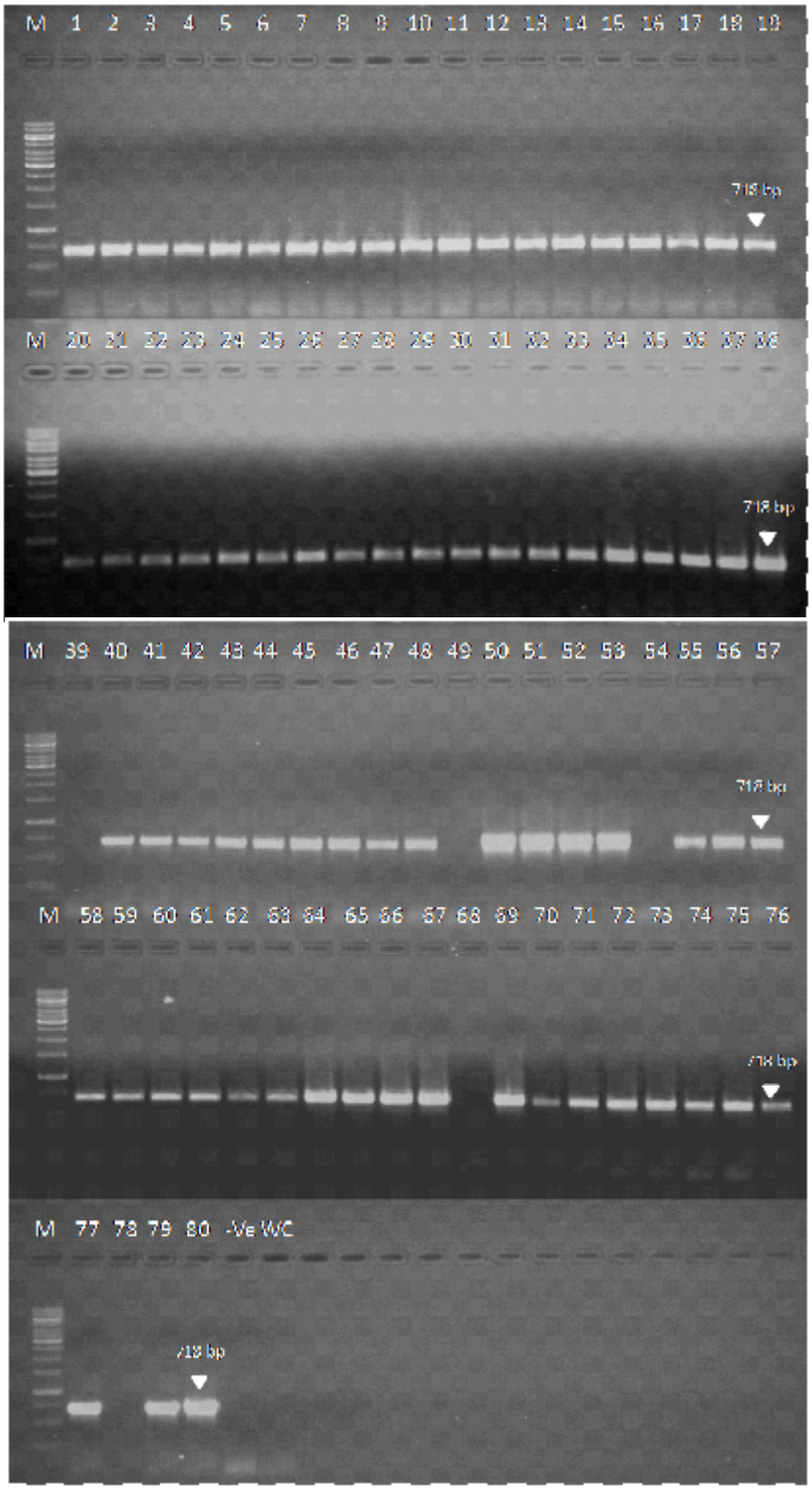

Fig 2. Two step RT-PCR screening of ScMV infected samples of sugarcane. M- 1 kb DNA ladder, 1 to 50- ScMV infected genotypes, 51 to 80- ScMV infected clones, -ve- uninfected Co435 negative control and WC- water control

pGEMT T/A cloning vector. The plasmids were isolated and sequenced by using universal primer T-7 promoter. Sequence data obtained from two clones of each isolate were BLAST at NCBI. The BLAST results represented maximum 98% sequence similarity with Sugarcane mosaic virus isolate S-9 coat protein mRNA, partial cds (EU650180.1). Both the sequenced ScMV isolates from this study were found to be very similar to each other and with ScMV (EU650180.1) showing less than 2% sequence variation (at nt levels). Thus in addition to PCR amplification the viral detection was also supported by sequencing of the amplicons. The BLAST sequences showing more than 95% homology were enlisted in Table 3.

DISCUSSION

Sugarcane mosaic virus (ScMV) is one of the most widely distributed and important pathogens of sugarcane worldwide, causing the disease known simply as mosaic. ScMV is an important pathogen of sugarcane (*Saccharum* inter-specific hybrids) and maize (*Zea mays*) worldwide, causing significant yield losses in susceptible varieties[15]. The virus belongs to species of the genus *Potyvirus*, family *Potyviridae*, and infects various cultivars of sugarcane. Mosaic is identified primarily by its leaf symptoms. The most distinctive symptom is a pattern of contrasting shades of green, often islands of normal green on a background of pale green or yellowish chlorotic areas on the leaf blade. Infected plants appear paler and more yellow than healthy plants. The symptoms are most easily seen in young rapidly growing leaves and the symptoms tend to fade as the leaves age. There is an increasing need for the improvement of current methods for rapid and specific detection of plant viruses of agriculturally importance[16]. 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 fact of viral disease it is the most important tactics to develop the technique for detection of ScMV detection.

