Detection and cloning of P1 gene of *Sugarcane mosaic virus*

Aroosa Maqsood

00000170723

DEPARTMENT OF PLANT BIOTECHNOLOGY

ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY
ISLAMABAD, PAKISTAN

2018

Detection and cloning of P1 gene of *Sugarcane mosaic virus*

Aroosa Maqsood

00000170723

Master of Science in Plant Biotechnology

Supervisor

Dr.Muhammad Qasim Hayat

Assistant Professor

DEPARTMENT OF PLANT BIOTECHNOLOGY

ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY
ISLAMABAD, PAKISTAN

2018

Detection and cloning of P1 gene of *Sugarcane mosaic virus*

By

Aroosa Maqsood

00000170723

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Plant Biotechnology

Thesis Supervisor:

Dr. Muhammad Qasim Hayat

Assistant Professor

Thesis Co Supervisor:

Dr. Muhammad Tahir

Assistant Professor

DEPARTMENT OF PLANT BIOTECHNOLOGY

ATTA-UR-RAHMAN SCHOOL OF APPLIED BIOSCIENCES

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY ISLAMABAD, PAKISTAN

2018

FORM TH-4

National University of Sciences & Technology

MS THESIS WORK

We hereby recommend that the dissertation prepared under our supervision by:**Aroosa Maqsood** (registration no)00000170723

Titled: **Detection and cloning of P1 gene of Sugarcane mosaic virus** be accepted in partial fulfillment of the requirements for the award of Master of Science in Plant Biotechnology degree with (_____grade).

Examination Committee Members

1. Dr Muhammad Tahir **(Co supervisor)** Signature: _____
2. Dr. Najam Us Sahar Sadaf Zaidi Signature: _____
3. Dr. Muhammad Faraz Bhatti Signature: _____

Supervisor's name: Dr. Muhammad Qasim Hayat Signature: _____

Date: _____

Date: _____ _____

Head of Department

COUNTERSINGED

Date: _____ _____

Dean/Principal


بسم الله الرحمن الرحيم

THESIS ACCEPTANCE CERTIFICATE

Certified that final copy of MS/MPhil thesis written by Ms **Aroosa Maqsood,** (Regiatration No. **00000170723**), of Atta-ur-Rehman School of Applied Biosciences, NUST (School/College/Institute) has been vetted by undersigned, found complete on all respects as per NUST Statutes/Regulations, is free of plagiarism, errors, and mistakes and is accepted as partial fulfillment for award of MS/MPhil degree. It is further certified that necessary amendments as pointed out by GEC Members of the scholar have also been incorporated in the said thesis.

Signature: _____

Name of Supervisor _____**Dr. Muhammad Qasim Hayat**

Date: _____

Signature (HOD): _____

Date: _____

Signature (Dean/Principal) _____

Date: _____

DECLARATION

I certify that this research work titled **Detection and cloning of P1 gene of Sugarcane mosaic virus** is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

Aroosa Maqsood
00000170723

PLAGIARISM CERTIFICATE

It is to confirm that MS thesis entitled "**Detection and cloning of P1 gene of Sugarcane mosaic virus**" of **Ms. Aroosa Maqsood** Reg No. **00000170723** has been examined by me. I undertake that,

1. Thesis has significant new work/knowledge as compares to already elsewhere. No sentence, table, equation, diagram, paragraph or section has copied verbatim from previous work except when except when placed under quotation marks and duly referenced.

2. The work presented is original and own work of author i.e. there is no plagiarism. No idea, results or works of others have been presented as author's own work.

3. There is no fabrication of data or results such that the research is not accurately represented in the records. The thesis has been checked using Turnitin, a copy of the originality report attached and focused within the limits as per HEC plagiarism policy and instruction based from time to time.

_____

(Supervisor)

Dr. Muhammad Qasim Hayat

Assistant professor.

ASAB, NUST

COPYRIGHT STATEMENT

ACKNOWLEDGEMENTS

I am thankful to my Creator, **Allah Subhana-Watala** to have guided me throughout this work at every step and for every new perception which ALLAH setup in my mind to improve it. Indeed, I could have done nothing without ALLAH's guidance. Whosoever helped me throughout the course of my thesis, whether my parents or any other individual was His will, so indeed none be worthy of praise but ALLAH. Peace and blessings of Allah be upon last **Prophet Muhammad (Peace Be upon Him).**

I want to place my humble thankfulness to my honorable mentor, prestigious leader, my supervisor **Dr. Muhammad Qasim Hayat** for encouraging me to deal with issues related to my research during last two years. I can safely say that I have been extremely lucky to have a supervisor like him who responded to my questions and queries so promptly. His attention, constructive suggestions and cheerful enthusiasm helped me greatly throughout my experimental and thesis work. My success beholds your support.

I feel my heartfelt gratitude towards my teacher and Co supervisor, **Dr. Muhammad Tahir** for his sincere guidance and motivations throughout my MS. I am thankful to you sir for sharing with us "The key to Success" that inspired me to achieve my goals. I am grateful to Principal ASAB, **Dr. Peter John** and Rector NUST, **Lieutenant General Naweed Zaman** for providing us righteous place and resources to carry out the project.

I profound special thanks to my GEC committee members **Dr. Najam Us Sahar Sadaf Zaidi** and **Dr. Muhammad Faraz Bhatti** for their guidance and support.

I would extend my thanks to the Higher Education Commission (HEC) for financial assistance through NRPU project no 5968 entitled as "**Sugarcane productivity by development of transgenic virus resistance through expression of hairpinRNA (hpRNA) carrying sugarcane mosaic virus derived sequences"**

I express my heartfelt gratitude to all my friends and fellows for their support and encouragements throughput the research

Lastly, a special thanks to my mother for her infinite prayers and my sisters Raheela, Aneela, Amna, Bushra and my brother Abdul Wahab for their endless support, unconditional love and encouragement throughout my studies

Aroosa Maqsood

Dedication

Dedicated to my beloved Ami for her affection, prayers and love

A**BSTRACT**

Sugarcane is the main cash crop of the Pakistan. Pakistan is the seventh largest Sugarcane producing country. Major loss of sugarcane yield is due to *Sugarcane mosaic virus* which belongs to family *potyvirid*, largest family of plant viruses containing single stranded positive sense RNA.Three major proteases are included in the genome of the SCMV i.e. P1, Hc Pro, N1a, P2-1 helper component. P1 is the major non conservative protein of the SCMV. Non-conservative nature of the P1 gene of the sugarcane is responsible for determining host range of the sugarcane. The Identification and cloning of the P1 gene of the *Sugarcane mosaic virus* will be helpful for screening of the infected sugarcane cultivars of the Pakistan

LIST OF ABBREVIATIONS

Approx	Approximately
$CaCl_2$	Calcium chloride
cDNA	Complementary DNA
CP	Coat protein
DNA	Deoxy ribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
g	gram
H2O	water
HCL	Hydrochloric Acid
i.e.	id est mean that is
ICTV	International Committee on Taxonomy of Viruses
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kb	kilobases
LB	Luria Bertani

M	molar
MDMV	Maize Dwarf mosaic virus
MgCl2	magnesium chloride
min	minute
ml	milliliter
M-MuLV	Moloney Murine Leukemia Virus
MRC	Molecular Research Center, Inc
NaCl	sodium chloride
nm	nano meter
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RPM	Revolutions Per Minute
rpm	revolution per minute
SCMV	Sugarcane mosaic virus
ssDNA	single stranded DNA
SSMV	Sugarcane streak mosaic virus
TAE buffer	Tris-acetate-EDTA
TBE	Tris Borate EDTA

TE buffer	Tris-EDTA buffer
ug	microgram
ul	microliter
UV	ultraviolet
V	volt
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Contents

THESIS ACCEPTANCE CERTIFICATE vi
DECLARATION vii
PLAGIARISM CERTIFICATE viii
COPYRIGHT STATEMENT ix
ACKNOWLEDGEMENTS x
ABSTRACT xiii
LIST OF ABBREVIATIONS xiv
INTRODUCTION 1
Sugarcane 1
Importance of Sugarcane 1
Stress factor affecting Sugarcane yield 2
Sugarcane mosaic virus 4
Symptoms of Sugarcane mosaic virus 5
Host range of the Sugarcane mosaic virus 6
Genomic organization of the *Sugarcane mosaic virus* 8
Proteases of the Sugarcane mosaic virus 11
LITERATURE REVIEW 14
OBJECTIVES 26
MATERIALS AND METHODS 27
Laboratory precautionary measures for virology lab 27
Sterilization 27
Media and solution 28
Nutrient agar media 28
LB media 28
TE buffer 1X 28
TAE buffer solution 50X 28
TAE buffer solution 1X 29
Glycerol solution 10% 29
Resuspension solution 29
Neutralization solution 29

