VARIETAL RESPONSE OF SUGARCANE AGAINST THE INFECTION OF SUGARCANE MOSAIC VIRUS (SCMV) IN PUNJAB PAKISTAN

Babar Hussain Babar*, Akhtar Hameed** and Muhammad Rizwan Khurshid*
*Agronomic Research Institute Faisalabad
**MNS University of Agriculture Multan
Email: babarhbabar@gmail.com

ABSTRACT

Sugarcane mosaic virus (SCMV) is among many viruses that infect sugarcane, cause yield loss, and become serious disease agents on sugarcane plantations. Since the morphological symptoms of SCMV are like other symptoms caused by Sugarcane streak mosaic virus (SCSMV) or nitrogen deficiency, the detection of SCMV is important through accurate diagnostic-like ELISA or RT-PCR. This research aimed to study the causative mosaic pathogen of SCMV in Punjab, Pakistan, including mosaic development. The results showed that the mosaic symptom is present in all sugarcane plantations with 78% and 65% disease incidence and severity, respectively. Moreover, the detection procedure based on an amplification of cDNA of the coat protein gene sequence confirmed that SCMV was the causative agent of mosaic disease on sugarcane. Re-inoculation of healthy sugarcane plants with plant sap from a symptomatic leaf from the field showed similar mosaic or yellowish chlorotic areas on the leaf blade and appeared on the fourth leaves upward from the inoculation leaf, in addition to showing different levels of peroxidase but not total phenol. Mosaic also correlated with the amount of total chlorophyll. Although Sucrose phosphate synthase (SPS) protein accumulation and activity were at a lower level in infected leaves, sucrose accumulation was at a higher level in the same leaves.
Keywords: Sugarcane mosaic virus; sugarcane; RT-PCR; plant response

INTRODUCTION

Sugarcane or Saccharum spp., family Poaceae, is a widely cultivated crop that provides sugar across the globe. In Pakistan, sugarcane is widely cultivated on Punjab, particularly in Central and South, and is the highest contributor to the national sugar production. During cultivation, this production is unstable due to several problems, including mosaic disease. Putra et al. [1] reported that sugarcane loss due to mosaic disease is about 20% with 50% of incidence. In Pakistan, mosaic-like symptoms are present with various possible causative agents, including nutrient deficiency and plant viruses [1,2]. Typically, mosaic disease in the affected sugarcane shows yellowing and chlorosis on leaves, resulting in yield loss for both crop yield and sugar production delete this reference, it is not relevant here. On the other hand, mosaic symptoms caused by (SCSMV). These viruses have been reported as dominant pathogens infecting sugarcane in several countries [3]. Although several viruses may infect and show similar mosaic symptoms on sugarcane, it has been reported that the most widespread and dominant mosaic pathogens on sugarcane in Pakistan are SCSMV, SCMV, or both [1].

Thus, it is critical to accurately identify the causative agent of mosaic on sugarcane in Punjab, Pakistan through biological, molecular, and serological assays [4], prior to deciding upon management and control strategies. Many reports on detecting the causative agent of mosaic on sugarcane have been conducted by a single or double methods such as RT-PCR [5] or a serological test [6]; however, each method

presents its own disadvantages and advantages concerning accuracy and reliability.

A potyvirus, such as SCMV, is a single-stranded RNA virus with a simple genome structure encoding 10 mature proteins, specifically (from N-terminal to C-terminal) the first protein (P1), the helper component proteinase (HC-pro), the third protein (P3), the first 6K protein (6K1), the cylindrical inclusion protein (CI), the second 6K protein (6K2), the viral protein genome-linked (VPg), the nuclear inclusion a protein (NIa), the nuclear inclusion b protein (NIb), and the coat protein (CP) [7]. In addition, genetic structure of SCMV, interspecific recombinants can be identified with two recombination patterns at the P1 coding region, depending on the hostplant of the virus. For example, SCMV from sugarcane (NRA) has recombination at six sites (at P1, HC-Pro, CI, NIa-Vpg, and NIa-pro coding regions), while SCMV from maize has four recombination sites (at P1, HC-Pro, NIa-Pro, and NIb coding regions). Interestingly, there is an Open Reading Frame (ORF) that overlaps P3, namely PIPO, expressing P3N-PIPO which is known to colocalize to plasmodesmata, where it acts to mediate cell-to-cell spread of the virus [8].