Sugarcane mosaic virus can be detected by using DAS-ELISA technique by using primary antibody and secondary antibody of the dilution stock 1:200. DAS-ELISA was reported to be sensitive and suitable for specific detection of many plant viruses[8]. DAS–ELISA was successful in the detection of ScMV infecting black pepper[17]. DAS-ELISA procedure could detect putative sugarcane mosaic virus infection from infected symptomatic sugarcane samples collected from different fields of Vasantdada Sugar Research Institute, Pune. A total of fifty genotypes and thirty promising genotypes of sugarcane, including ScMV positive and negative control were used in the tests. Among these, 44 genotypes and 12 promising clones of sugarcane showed positive reaction in DAS-ELISA. The total 70 % of collected samples were positive for ScMV infection through DAS-ELISA. The variations in the OD values obtained with different samples indicate the varying concentration of the virus. However the severity of the disease symptoms could not be correlated with the OD values obtained. As sugarcane is vegetatively propagated, it is essential to develop methods for producing and certifying virus-free planting material. In the present study, though the healthy sugarcane samples gave a faint color indicating background reaction, the infected samples could be distinguished from the healthy samples with an increase in the OD values. However, some of the samples exhibiting symptoms of the disease gave an OD close to that of the healthy samples. This might be due to low titer of the virus in these samples. Tosic[6] demonstrated that serological detection method such as ELISA can exactly distinguish ScMV from other mixed infecting ScMV subgroup viruses. Also Balamuralikrishnan[7] revealed that antibody based techniques can be employed for screening ScMV. Hence more sensitive and specific detection technique like PCR may be necessary for samples that were negative in DAS-ELISA. However due to its cost effectiveness and adaptability in testing large number of samples, ELISA can be used for primary

screening of samples.

Recently, the application of the RT-PCR technique has resulted in clear and fully reproducible method for diagnostic assay of virus infected leaf material. This is well ensured for its eventual application as a reliable and definitive test for ScMV regardless of strain. Work is in progress to establish how universally the test can be applied for the diagnosis of ScMV. Hence, to validate virus free nature of different varieties and clones of sugarcane plants through molecular method, two steps RT-PCR was employed. RT-PCR has some advantages over other serological analysis like ELISA because it requires only small amount of samples and full analysis including PCR can be done in less than 24 Hours[18]. Specific amplification of target product depends on selection and designing of specific primers[19]. It has the potential to detect most of the variants of ScMV, from different verieties. Therefore, in the present study presence of ScMV was successfully analyzed through two steps RT-PCR. The result revealed successful validation the collected and inoculated plants but not from healthy plants similar results in RT-PCR with F3/R3 ScMV specific primers were obtained by Balamuralikrishnan[7]. There were four genotypes Co07009, Co05132, Co09009, Co09006 and fifteen clones (TC2543, CoVSI05058, 153-1, 70-6, CoVSI08033, CoVSI09121, CoVSI08017, 152-8, VSI434, CoVSI07012, 08-20, 12-25, VSI08121, CoVSI08122 and 169-7) which were found negative through DAS-ELISA serological assays but shown the successful amplification in two steps RT-PCR. Total 93.75 % of collected samples were positive through RT-PCR. This result indicates the stringency of RT-PCR (93.75%) over the DAS-ELISA (70%) for ScMV detection. Thus RT-PCR is more reliable method over the DAS-ELISA. DAS-ELISA and RT-PCR screening of sugarcane samples collected from VSI field revealed that most of them were infected with ScMV regardless of the presence of visible symptoms. The possible reasons for the high incidence of viral infection in our collections might be; susceptibility of sugarcane varieties[20], high densities of aphid populations, which transmit sugarcane mosaic disease[21], and also the presence of viral inoculums reservoirs available near sugarcane-growing areas[22].

The BLAST results clearly indicates that the amplified fragments are from viral genome which supports the RT-PCR detection of ScMV in different genotypes and there clones. Although sequence homology among both ScMV strains/ variants is usually very high (~98%), but they show different biological properties such as variation in symptom severity and patterns as well as host range. The molecular basis of these variations is still unclear although the difference of sequences between these strains can be established. If more sequences of different strains of ScMV can be obtained from a region, it may be possible to identify the substitution which modify gene functions and give rise to the variations of biological properties. This information will particularly be helpful for understanding the functions of virus genes and hopefully will lead to the development of new methods for the control of ScMV. Studies are now underway to clone and sequence rest of the genome of these two isolates and to extend this survey for other cultivars of sugarcane grown in the country.

In the present study the, a two step RT-PCR based protocol for ScMV was developed and was compared to a DAS-ELISA based ScMV detection. The results clearly showed that both protocols detect virus when the titer level is high. As it had been reported earlier by several researchers[19, 23, 24] RT-PCR with a pair of Coat protein specific primers could detect the presence of very small amount of infection in field samples, which was not detected by DAS-ELISA. This method will also help to correctly identify resistant plants in population so that they could be separated for further multiplication through both *in-vitro* as well as *in vivo*.

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