Lysis solution 29
Ampicillin 100mg/mL 30
Glycerol solution for preservation of stock 30
Sample collection 31
Total RNA extraction 31
Analysis of RNA 32
Agarose gel electrophoresis 32
Quantification of RNA 32
cDNA synthesis 32
Polymerase chain reaction 33
Agarose gel electrophoresis 33
Gel elution of PCR product 35
Gel electrophoresis 35
Ligation 36
Preparation of the DH5α competent cells by $CaCl_2$ Method 36
Transformation with DH5α competent cells 37
Plasmid preparation 37
Confirmation of the clone through Restriction digestion 38
Directional cloning 38
RESULTS AND DISCUSSION 39
Sample collection 39
RNA isolation 44
Complementary DNA (cDNA) synthesis 44
Polymerase chain reaction 45
Gene clean 46
T/A cloning 47
Screening of clone through plasmid isolation and Plasmid PCR 48
Confirmation of the clone through restriction digestion. 49
REFERENCES 1

CHAPTER: 1

INTRODUCTION

Sugarcane

Sugarcane *Saccharum officinarum* belong to C4 perennial grass of the family *Poaceae*. It is second largest cash crop of Pakistan, while Pakistan is the 7th largest sugarcane producing country. Sugarcane is being cultivated on 0.966 million hectares which almost contributes about 3.6 % of Gross domestic production (GDP) For Pakistan. **(Qureshi, 2005)**.Sugarcane is tropical grass which is cultivated in different areas of the Pakistan according to the different environmental conditions such as temperature, soil texture and water. Sugarcane is extensively grown in all provinces of Pakistan i.e. Punjab, Khyber-Pakhtunkhwa and Sindh. Sugarcane varieties which are grown worldwide are basically hybrids which are the combination of the two-parental species i.e. *Saccharum officinarum* and *Saccharum spontaneum* **(Cox et al. 2000)**

Importance of Sugarcane

Sugarcane is the main cause to produce sucrose. Sucrose is the most needed carbohydrate for entire world. Sucrose account for approximately 40 percent of the dry dead matter product of the sugarcane. On the industrial point of view sucrose is being produced from two industrial crops.one of them is sugar cane *Saccharum officnarum*. approximately sugarcane is responsible for the 70 percent production of the sugar, while

rest of 30 percent of the sugar is being produced from the sugar beet *Beta vulgaris*. Sugarcane is used to produce crystal white sugar, gur and shkka. Production of sugar also have an important role in the national and international economy of Pakistan, for example production biofuel, fibre, organic fertilizers, Molasses, ethanol, bagasse.**(Qureshi, 2005).** The sugarcane industry also provides approximately 4 billion rupees under the head of general sales tax. Sugar industry contributes substantially to the rural economy of the Pakistan as the mills are in rural areas of Pakistan. **(Anonymous, 2005)**. Primary product of the sugarcane industry is sugar, and this Sugarcane industry is directly or indirectly linked to the approximately 25 other industries. The by-product of the sugarcane industry is bagasse is burnt to produce energy, because bagasse has potential to produce more energy. By product of sugarcane industry are also used to produce the molasses, fibres, paper and pulp. Other by product of the sugarcane industry for example molasses, ash and mud are source of reactant for other major industries. **(Taupier and Bugallo 2000)**

Stress factor affecting Sugarcane yield

There are different types of the stress factors which are responsible for the affecting the sugarcane productivity worldwide. There are two major types of the stress factors i.e. biotic (living) stress factors and abiotic(non-living) stress factors. Abiotic stress factors consist of fluctuation ion in the temperature due to any type of the climatic change, change in water conditions, alkalinity and salinity conditions, which overall affect the productivity of the sugarcane. **(Bock and Baily, 1989).** Biotic factors mostly include viruses, bacteria, nematodes, fungi, and phytoplasma. On an average almost 50 diseases of sugarcane crop have been in record to be caused by viruses and phytoplasma.

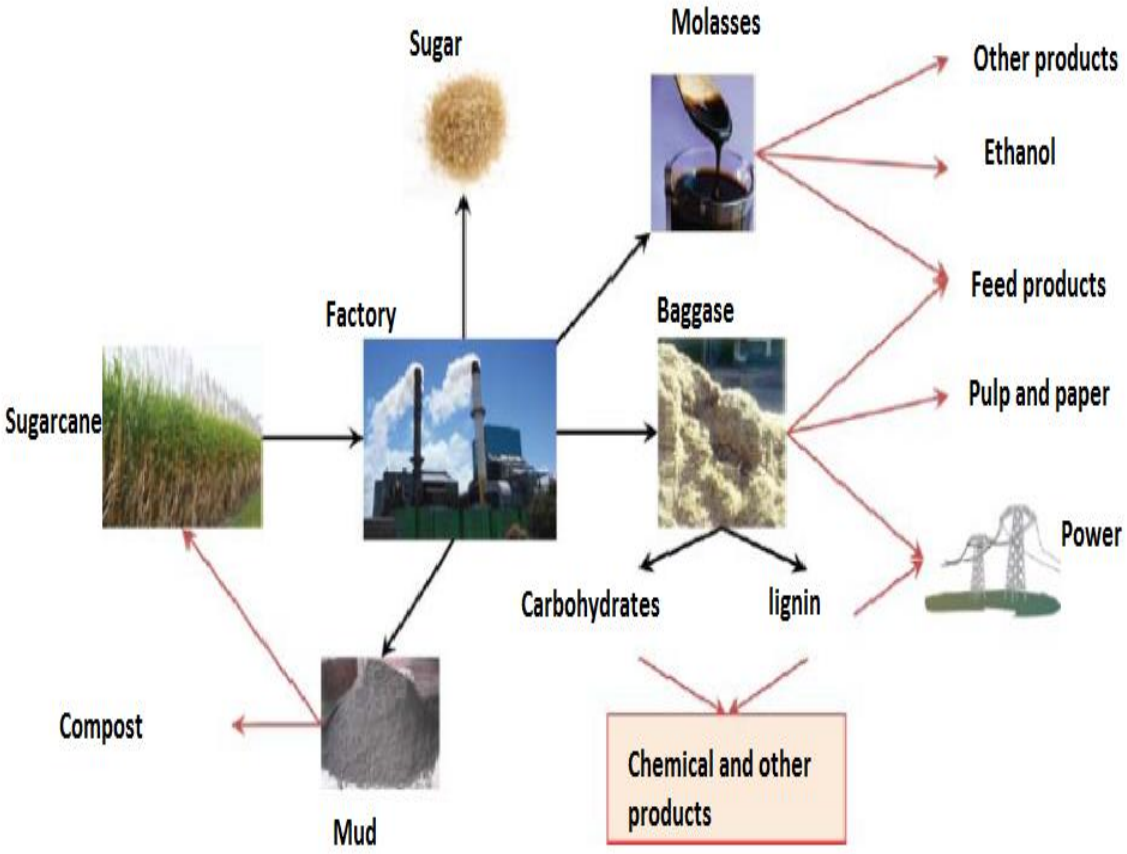


Figure 1: Diagrammatic representation showing importance of sugar industry and explaining how sugar industry is related to other industries. **(Rein et al, 2007*)***

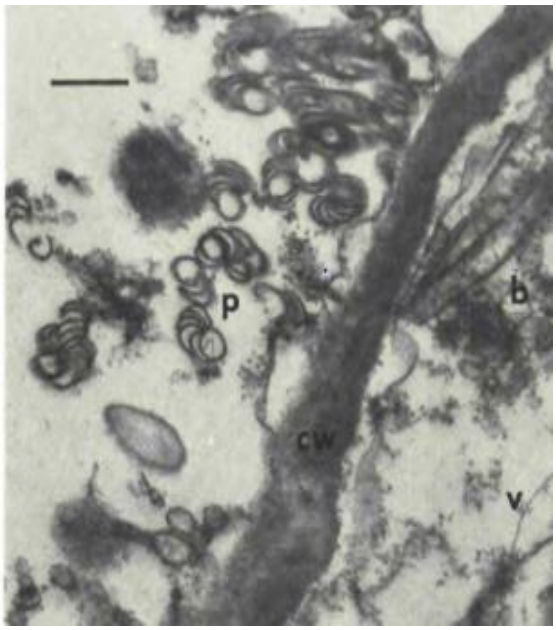
Sugarcane mosaic virus

Viruses are known for the ability to destroy and invade in various range of organisms, such as fungi, animals, plants, algae, bacteria, and viruses. Plant viruses make a systemic infection in their hosts and persist throughout the life of the infected plants. Mosaic is one of the most major and important disease of the sugarcane which is worldwide distributed. Causative agent of the Mosaic in sugarcane is sugarcane mosaic virus, but there are also other viruses which are responsible for mosaic in sugarcane for example *Sugarcane streak mosaic virus.* **(Viswanathan and Rao, 2011)** Sugarcane mosaic virus is the cause of almost 6-10% sugar yield loses and 10-32% cane reduction in overall Pakistan every year **(Anwar,2005)** *Sugarcane mosaic virus* (SCMV) belongs to the family Potyvirid. Family potyvirid is the largest group of the plant viruses which contain approximately 30 percent of the plant viruses and 200 species of the plant viruses **(Wylie *et al.*, 2017).** *Sugarcane mosaic virus* is the very major hindrance in the selection and screening of the breeding cultivar.