During a virus infection, there are two possibilities of host-virus interaction. In the compatible interaction, the infection affects physiological, biochemical, and metabolic processes or changes in the plant, leading to symptom development due to systemic infection, activation, and suppression of global gene expressions in the host [9]. In the incompatible interaction, the virus infection triggers specific molecular interactions between the plant resistant (R) gene and viral avirulence (Avr) proteins, leading to the activation of a cascade of genes to induce defense mechanisms in the plant. Several reports have demonstrated that various alterations in the plant as a response to virus infection have been indicated by some biochemical changes such as defense-related enzymes, carbohydrate accumulation, or photosynthetic and photo-assimilation activity.

MATERIALS AND METHODS

Sugarcane leaf samples, disease assessment, and plant inoculation

Sugarcane leaves, from both symptomatic (mosaic) and non-symptomatic plants, were collected from Sugarcane Research Institute Faisalabad, and were assessed for disease incidence and severity. Briefly, disease incidence was assessed by calculating the number of symptomatic plants per total observed plants in the field, while disease severity was calculated by estimating the percentage of leaf area with mosaic symptoms using the following scoring system: 1 = no symptoms, 2 = 0.1%–2.5% leaf area showing symptoms, 3 =2.6–5%, 4 = 5.1–10%, 5 = 10.1–20%, 6 = 20.1–35%, 7 = 35.1–50%, 8 = 50.1–75%, 9 = 75.1–100%. Samples were either directly processed for RNA isolation or stored at −80 ◦C to avoid the degradation of RNA by RNAse. For the inoculation experiment, leaves from the symptomatic plant (cultivar NXI-1T) were homogenized with a mortar in 2 mL of phosphate buffer 0.1 M pH 8.0 (ratio 1:10) containing 2% of PVP (Polyvinylpyrrolidone). Plant sap was filtered and inoculated directly onto leaves of 6-week-old sugarcane PS 881 cultivar (seeds were obtained through tissue culture treated with 40 ppm of ribavirin and were confirmed to be healthy through RT-PCR) with carborundum as an abrasive. Inoculated leaves were then rinsed with ddH2O water to remove unnecessary material before incubation in a dark room overnight, prior to incubation in greenhouse.

Total plant RNA extraction and reverse transcriptase polymerase chain reaction

Frozen leaf samples (200 mg) were placed in liquid nitrogen and ground in a mortar. Total RNA was extracted using RNAeasy Mini Kit (Qiagen, Venlo, The Netherlands). The contaminant DNA was eliminated by DNAse (Merck KGaA, Darmstadt, Germany) treatment for 2 h. The quality of total RNA was checked in denaturing agarose gel electrophoresis and the quantity was determined using NanoVue Plus-UV Spectrophotometer.

First strand cDNA was synthesized from purified RNA. The mixture: 2 µg of purified RNA, 200U of M-MLV reverse transcriptase, 50 pmol of antisense primer (dT)

and 1 mM dNTPs, was incubated at 42 ◦C for 1 h. The mixture was then heated at 70 ◦C for 10 min to stop the reaction. The cDNA was then PCR amplified using the synthesized primers (Bioneer, Daejeon, South Korea). The PCR reaction mixture contained 25 µL of 2×PCR Master mix Solution (i-Taq, iNtRON Biotechnology, Kyungki-Do, South Korea), 2 µL (100 ng) of template cDNA, and 1.5 µL of 10 pmol of pair primer. Primers used in this experiment were designated to amplify the coat protein sequence of SCMV using forward primer SCMV-F: 5'-TTT TCA CCA AGC TGG AA-3' and reverse primer SCMV-R: 5'-AGC TGT GTG TCT CTC TGT ATT CTC-3' [10], while for SCSMV using forward SCSMV-CPF2 5'-TCA TMT CTT CAT CRG CCG C-3' and reverse primer SCSMV-CPR2 5'-ATC TTC YCT ACG CAG GTC CG-3' [11]. PCR was performed by pre-denaturing at 94 ◦C for 2 min, followed by 40 cycles at 94 ◦C for 1 min, 65 ◦C for 1 min, and 72 ◦C for 1 min, and lastly one cycle of final extension at 72 ◦C for 10 min. The 10 µL of PCR amplified product was analyzed by electrophoresis on 1% agarose gel.