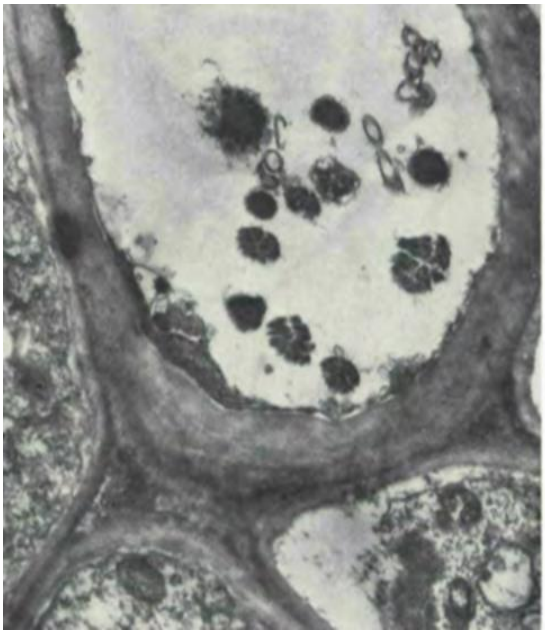
Figure 2: Long flexuous filamentous SCMV particles **(Raoet al. 1996a)**

Symptoms of *Sugarcane mosaic virus*

Symptoms of *sugarcane mosaic virus* include chlorotic area in young and developing leaf blades of sugarcane along the pattern of parallel venation of the leaf blades, sometime reddening also appear at the edge of the leaf margin. Contrasting shades of dark green and yellow colour can also be observed on leaves. **(Croft et al 2000).**



A



B

Figure 3: A: pinwheel and bundle type of cytoplasmic micro inclusion bodies in the mesophyll and epidermal cells of infected leaf sample **B:** cytoplasmic inclusion bodies due to sugarcane mosaic virus in sieve cells of infected leaf sample

Leaf curling, plant dwarfism and deterioration of flower and fruit also appear at the late stages of the infection. **(Handley et al., 2001).** Sugarcane mosaic virus symptoms are most prominent in the young and dividing tissues of the plants**.** Irregular moulting and stunt growth of the younger plant has also been observed in young leaves. Later stage of the infection Chloris and necrosis are also observed in the leaves. Chloris and necrosis pattern can be seen along the pattern of the venation. As sugarcane belong to family *Poaceae* which exhibit parallel venation, so chlorotic and necrosis pattern can be seen along the pattern of the venation. **(Koike and Gillaspie, 1989).** While most important and significant host of the Sugarcane mosaic virus is sugarcane species which induce the different type of symptoms in the host as shown in **Fig 3**

There are almost six various strain of the Sugarcane mosaic virus which are responsible for infection in Sugarcane crop. Mixed infection due to the combination of the two or more than two strains have also been observed in the sugarcane.

Host range of the *Sugarcane mosaic virus*

Mostly Sugarcane mosaic virus infection is the restricted to the gramineous host i.e. sugarcane *(saccharum officinarum),* Sorghum *(Sorghum bicolor),* barley *(Hordeum vulgare).*some wild grasses as well as some ornamental plants growing near the major crop field are also infected with *Sugarcane mosaic virus.*

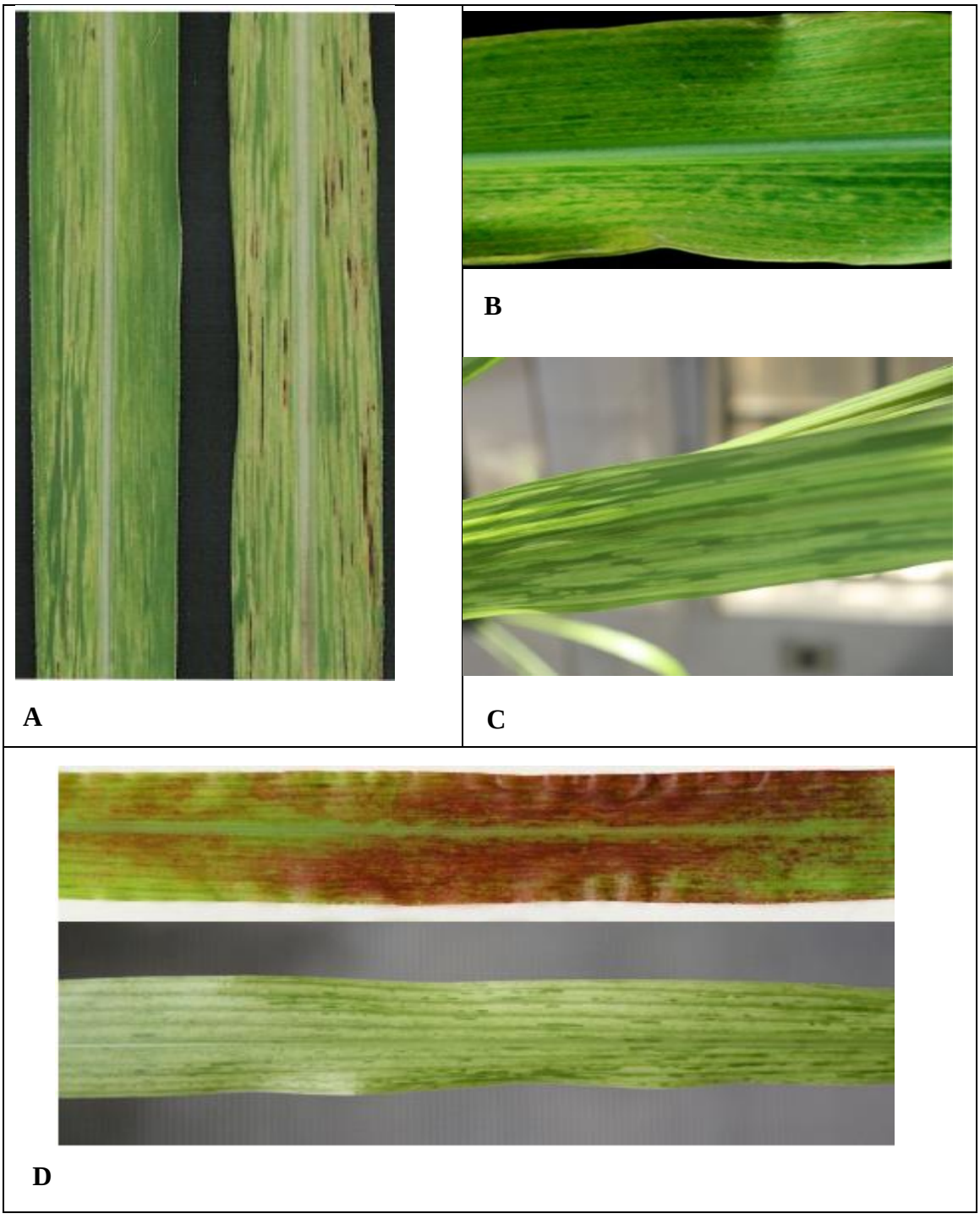


Figure 4: sugarcane plants showing infection of the *Sugarcane mosaic virus* on infected leaves**. A:** severe mosaic and partial margin necrosis symptoms can be observed on the leaves**, B:** mosaic on leaves: alternating contrat of yellow and green **D**: reddening on the sorghum leaves due to *Sugarcane mosaic virus* **(Gonçalves et al, 2012)**

Genomic organization of the *Sugarcane mosaic virus*

Sugarcane mosaic virus (SCMV) is transmitted by aphid, mites and white fly by non-persistent manner. Genome contain single stranded positive(+) sense RNA containing 5 end viral genome(VPG) linked protein and 3 end poly A tail, while RNA consists of nucleotide sequence of about 1000bp. that is translated into single major polyprotein by proteolysis genome like other members of potyvirid contain a single major open reading frame that is autoproteolytic cleavage into ten mature major proteins abbreviated as P1(peptidase1), P3, HC-Pro(helper component), 6K1, CI, 6K2, , NIa- Pro, VPg Nlb, and CP(coat protein). **Viswanathan *et al.* (2009a).** all these proteins have different role in the entire life cycle of the SCMV mentioned briefly below

Sequence of P1 protein is much more variable as compared to the other proteins of the Sugarcane mosaic virus. And their actual role in entire virus life cycle of the SCMV is not yet clear. Lot of evolution driving events are responsible for the diversification of the P1 gene i.e. random gene duplication and recombination events. All these events are responsible for the adaptation of the *Sugarcane mosaic virus* (SCMV) to different host range. The recombination events in virus i.e. *Sugarcane mosaic virus* (SCMV) also dependent upon the type of host for example recombination events in *sugarcane mosaic virus* (SCMV) isolated from sugarcane *(saccharum* spp), are different than that of SCMV isolated from Zea Mays (Maize).