Estimation of total chlorophyll, phenol, and peroxidase activity

Total chlorophyll was estimated by following the procedure of [21]. Two hundred and ten milligrams (210 mg) of finely cut fresh leaves were ground with 2.1 mL of 80% acetone. This mixture was then centrifuged at 3000 rpm for 10 min. The supernatant was carefully transferred, and the procedure was repeated till the residue became colorless. The absorbance of the solution was read at 645 nm and 663 nm against the solvent (acetone) blank in 1 mL of supernatant using a spectrophotometer (UV-VIS double Beam, Hitachi, Japan). The concentrations of chlorophyll a, chlorophyll b, and total chlorophyll were calculated using the following equation: Chlorophyll a was calculated as (12.7(A663) – 2.69(A645)) × 0.5, while Chlorophyll b was calculated as 22.9(A645) − 4.69(A663).

The total phenolic content in the leaf was estimated using the Folin–Ciocalteau method with slight modification. Briefly, extracts (200 µL), 50% of Folin–Ciocalteau's reagent (100 µL), and distilled water (750 µL) were mixed and incubated in a tube for 3 min, and then 2% of Na2CO3 (300 µL) was added to the solution. The reaction mixture was mixed and incubated at 28 ◦C for 10 min. The mixture was then heated at 45 ◦C for 20 min prior to determining its absorbance at 755 nm. The results were compared to a gallic acid calibration curve and total phenolic content in the extraction of sugarcane was expressed as mg of gallic acid equivalents per gram of extracts per total protein. Peroxidase activity was spectroscopically evaluated by measuring the absorbance of the reaction at 420 nm every 20 s for 2 min. Briefly, leaf extracts (5 µL) and 0.05 M of pyrogallol (150 µL) were mixed in a microplate, and then 1% of H2O2 (25 µL) was added and mixed before reading the absorbance using a spectrophotometer. All evaluations were performed in triplicate.

Analysis of sucrose phosphate synthase, rubisco, and sucrose accumulation in leaves

Sucrose phosphate synthase (SPS) and rubisco were determined through Western blot analysis. Sodium dedocyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with equal amounts of leaf extracts (15 µg/mL of total protein content). Proteins were denatured and electro phoretically transferred to nitrocellulose membrane at 4 ◦C for 2 h. The membrane was then washed three times with Tris Buffer Saline (TBS). The SPS and rubisco protein abundance were evaluated by detection of SPS and rubisco using specific polyclonal antibodies and visualized using chromogenic dye in conjunction between 25 µL of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 50 µL of nitro blue tetrazolium (NBT) for every 10 mL of alkaline phosphate buffer.

Quantitatively, SPS activity was estimated by following. Leaf extract was cleaned up using Sephadex G-25 and subjected to an enzyme activity assay. Twenty-five microliters (25 µL) of crude enzyme were mixed with 20 µL of buffer (composed by 86 mM MOPS-NaOH (pH 7.5), 26 mM MgCl2, and H2O), 10 µL of substrate (70 mM fructose-6-phosphate), 10 µL

of 70 mM uridine diphosphate glucose, and 5 µL of 70 mM glucose 6-phosphate as the activator. One portion of reagent (composed by 125 µL of 0.1% resorcinol and 375 µL of 30% HCl) was then added to the mixture and incubated at 80 ◦C for 8 min before measuring the absorbance at 520 nm. Sucrose from the leaf extract was quantified by following Seliwanoff's method. Seventy microliters (70 µL) of 1 M NaOH were homogenized with 15 µL of leaf extract and heated at 100 ◦C for 10 min. After cooling, the solution was mixed with 250 µL of 0.1% resorcinol (in 95% of ethanol) and 750 µL of 30% HCl following incubation at 80 ◦C for 8 min, prior to determining the absorbance using a spectrophotometer (UV-VIS double Beam, Hitachi, Japan) at 520 nm. Each sample was analyzed in triplicate against the concentration of sucrose as a standard curve.