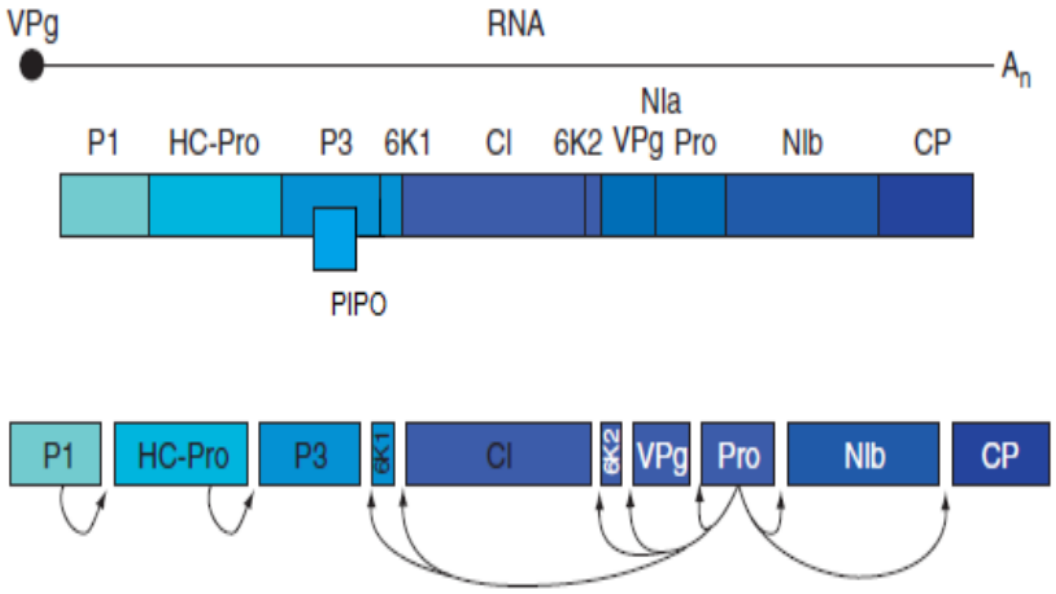


Figure 5: Genomic map of the *sugarcane mosaic virus.* **(Rodamilans et al, 2013)**

Genome contain a single stranded positive (+) sense RNA shown in **Fig 5** which is represented by single line. RNA contains 5 prime genome linked protein which is covalently attached while poly A is attached at 3 prime terminals of the genome. It consists of single open reading frame which is translated into the single polyprotein. But at next level this single poly protein is cleaved with the help of the viral peptidases into the individual proteins. There are three major peptidases which are present in the genome of the Sugarcane mosaic virus, while a recently discovered major open reading frame i.e. pretty interesting potyvirid open reading frame (PIPO) can also be seen in genomic map **(Rodamilans et al, 2013)**

Although mutation and change in genetic sequence of P1 protein does not drastically affect the transmission mode of the Sugar cane mosaic virus. **(Verchot and Carrington, 1995a, 1995b)** Coding amino acid sequence of the P1 protein is present at the start of the genome. Some members of the potyvirid family encodes only one P1 peptidase while some have two copies of the P1 which are responsible for different types of the proteolytic specifications. **(Rodamilans *et al.*, 2013**). P1 protein is most diverse and divergent protein which vary in length and its amino acid sequence. Non-conservative nature of P1 protein of SCMV is responsible for the SCMV adaptation to various host. The first N terminal peptidase which is taking a major part in processing of polyprotein of sugarcane mosaic virus remain least studied.

P1 peptidase along with its helper components proteinase i.e. Hc pro plays an important in potyvirus transmission as proved by mutagenesis experiments. Coding region of P1 peptidase was cloned by mutation in the region. Experiments only mutation in P1 region does not affect the virus transmission. P1 peptidase along with HC pro is responsible for the virus infectivity and infection. (**Verchot and Carrington 1995b)** the response of P1 protease which are observed in modulating replication of the genome is in actual the result of the ability of the p1 peptidase in counter defence mechanisms of the host immunity. **(Jupin et al, 2017)**

Hc peptidase (Hc Pro) is a major multifunctional protein of the *Sugarcane mosaic virus* (SCMV). The helper component peptidase is functional in its dimeric and polymeric foam and it also exhibit three conserved structural motifs. N terminal domain of Hc pro

contain the conserved domain of KITC amino acid which is helpful for the association of the Hc pro with aphid stylet. Mutagenesis experiment prove that amino acid Lysine (K) is more helpful for the interaction of the virus with aphid stylet as compared to other three amino acids. **(Blanc *et al*., 1998**) Hc pro is also responsible for the formation of the characteristic amorphous and cytoplasmic inclusion bodies in the cytosol and cytoplasm of the infected cells. **(Dolja *et al*., 1993).**

Proteases of the *Sugarcane mosaic virus*

As plant viruses have a small genomic size and have confined genetic space in the cells, so plant viruses use several alternative ways to produce the protein for completing their life cycle**. (Miras et al., 2017).**one of the important strategy is the production of the polyprotein, that are mostly processed with the help of the viral protease into the smaller proteins. This strategy is helpful to produce different type of protein required for establishing a viral infection from single molecule and by the single set of translational and transcriptional control elements saving time and space**,** thus gene expression of the polyprotein is extremely dependent on the major proteolytic activity of the viral proteases**. (Konvalinka et al., 2015).**

Apart from proteolytic cleavage plant viral protease also perform some major functions **(Liu et al., 2009)** for example:

1. Suppression of the RNA silencing
2. Host range determination
3. Aphid based transmission

4. Cell to cell movement of the virus particle.
5. Systematic movement of the viral infection throughout the plant cells
6. Viral accumulation in the cells
7. Virus particle maturation in the host adapted cells.
8. Counter defence mechanism on host

Among all the plant viruses' family potyvirid have high variety of the protease and protease activity. Potyviral genome have a single stranded positive (+) sense RNA that encodes four different types of the proteases **(Revers and García, 2015)**, with different specific functions.

1. P1 also subclassified into P1a andP1b
2. Helper component protease
3. N1a proteases
4. P2-1 helper component like proteases

In the lifecycle of the sugarcane mosaic virus polyprotein processing is a highly regulatory mechanism. **(Ivanov *et al.*, 2014).** Processing of the potyviral polyprotein is highly coordinated with the virus replication, assembly of the viral particle and movement across the cells as well as systemic infection of the virus along whole plant. Based on the certain features viral peptidases can be separated from the host proteases based on which they act as biotechnological tool, i.e**.** (**Tran et al., 2017)**

1. Viral proteases are smaller
2. Viral proteases possess certain sequence similarities that are restricted to the active

sites.

3. Viral proteases are multifunctional which are helpful for their adaptations.
4. Viral proteases are very much specific as compared to the host cells proteases.

Polyprotein processing is a highly modulated process. Among all the proteases studies in the life cycle of the potyviral, N1a proteases is the most characterized proteases which help in modulation during replication of the virus particles. N1a proteases is a cysteine protease that release different intermediate and final processed product in the infection cycle of the virus. N1a protease of the family potyvirid also involved in the host range determination. These processed protein products are involved for the establishment of the replication complex **(Cui and Wang, 2016).**

CHAPTER 2:

LITERATURE REVIEW

Sugarcane mosaic virus belongs to family Potyvirid and genus potyvirus. *Sugar cane mosaic virus* is a serious threat for sugarcane *Saccharum officnarium* crop as well as other monocots crop e.g. *Zea mays* (maize), *Sorghum bicolor* (sorghum) and other *Poaceae* species. Disease is spreading fast due to the cultivation of infected varieties and presence of various type of pests which are responsible for the systemic infection of the virus. A lot of efforts are being carried out for controlling the virus, but high rate of resistance and recombination are hindrance. Some of the work carried out related to this field and technique are mentioned below for better understanding:

Apriasti et al. (2018) reported that full length sequence details of the CP (Coat protein) gene is essential for disruptions of the viral packaging and assembly because N terminal sequence of coat protein sequence is responsible for binding with viral ribonucleic acid. They amplified the full-length sequence approximately 980bp sequence of the coat protein (CP), and used the agrobacterium mediated gene transformation method for the formation of the transgenes using coat protein (CP) sequence. Artificial infection in transgenic lines show better resistance and tolerance to the infection and this infection was also observed in next generation proving that complete full-length sequence is necessary or viral assembly as well as viral packaging.

Gustafson et al. (2018) identified key genomic regions in *Sugarcane mosaic virus(SCMV)* and *Maize dwarf mosaic virus(MDMV)* which are responsible for resistance in host cells. They used the artificial inoculation method for observing the behavior of the virus particle in the host cell. Inbred line off three leaf stage was used in experiment. Leaf were artificially inoculated with SCMV and then calculation was done at 7, 10, 14, 21 days showing that inbred with the absence alleles were more susceptible to the viral infection.

Xia et al (2018) elaborate the miRNA mediated regulatory network in maize in response of the *Sugarcane mosaic virus* infection. miRNA mediated regulatory network is more complex network, in total 154 already known miRNA while 213 novel miRNAs were profiled together. The behavior of these miRNAs was different upon infection with *Sugarcane mosaic virus* particles. With the help of the degradome analysis three novel miRNAs was identified, while northern blot analysis and the RT PCR quantitative real time polymerase chain reaction prove that these target miRNAs are mostly infected after the twelve days of the post inoculation infection.

Shan et al (2018) elaborated the new insights for the evolution and adaptation of the potyvirus, potyviral protein P1 cleave itself from the other viral proteins and it exhibit the great sequence variability which is greatly helpful for the adaptation of the this virus to different host range. Invitro cleavage analysis of the potyviral protein P1 from the polyprotein shows that N terminal domain is relevant to the host virus association and adaptation.

Sankaranarayanan et al. (2018) reported that Hubei poty like virus should be considered as strain of the Sugarcane mosaic virus instead of considering it as a new potyviral specie. Recombination event and specie demarcation tool prove that it is more likely to be placed as strain of SCMV. Previously Hubei poty-like virus 1 (HuPLV1) as a new member of the family *Potyvirid* based on it recombination and phylogenetic analysis compared to *Sugarcane mosaic virus(SCMV)* and *Bean yellow mosaic virus (*BYMV)

Xu et al. (2018) Studied the expression patternof *Sugarcane mosaic virus* in Maize at translational level, which clearly demonstrate the physiological effect of the *Sugarcane mosaic virus* on Maize at transcriptional as well as translational level. Both SCMV single stranded RNA and proteins are accumulated in the host cells upon starting of the systemic infection of the viral particle. Upon this stage symptoms of mosaic start appearing on infected leaves. At the early stage of the virus infection SCMV particles use very little host resources for the establishment of the infection. Global gene expression analysis and various gene oncology (GO) experiments helps to analysis the physiological changes upon SCMV infection that at both transcriptional as well as translational levels metabolism and photosynthesis rate decreased in host cells. *Sugarcane mosaic virus* occupy very translational resources of the host plant i.e. Maize.

Tang et al. (2018) reported for the very first time full length sequence of the two *Sugarcane mosaic virus* isolates infecting Canna *spp***.,** comprising of 9576 nucleotides which have single open reading frame which encode a polyprotein of 3063 amino acids.

Aslam et al. (2018) used the virus based short hairpin RNA (sh RNA) from conserved region of coat protein (CP) sequence of sugarcane mosaic to initiate resistance in transgenic lines of sugarcane. About 456 base pair sequence of the Coat protein (CP) was used as target. A stable short hairpin loop containing stem and loop structure was used as a tool. Molecular identification and southern blot analysis confirmed the presence of Anti SCMV particles in the transgenic lines. Upon mechanical inoculation 80% of the sugarcane varieties show resistance to SCMV.

Darsono et al. (2018) used the immunodiagnostic method to produce the polyclonal antibody using the recombinant sequence of the coat protein (CP) of the *Sugarcane mosaic virus*. Polyclonal antibody exhibits great sensitivity and specificity for detection of *Sugarcane mosaic virus*, as it can detect the recombinant protein at the concentration of 10ng.purified recombinant protein was used as antigen to produce the polyclonal antibody**.**

Valli et al (2018) described the independent detailed role of the HC Pro for cell to cell movement of virus, maturation of the poly protein as well as RNA silencing expression**.** They explain that plant viral protease apart from catalytic cleavage of the polyprotein play major role in viral infection throughout the life cycle of the virus. Plant viral proteinase are responsible for the replication, assembly, aphid-based transport and finally maturation of the virus particle in the hot cell. Processing of the host protein through proteases also change the function and expression of the proteases itself.

Jupin *et al*. (2017) studied the specific role of plant viral proteases from escaping the host plant defense mechanism. Plant viral proteases not only help in modulating the replication of the genome but also responsible for the facing the host defense mechanism, so plan viral proteases can be considered as playing an important role in countering plant defense response against viruses i.e. P1 protease of the plum pox virus help in the altering defense mechanism of the host plants, whose affect can be observed in the replication of the virus particles.

Peng *et al* (2018) stated that N region of the coat protein (cp) is highly variable and thus it can be used to produce polyclonal antibody, because serological identification and detection of the potyviruses yield false positive results. Specific segment of the turnip mosaic virus was obtained through prokaryotic gene expression and this segment was used for the preparation of the polyclonal antibody.

Addy *et al*. (2017) conducted experiments for the determination of the causative agent of the Mosaic disease. As morphological symptoms of the *Sugarcane mosaic virus* and sugarcane streak mosaic virus are similar. Inoculation of the healthy plants with sap of the infected plants shows the symptoms of the mosaic in healthy leaves blades. Causative agent of the *Sugarcane mosaic virus* was also confirmed by amplification of the complementary DNA by polymerase chain reaction.

Moradi *et al.* (2016) analysis the complete genome sequence of 24 *Sugarcane mosaic virus* (SCMV) isolated from different host i.e. sugarcane, Maize, and sorghum. Genome of RNA contain single open reading frame that is cleaved into ten mature proteins ie P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, Nlb, and CP

Mollov *et al.* (2016) reported the occurrence of *Sugarcane mosaic virus* (SCMV) in grass species., Columbus grass *(Sorghum almum). S. almum* was present near to the Sugarcane field which serve as alternative host for the propagation of the *Sugarcane mosaic virus* (SCMV). Relationship between the strain of SCMV present in sugarcane and the that od *S. almum* is helpful for tracing the epidemiology of the disease.

Xie *et al*. (2016) reported various strain of *Sugarcane mosaic virus* (SCMV) in various selected area of China. The negative selection and the recombination event are the major causes for incidence of mosaic disease in China. *Sugarcane mosaic virus* exit in several strains, which are strongly associated with the host species. Epidemiological of *Sugarcane mosaic virus* is helpful for sustainable control of virus.

Putra *et al* (2015) described for the first-time incidence of the Sugarcane streak mosaic virus in Indonesia. Host plant of *Sugarcane mosaic virus* are very similar to the *sugarcane streak mosaic virus* i.e. sorghum, *Zea mays* (maize) and sugarcane. Different types of weed also serve as alternate secondary host for the virus.

Guo *et al.* (2015) use the RNA interference technology to produce the resistant transgenic sugarcane plants. Conserved region of the coat protein sequence was used for the formulation of the hairpin constructs. Resistance was assured by artificial inoculation of the *sorghum mosaic virus* which is a strain of *Sugarcane mosaic virus*. Transgenic plants provide different levels of resistance towards virus

Konvalinka *et al.* (2015) explained phenomenon of polyprotein processing in the virus life cycle. Plant virus have smaller genome size and confined smaller and confined place in the host cell where they must complete their life cycle for establishing a systemic infection. Polyprotein processing is one of the adaptation. in which virus release a smaller protein product with the help of the virus encoded proteolytic enzymes using the single parent molecule as well as single transcription and translation control machinery, so that partially processed viral proteins released in as control manner for establishing virus infectivity, however gene expression of the polyprotein completely depends upon the several protease activities.

Keizerweerd *et al.* (2015) developed the reverse transcription loop-mediated isothermal amplification (RT-LAMP) mediated method for the diagnostics of the *Sugarcane mosaic virus.* Whereas RT-LAMP shows less sensitivity as compared to the conventional diagnostic method e.g. real time RT PCR.

Ali *et al.* (2014) performed experiments for the analysis of the diversity of the *Sugarcane mosaic virus* from sugarcane varieties of Pakistan. They try to find out the correlation between the isolates of Pakistan with rest of the world. SCMV was detected using the RT PCR. They also report the novel *Sugarcane mosaic virus* in Pakistan named as SCMV-PK. Total 102 leaf samples from nine cultivars infected with *Sugarcane mosaic virus* (SCMV) were subjected to the RT (real time) PCR for the confirmation of the virus. All samples were positive for the *Sugarcane mosaic virus.*

Pasin *et al.* (2014) proved that although P1 protease act as non-essential factor in transmission and systemic infection of the *Sugarcane mosaic virus* (SCMV), but its

separation from the polyprotein impair functionally HC pro for downstream RNA silencing suppressor. Bioinformatic analysis and several in vitro experiments was used for mapping the C terminus domain, prove that P1 alone is not performing any special function in virus life cycle, but it is responsible for the cleavage of the HC pro and thus plays a major role in helping Hc pro for performing its function.

Thorat *et al.* (2014) use the DAS-ELISA (Double Antibody Sandwich -Enzyme linked Immunosorbent Assay) technique for the identification of the *Sugarcane mosaic virus in* the infected sugarcane leaves blades. DAS ELISA and RT PCR both can be used for the identification of the *Sugarcane mosaic virus*, but RT PCR is more reliable as compared to the other techniques

Rohožková *et al* (2011) elaborated the mysterious role of P1 peptidase. Despite the diversity of the P1 peptidase, it is very much important to identify the structure of this protein. Analysis of the structural conserved motifs (DAG)which are present at P1 are responsible for P1 interaction.

Yasmin *et al.* (2011) reported the sugarcane viruses i.e. *Sugarcane mosaic virus (SCMV), sugarcane bacilliform virus (SCBV), Maize dwarf mosaic virus* (MDMV) and Maize streak virus (MSV)in Punjab. They recorded that highest rate of infection is caused by the Maize streak virus. Their further analysis also confirmed the presence of *Sugarcane mosaic virus* in seventeen species of the Pakistan, which act as alternative host of the virus. **Viswanathan *et al.* (2009a)** studied the phylogeny of coat protein sequence of 30 Indian SCMV isolate. Phylogeny was studied based on the variable as well as concerned region

of coat protein. phylogenetic analysis does not show any similarity to the previous strain of SCMV in India.

Mbanzibwa *et al.* (2009) analyzed the complete RNA genome sequence of the Cassava brown streak virus (CBSV). Its amino acid sequence lacks the helper component (Hc Pro) but contained the amino acid sequence of the P1 protein which is strongly involved in the suppression of the RNA silencing. High variability and diversity is the characteristic feature of the family potyvirid.