RESULTS AND DISCUSSION

Mosaic disease incidence, severity, symptom development, and its pathogen

We studied five sugarcane cultivars from three different regions of sugarcane farms in Punjab, Pakistan, including Co 1148, SPF-238, SPF-213, CoJ 84 and L-118. All cultivars were showing mosaic symptoms on leaves with different incidence and severity. Our data indicated that COKRO was the most resistant cultivar with 26% and 16.9% of disease incidence and severity, respectively, while NXI 1T and PS 881 were the most susceptible cultivars with about 78% and 63% of disease incidence and 53% and 60.13% of disease severity. The field symptomatic plants showed mosaic and yellowing along the sugarcane leaves. Since there are some plant viruses which can infect sugarcane (such as SCMV, SCSMV, or SrMV) with the ability to induce very similar mosaic symptoms, we conducted RT-PCR analyses to diagnose the possible causative virus. The data confirmed that all symptomatic plants (both from the field and re-inoculated plants) produced a specific size of band. All samples (symptomatic plants and re-inoculated plants) showed a particular band at about 900 bp. In addition, to confirm that the plant sap contained only one virus, we then detected the possible presence of widely distributed viruses in sugarcane using either SCMV or SCSMV pair primers. In addition, the observation of symptom development of inoculated plants showed that the first mosaic symptom appeared at 24 days post-inoculation (dpi) on the fourth leaf above the inoculation site and became clearer at the fifth leaf. This observation indicated that infectious agents such as the virus cause the mosaic on sugarcane.

Sugarcane response and its alteration during infection by *SCMV*

During infection, we observed some properties of sugarcane such as total chlorophyll, peroxidase activity, and total phenol in leaves. Our results showed that total chlorophyll was drastically reduced in inoculated leaves, indicating that SCMV infection may alter or inhibit chlorophyll formation, while peroxidase activity and total phenol content had not significantly increased. Interestingly, the results showed that SPS activity was in contrary to the sucrose content in the leaves. SPS activity was drastically reduced in inoculated leaves by about 40%, while the sucrose content significantly increased in inoculated leaf by about 25%. To understand the possible reason for a reduction in SPS activity, we analyzed the SPS content in sugarcane leaves. Western blot analysis indicated that SPS was produced abnormally in inoculated leaves, but not rubisco. The abnormality of production of SPS was indicated by a smaller SPS signal detected using SPS polyclonal antibody, while the internal control (rubisco, both large sub-unit (LSU) and small sub-unit (SSU)) showed a comparable amount. One of the causes of mosaic on sugarcane is virus infection, specifically a potyvirus group such as Sorghum mosaic virus (SrMV) and Maize dwarf mosaic virus (MDMV) including SCMV. Infection of SCMV presents as irregular, light-green mosaic or a yellowish or chlorotic effect along the veins and causes yield loss on several susceptible plants. It is difficult to identify a particular causative virus because of the pattern similarity of symptoms. Researchers have

used several tools to detect these pathogens by examining virus particles using electron microscopy [12], enzyme-linked immune-sorbant assay (ELISA) [11], or by reverse transcriptase polymerase chain reaction (RT-PCR) [20] combined with DNA sequencing, particularly on the coat protein gene fragment. Moreover, according to the coat protein sequence, the virus is also easily grouped into strain, because the sequence has unique parts among strains of SCMV related to their hosts [14], and more specifically, it has unique parts at the N-terminal amino acid residue of coat protein which is the second trypsin cleavage site and the residues which contain repeat sequence motifs [13]. In this research, we amplified the 900 bp cDNA fragment and suggested that the causative agent of mosaic in sugarcane was Sugarcane mosaic virus.