Mohammadi *et al*. (2009) used the Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA) and Dot Immunobinding Assay (DIBA) techniques for the detection of the *Sugarcane mosaic virus* (SCMV) in sorghum. *Sugarcane mosaic virus* (SCMV) was detected in all collected infected samples of the sorghum

Chen *et al.* (2008) described the role of N1a protease in the determination of the host range for potyvirus. Mutation in the single amino acid of the N1a change the host range of the virus particle form cucrbitaceae to the Papaya. Modulation of the N1a protease activity is the host specific.

Valli *et al.* (2007) described the process of recombination and gene duplication for the analysis of the diversity of the P1 protein. The irregular distribution of the C terminal motif in P1 *of potyviridae* shows irregular evolutionary diversification.

Gemechu *et al.* (2006) cloned and sequence the coat protein (CP) gene of *Sugarcane mosaic virus* screened from sugarcane and *Zea mays* (Maize). immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) was used for the cloning of the respective gene Three sequences of CP was compared for the phylogeny**.**

Adam *et al.(*2005a) studied the sequence analysis of the potyviral genome. Providing the sequence of the conserved motif present at the junction of the P1 and Hc pro site.C terminal conserved junction contain serine proteinase domain which is responsible for the autoproteolytic cleavage of the polyprotein at the junction of the P1 and Hc Pro. They also studied the intraspecific and intergenic recombination events, proving that intergenic recombination events along with interspecific recombination a events are responsible for the potyvirus evolution and adaptation to different type of the host. Their analysis prove that extensive and uneven duplication and recombination events are responsible for the diversification of the P1 gene within the P1 gene high.

Adam *et al* (2004) mentioned the species demarcation criteria for the family *potyviridae*, based on the nucleotide identity and amino acid identity. Approximately 187 complete genome sequences of *potyviradae* family was used for phylogenetic analysis. For the entire open reading frame genus demarcation shows 46 percent nucleotide identity but this ratio does not separate Rymovirus from potyvirus. Taxonomic status of the rymovirus and other unclassified species was discussed briefly.Complete phylogeny of nucleotide sequence was base for taxonomy of the potyvirid.

Ullah *et al.* (2004) reported that despite of high variability in the in the N terminal sequence of the coat protein (CP), it is very much important for the transmission of the virus and for the adaptation of the virus. Despite of mutation in the N terminal region of the potyvirus, transgenic plant was unable to develop resistance, proving that variability and mutation in the N terminal region does not affect the transmission efficacy of the virus

Balamuralikrishnan *et al.* (2004) evaluated the different techniques for the diagnostics of the *Sugarcane mosaic virus.* They try to compare dot blot immunoassay, electron microscopy, reverse transcriptase polymerase chain reaction (RT PCR) for the identification of *Sugarcane mosaic virus* from the leaf tissue as well as from buds. They find out that reverse transcriptase PCR the effective way for the diagnostic of sugar cane mosaic virus as compared to the other techniques.

Sasaya *et al*. (2000) studied the transmission pattern of potyvirus by helper component proteinase. Hc pro gene was cloned and protein expression was performed. Hc pro has conserved domain of KITC. Alteration in the Lysine (K) residue drastically affect the binding capacity of the *Sugarcane mosaic virus* to aphid stylet.

Arbatova *et al.* (1998) described that P1 is responsible for the for the formation of cytoplasmic inclusion bodies in the *Sugarcane mosaic virus* infected leaves.

Rojas *et al.* (1997) studied the role of Coat protein CP gene in virus life cycle. Coat protein CP gene is responsible for the movement and encapsulation of the virus particles as well as responsible for the replication and duplication of the genome across the cells.

Verchot and Carrington (1995b) conducted mutagenesis experiments for the determination of function of P1. mutation in only P1 region does not affect the virus transmission affectively, but mutation in P1 region along with Hc Pro is more detrimental for virus amplification and systematic distribution, so tha**t** P1 behave as a non-essential accessory factor in the life cycle of the *Sugarcane mosaic virus* (SCMV**)**

Hancock *et al* (1995) described the phenomenon of replication slippage for the evolution of the potyviruses. Genome of potyviruses contained the multiple repetitive sequences which are helpful foe the virus transmission as well as for adaptation of various host range of viruses.

OBJECTIVES

- Detection of *Sugarcane mosaic virus*
- Partial characterization of the P1 gene of the *Sugarcane mosaic virus*

CHAPTER 3:

MATERIALS AND METHODS

Laboratory precautionary measures for virology lab

Precautionary safety measurements for working in plant virology laboratory has been followed throughout the project. Protective laboratory overall along with disposable gloves were used while handling with the toxic or carcinogenic chemicals such as ethidium bromide and mercarptoethanol. Chemicals having pungent smells or fumes were on open under the Laboratory fume hood such as Chloroform, mercarptaethanol, acetic acid, hydrochloric acid and phenol etc. before starting any experiment whole working area was cleaned using 70% ethanol or spirit. Ultraviolet exposure was minimized by using full sleeves lab coat, gloves and UV protected glasses. All waste material was disposed properly. For minimizing the chances of contamination all sensitive experiment such as transformation or plating was done under biosafety cabinet.

Sterilization

All the glassware and pestle mortar (used for RNA extraction) which were used in experiment were washed with detergent, and after drying they were autoclaved at 120°C.C for 20 minutes at 15 psi. After autoclaving glassware were dried at 55 to 60C in drying oven for approximately 2 to 3 hours.

Media and solution

Nutrient agar media

Yeast extract	2.5g
Tryptone	5g
Agar	7.5g
NaCl	5g

Volume was made up to 500mL using sterile water and media was autoclaved at 120°C.C for 20 minutes.

LB media

Yeast extract	2.5g
Tryptone	5 g
NaCl	5g

Volume was made up to 500mL using sterile water and media was autoclaved at 120°C.C for 20 minutes.

TE buffer 1X

0.5MEDTA	200µL
1M tris HCL	1mL

Final volume was made up to 100mL using sterile water.

TAE buffer solution 50X

Tris base	60.5g
Glacial acetic acid	14.3mL
0.5M EDTA	25mL

All above solution was mixed and volume was raised with autoclaved water up to 250mL

TAE buffer solution 1X

TAE 50X buffer	20mL
Autoclaved water	980mL

1X TAE buffer was used for gel electrophoresis.

Glycerol solution 10%

Glycerol solution 100%	100mL
Autoclaved water	900mL

Solution was autoclaves at 120°C.c for 20 minutes. 10% glycerol solution was used for the preparation of the competent cells

Resuspension solution

IM Tris	1.25mL
0.5M EDTA	1mL
0.5M glucose	5mL

Total volume was made up to 50mL using autoclave water

Neutralization solution

5M potassium acetate	30cmL
Glacial acetic acid	5.5mL

Total volume was made 50mL using autoclaved water

Lysis solution

1M NaOH	2mL
10% SDS solution	1mL

Total volume was made 10mL using autoclaved water

Ampicillin 100mg/mL

Ampicillin powder	1g
Autoclaved water	10mL

Antibiotic solution was sterilized using the syringe filter

Glycerol solution for preservation of stock

100% Glycerol solution was autoclaved and 200µL was poured into cryogenic storage vails. These vails for used for the preservation of the stocks.

Sample collection

Sample collection was from selected areas of the Pakistan. Sugarcane leaves sample having symptoms of the *sugarcane mosaic virus* as well as healthy leaf sample as a control was collected. Fresh leaf sample were stored into plastic zipper bag and were stored at -80°C. for further use.

Total RNA extraction

Ribonucleic acid (RNA) isolation was done using the using the TRIzol method (Piotr Chomczyński and Sacchi, N. 1987). Approximately 0.1 g sugarcane fresh leaf sample was grinded with the help liquid nitrogen using pestle and mortar. Powder leaf sample was transferred into 1.5mL eppendrof tube. 1mL of TRIzol reagent (guinidum isothiocynate and Phenol solution) (TR 118 MRC) was added into the powder sample. Cell disruption was done by incubating the micro centrifuge tubes at ice for 5 minutes. Phase separation was done using 200µL of chloroform. Resulting slurry was shake well for 10 to 20 times. Sample was incubated 10 minutes on ice for maintain RNA integrity. Sample was centrifuge at 12,000g for 15 minutes at 4°C. Because of centrifugation two phases will appear, upper aqueous phase containing total ribonucleic acid and lower organic phase. Aqueous phase was then transferred into new Eppendorf tube. RNA was precipitated by the addition of 500µL of ice chilled isopropanol. Incubation was done at 4 for 15 minutes. Pellet was obtained by centrifugation of the sample at 12,000g for 10 minutes. Supernatant was removed and then pellet washing was done with 1mL of 75% ethanol. After removing

the ethanol pellet was dried at 37C and dissolved into approximately amount of the 1X TE (1mM EDTA, 10mM Tris HCL) buffer sample was stored at -80 for further use.