A similar pair-primer has also been used following confirmation by sequence analysis, which revealed that a particular band amplified by using the primer was responsible for the coat protein of SCMV [8]. In addition, our results showed that mosaic development depended on sugarcane cultivars, indicating that plant response might influence symptom development. Infection of SCMV may incite different responses from different cultivars, host species, resulting in variation of symptom appearance or incubation time.

Incubation of SCMV on maize, sorghum, and sugarcane varied about 4–15 dpi and was longer when transmitted through the seed (about 25–30 dpi [27]. SCMV is a plant pathogenic virus that systemically transmits and presents mosaic on younger leaves [15]. Our results showed that the mosaic appeared at the fourth leaf and younger leaves above the inoculation site and showed mosaic symptoms such as yellowing and chlorotic effects on leaves. This phenomenon indicates that virus infection develops in the plant systemically. During infection, the virus replicates and transmits into upper or younger leaves but requires and interval to produce mosaic symptoms. Our data showed that mosaic due to SCMV infection exhibited for the first time at the fourth leaf and became contrasted at the fifth leaf above the inoculated leaf. Moreover, virus infection related to chloroplast is responsible for some changes such as chlorophyll pigmentation, photosystem efficiency, or photo-assimilate accumulation [16].

Peroxidase is an enzyme in plants that occurs in response to some stimuli such as pathogen infection, chemical agents, or mechanical agents [16]. This was supported by our data that the plant cultivar which we used in this study was the most susceptible cultivar.Peroxidase activity increased in SCMV-infected sugarcane indicating that infection affects sugarcane physiology by inducing activity of catalase resulting in higher activity of peroxidase to produce H^2O^2. We suggest that although the plants exhibited a response against SCMV infection, they were unable to inhibit the development of SCMV, resulting in the appearance of symptoms. During the infection stage, the virus may change post-transcriptional gene silencing, alter particles movement, and affect host biochemical and physiological changes [17]. Interestingly, we observed an unusual phenomenon between SPS activity and sucrose accumulation in leaves.

We suggest that the lower activity of SPS in infected leaves occurred because of the inhibition of the plant to produce normal levels of SPS protein. Less abundant SPS production caused lower SPS activity in leaves. However, the mechanism of how SCMV infection affects SPS protein biosynthesis remains unclear. Since SPS plays a crucial role in sucrose biosynthesis, incorporating with Sucrose Phosphate Phosphatase (SPP), the increased activity of SPS would result in a higher sucrose accumulation [17]. We suggest a lower SPS activity, but higher sucrose accumulation may occur during virus infection, resulting in the reduction of total chlorophyll, which consequently leads to lower light absorption and abnormal phloem functionality [17]. The lower activity of SPS may be due to the higher sucrose accumulation itself by downregulating SPS by inhibiting the enzyme activity, but not its expression. This suggestion was supported by [11] and, in that sucrose reduced SPS activity by

inhibiting and inactivating the enzyme. Sucrose is the main photo-assimilate translocated from source to sinks via phloem. Plant viruses remain in simplest and need to move systemically via phloem (for long distance), by which a virus-encoded protein facilitates its movements and alters the size of plasmodesmata, leading to the impairment of photo-assimilate trafficking, including sucrose. Modification or alteration of phloem in infected leaves affects the translocation of sucrose from source to sink on potyvirus infection in melon by Cucumber mosaic virus (CMV).

CONCLUSION

This study confirmed that Sugarcane mosaic virus (SCMV) was the causative agent of mosaic on sugarcane observed in Punjab, Pakistan. Symptom of mosaic appeared on the fourth leaves upward from the inoculation leaf, in addition to showing some changes in those leaves including peroxidase, chlorophyll, as well as sucrose phosphate synthase (SPS).