Analysis of RNA

Agarose gel electrophoresis

RNA was visualize using the agarose gel electrophoresis.1% agarose gel was prepared by adding 0.4g of the agarose into 40mL of the 1XTAE buffer. Solution was heated until a clear phase is obtained.4µL of the ethidium bromide was added when gel reached to room temperature. RNA sample was loaded into the solidified gel along with suitable marker. Apparatus was run for 40 minutes at 80V.transalminator was use for visualization of the RNA bands.

Quantification of RNA

Quantification of the RNA was done using the spectrophotometer (Nano drop Thermo scientific 2000), according to the manufacturer instructions. RNA concentration and purity were observed by observing the reading of concentration and 260nm and 280nm ratio. Good quality RNA has approximately 1.8 to 2 ratio of 260/280 ratios respectively.

cDNA synthesis

RNA to complementary DNA strand synthesis was done by following steps. Total reaction mixture containing 25ng RNA, 3.5mM gene specific reverse primer., 1µL of the M-MuLV reverse transcriptase enzyme, 2µL of the 10X MuLV buffer, 0.7mM dNTPs, nuclease free water, was incubated at 42°C for 1 hour. Enzyme was denatured at 65°C for 20 minutes.

Polymerase chain reaction

Total 25µL reaction containing 10X buffer, I unit of Taq DNA polymerase,10X Taq buffer [25mM MgCl2 and (NH4)2SO4], 10Mm primers P1F and P1R and 2.5µL of cDNA. reaction mixture of PCR was placed in the thermo cycler at following optimized conditions: initial temperature at 94°C for 1min, followed by 30 cycles of denaturation at 94°C for 30sec, annealing at 50°C for 30 sec and extension for 1min at 72°C.final extension was 72°C for 7minutes.PCR product was visualized on 1% agarose gel electrophoresis

Agarose gel electrophoresis

Amplified product was visualize using the agarose gel electrophoresis.1% agarose gel was prepared by adding 0.4g of the agarose into 40mL of the 1XTAE buffer. Solution was heated until a clear phase is obtained.4µL of the ethidium bromide was added when gel reached to room temperature. PCR sample was loaded into the solidified gel along with suitable marker. Apparatus was run for 40 minutes at 80V.transalminator was use for visualization of the amplified bands

P1F	5’CTCGAGGATCCGATTGTGGACTCACGTGTC3’
P1R	5’GGTACCAAGCTTGATGTCGAGCACGTTCTTG3’

Table 1 Sequence of forward and reverse primer used for the amplification of the P1 region of the *Sugarcane mosaic virus*

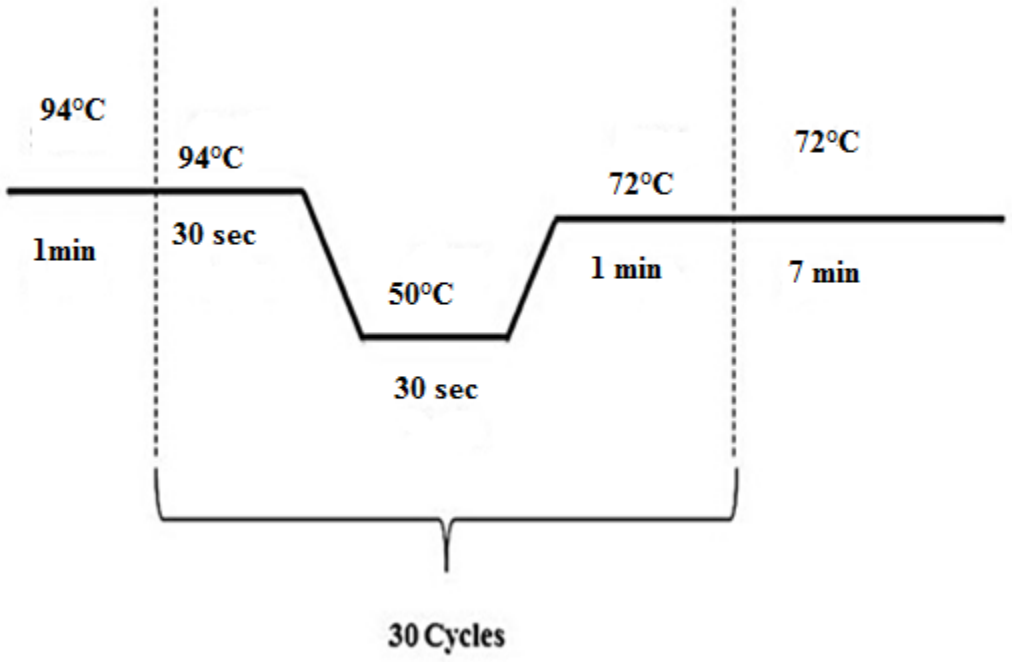


Figure 6: conditions for polymerase chain reaction used for the amplification of the P1 region of the *Sugarcane mosaic virus*

Gel elution of PCR product

Gel elution of PCR product for required gene was done using the Gene JET gel extraction kit (K0691). Approximately. 500bp of the required amplified segment was cut from the agarose gel using sterilized blade. Gel was placed into the pre-weighed 1.5mL of the Eppendorf tube.1:1 (volume: weight) binding buffer was added to the solid gel. Gel mixture containing binding buffer was incubated at 50-60°C for approximately 10 minutes. Micro centrifuge tube was mix by inversion every two to three minutes to increase the melting process.800µL of the solubilized gel solution was transferred into Gene JET purification column. After centrifugation of the 30s to 1-minute flow through was removed and column was placed into the same collection tube. Washing of amplified segment was done using the 700µL of the Washing buffer. Excess ethanol was removed and 40µL of the elution buffer i.e. TE was added to the center of the purification column. Column was placed at room temperature for 2 to 3 minutes. After centrifugation of 1 minute at high speed, eluted product was collected into new microcentrifuge tube. Purified amplified segment was stored at -20°C for further use.

Gel electrophoresis

Gene clean was visualize using the agarose gel electrophoresis.1% agarose gel was prepared by adding 0.5g of the agarose into 50mL of the 1XTAE buffer. Solution was heated until a clear phase is obtained.5µL of the ethidium bromide was added when gel reached to room temperature. sample was loaded into the solidified gel along with suitable marker. Apparatus was run for 40 minutes at 80V.transalminator was use for visualization of the gene clean

Ligation

Ligation of the purified PCR segment was done in pTZ57R/T following the stranded manufacturer protocol (InsTAclone ™ PCR cloning kit, Fermentas). Total 25µL of the ligation mixture containing 2.5X of the ligation buffer, 350-400ng of DNA, of the pTZ57R/T vector, 4.5 units of the T4 DNA ligase. Total 25µL of the ligation mixture was incubated at 4°C overnight for the completion of the ligation process

Preparation of the DH5α competent cells by $CaCl_2$ Method

DH5α cells were prepared by using calcium chloride method (Mandel and Higa, 1970; Cohen et al., 1972). A single colony of E. coli DH5α strain was inoculated in 50mL of falcon tubes containing 10mL of the Lauria Bertani(LB) media. Inoculum was incubated in shaking incubator at 37°C at 150rpm for overnight. Next day bacterial culture was refreshed by adding 1mL of the culture into 250mL of the flask containing 100mL of the Lauria Bertani media. Culture was incubated at 37°C for approximately 2-3 hours or until the optical density of the culture reached to 0.4 to 0.6. Culture was shifted to two falcon tubes containing 50mL of the culture into each falcon tube. Bacterial culture was pelleted by centrifugation at 5000rpm for 10 minutes. Supernatant was removed, and bacterial pellet was incubated on ice with ice cold 50Mm of anhydrous $CaCl_2$ solution for 40 minutes. After incubation cells were pelleted by centrifugation at 5000rpm for 10 minutes. After centrifugation pellet was dissolved into 1mL of the $CaCl_2$ solution.Aliquotes of 100µL cells for made. Cells were stored -80°C for further use.

Transformation with DH5α competent cells

Heat shock method has been followed for the transformation of the DH5 α cells (Mandel and Higa, 1970; Cohen et al., 1972). 25µL of the ligation mixture containing gene of interest was mixed with 100µL of the chemical competent cells. Incubation was done on ice for approximately 40 minutes. Heat shock was given for exactly 90 seconds at 42°C in waterbath.After heat shock mixture was placed immediately on ice for 2 to 5 minutes. Transformation mixture with mixed with 1mL of the LB broth media and was kept at 37°C for shaking. After shaking, media was spread on plates containing nutrient agar, ampicillin, IPTG(100Mm) and X-Gal(20mg/mL) (for the selection of blue white colonies). After spreading under the biosafety cabinet plates were incubated upside down overnight at 37°C.

Plasmid preparation

Transformed plates having white colonies (containing our gene of interest) was inoculated into 50mL of falcon tubes containing 10mL of the LB media along with appropriate amount of the antibiotic. Next day approx. 4.5mL of the culture was centrifuged at maximum speed. Supernatant was discarded and removed, and pellet was re suspended into 250µL of the suspension solution. After resuspending the pellet 250µL of the lysis solution was added and then micro centrifuge tube were mixed by inversion 4-5 times until the entire solution becomes slightly viscous and clear. After lysis 350µL of the neutralization solution was added, immediately inversion was done for 4-5 times until white precipitate seen. Centrifuge it for 5 minutes. Supernatant was transferred into supplied Gene Jet column. Centrifugation was performed for 1 minute, Supernatant in the

collection tubes was separated and column was washed two times with washing solution. Appropriate amount of the TE buffer was added into the middle of the spin column. Centrifugation was done for 30 to 60 seconds. Plasmid was stored into new microcentrifuge tube for further use.