REFERENCES

Putra, L.K.; Kristini, A.; Achadian, E.M.; Damayanti, T.A. Sugarcane streak mosaic virus in Pakistan: Distribution, characterization, yield losses and management approaches. Sugar Tech 2014, 16, 392–399.

Rao, G.R.; Chatenet, M.; Girard, J.G.; Rott, P. Distribution of Sugarcane mosaic and Sugarcane streak mosaic virus in India. Sugar Tech 2006, 8, 79–81.

Luo, Q.; Ahmad, K.; Fu, H.Y.; Wang, J.D.; Chen, R.K.; Gao, S.J. Genetic diversity & population structure of SMV infecting Saccharum spp. hybrids. Ann. Appl. Biol. 2016, 169, 398–407.

Arif, M.; Ali, M.; Rehman, A.; Fahim, M. Detection of Potato mop-top virus in soils and potato tubers using bait-plant bioassay, ELISA and RT-PCR. J. Virol. Methods 2014, 195, 221–227.

Reddy, C.V.S.; Sreenivasulu, P.; Sekhar, G. Duplex-immunocapture-RT-PCR for detection and discrimination of two distinct potyviruses naturally infecting sugarcane (Saccharum spp. hybrid). Ind. J. Exp. Biol. 2011, 49, 68–73.

Jiang, J.X.; Chen, Z.X.; Zhou, X.P. Production of a monoclonal antibody to sugarcane mosaic virus and its application for virus detection in China. J. Phytopathol. 2003, 151, 361–364.

Urcuqui-Inchima, S.; Haenni, A.L.; Bernardi, F. Potyvirus proteins: A wealth of functions. Virus Res. 2001, 74, 157–175.

Shalitin, D.; Wolf, S. Cucumber mosaic virus infection affects sugar transport in melon plants. Plant Physiol. 2000, 123, 597–604.

Clark, C.A.; Davis, J.A.; Abad, J.A.; Cuellar, W.J.; Fuentes, S.; Kreuze, J.F.; Gibson, R.W.; Mukasa, S.B.; Tugume, A.K.; Tairo, F.D.; et al. Sweetpotato viruses: 15 years of progress of understanding and managing complex diseases. Plant Dis. 2012, 96, 168–185.

Putra, L.K.; Ogle, H.J.; James, A.P.; Whittle, P.J.L. Distribution of Sugarcane mosaic virus in sugarcane plants. Australasian Plant Pathol. 2003, 32, 305–307.

Fu, W.L.; Sun, S.R.; Su, J.W.; Gao, S.J. A one-step real-time RT-PCR assay for the detection and quantitation of Sugarcane streak mosaic virus. Biomed. Res. Int. 2015, 2015, 569131.

Chaves-Bedoya, G.; Espejel, F.; Alcalá-Briseño, R.I.; Hernández-Vela, J.; Silva-Rosales, L. Short distance movement of genomic negative strands in a host and nonhost for Sugarcane mosaic virus (SCMV). Virol. J. 2011, 8, 15.

Gemechu, A.L.; Chiemsombat, P.; Attathom, S.; Reanwarakorn, K.; Lersrutaiyotin, R. Cloning and sequence analysis of coat protein gene for characterization of Sugarcane mosaic virus isolated from sugarcane and maize in Thailand. Arch. Virol. 2006, 151, 167–172.

Xie, X.; Chen, W.; Fu, Q.; Zhang, P.; An, T.; Cui, A.; An, D. Molecular variability and distribution of Sugarcane mosaic virus in Shanxi, China. PLoS ONE 2016, 11, e0151549.

Hull, R. Comparative Plant Virology, 2nd ed.; Elsevier Academic Press: British, UK, 2009.

Rani, P.U.; Jyothsna, Y. Biochemical and enzymatic changes in rice as a mechanism of defense. Acta Physiol. Plant. 2010, 32, 695–701.

Salerno, G.L.; Pontis, H.G. Studies on sucrose phosphate synthetase: The inhibitory action of sucrose. FEBS Lett. 1978, 86, 263–267.