Confirmation of the clone through Restriction digestion

Restriction enzymes was selected according to the restriction sites of vector and gene of interest.30µL of the reaction mixture containing 10X buffer, 5unit of restriction enzymes *HindIII, EcoRI,* according to the restriction site of the vector, and KpnI *and XhoI* according to the restriction sites of the primers were used for the confirmation of the clones.

Directional cloning

After confirmation of the clones through restriction enzymes of the gene of interest. Required segment was eluted from gel and were ligated into the pKANNIbal vector and was confirmed through restriction digestion

CHAPTER: 4

RESULTS AND DISCUSSION

Sample collection

Sugarcane infected sample were collected from different area of the Punjab and Khyber Pakhtunkhwa. Punjab is the major area for sugarcane production in Pakistan which covers approximately 62% of the total area under production. Average cane yield and area under production in Pakistan does not show any improvement in Pakistan from last decade. Mosaic is the major disease of the sugarcane. Typical mosaic symptoms include contrasting shades of leaf lamina in infected leaf with or without varying degree of reddening or necrosis along with lamina pattern. Infected leaves were collected into sampling bag and were store into -80°C for further use.

Following table shows the sampling details of the sugarcane from different sugar growing areas of the Pakistan

Serial no	Sampling date	Area	Symptoms
1	Sep, 2017	Rahim yar Khan,Punjab,PK	mosaic
2	Feb, 2018	Shakarganj, Punjab, PK	mosaic
3	March, 2018	Mardan, Pakhtunkhwa, PK	mosaic
4	June, 2018	Rahim Yar Khan, Punjab,PK	mosaic
5	June, 2018	Rahim Yar Khan, Punjab,PK	mosaic
6	July, 2018	Rahim Yar Khan, Punjab, PK	mosaic
7	July 2018	Bahawalpur, Punjab, PK	mosaic
8	Aug, 2018	Vehari, Punjab, Pk	mosaic
9	Aug, 2018	Shakrganj, Punjab, PK	mosaic
10	Sept, 2018	Rahim yar Khan,Punjab,PK	mosaic

Table 2: Table is showing the different targeted areas for the sample collection

.

Figure 7: Map of the Pakistan showing highlighted areas which was used for the sample collection

A

B

C

D

A

B

C

D

RNA isolation

Sugarcane leaf sample showing symptoms of the sugarcane mosaic virus were proceeded for RNA extraction. Total genomic RNA was extracted using TRIzol reagent. Figure 2 shows the result of RNA isolation on 1% Agarose gel. Gel picture shows the distinct bands of 28S, 18S and 5S band of RNA. Concentration of RNA was determined using the Nano drop reading

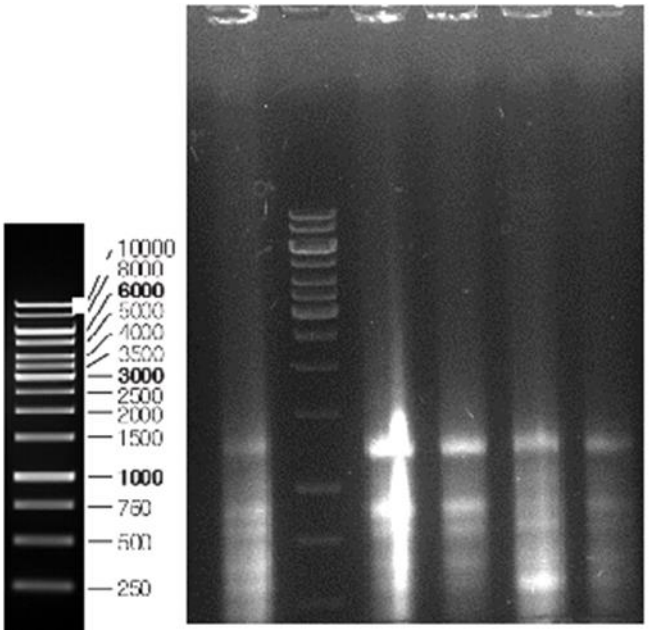


Figure 8: 1% Agarose gel of RNA isolation of five different sample of sugarcane showing 28S, 18S and 5S units of RNA

Complementary DNA (cDNA) synthesis

Complementary DNA strand was synthesized using the gene specific reverse primer by using M-MLV (Moloney-Murine Leukemia Virus) reverse transcriptase enzyme by following incubation steps. Complementary DNA strand was stored at -20 for further use.

Polymerase chain reaction

For the confirmation of the presence of the *Sugarcane mosaic virus* in symptomatic leaves sample of the sugarcane P1 (proteinase 1) specific primer were used. The P1 peptidase consist of a protease domain at its C-terminal region which cleaves itself from the neighboring helper component peptidase (HC-Pro) protein. Its other functions are presently unknown

Figure 3 shows the result of the polymerase chain reaction. Approximately 500bp gene segment was amplified using cDNA as template. Amplified segment was eluted using the Fermentas gene clean kit. The eluted segment was run into 1% agarose gel foe the confirmation.

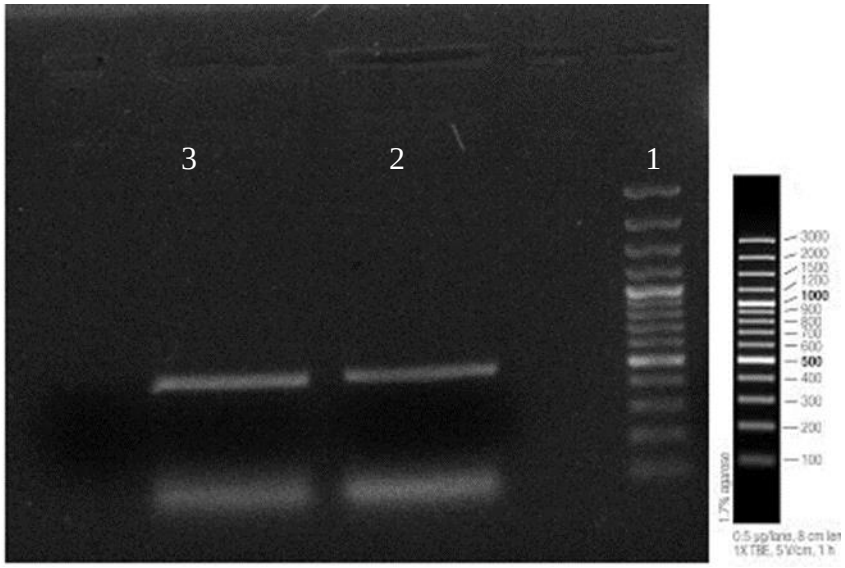


Figure 9: Result of polymerase chain reaction on 1% agarose gel showing amplification of the P1 gene of SCMV from infected sugarcane samples. Lane 1 represent the Thermo Fisher Scientific (SM 0321) 100bp stranded DNA marker. Lane 2 and 3 shows the amplified segment of the P1 gene

Gene clean

The amplifies PCR product was cleaned using the stranded silica bead kit. Amplified gene clean was run on 1% agarose gel for the confirmation of the cleaning procedure. Fig shows the result of gene clean

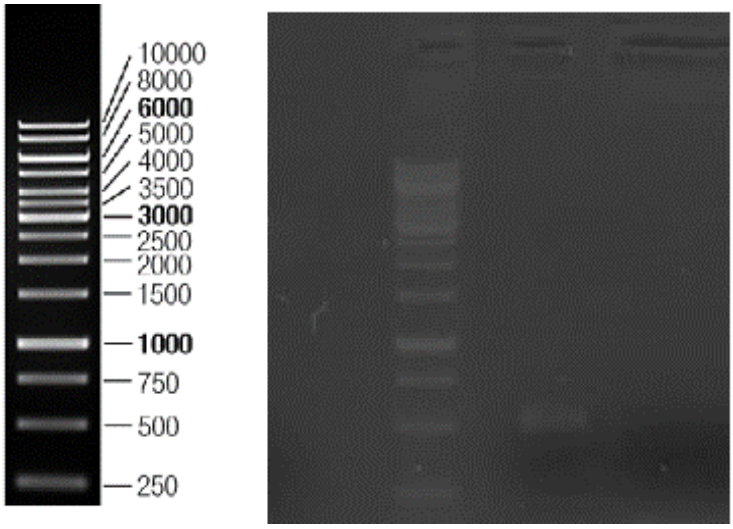


Figure 10: 1% agarose gel showing the amplified segment after gene clean along with Thermo Fisher Scientific (SM 0313) 1 kb stranded DNA marker

.

T/A cloning

Amplified product after gene elution was ligated into linear pTZ57R expression vector which ha ve3'-ddT overhangs for TA cloning of amplified products. After completion of the overnight ligation process, product was transformed into DH5alpha competent cells. Transformation plates were kept at 37C for screening of the clones. Next day clear blue white selection was observed. White colonies show clone with required insert

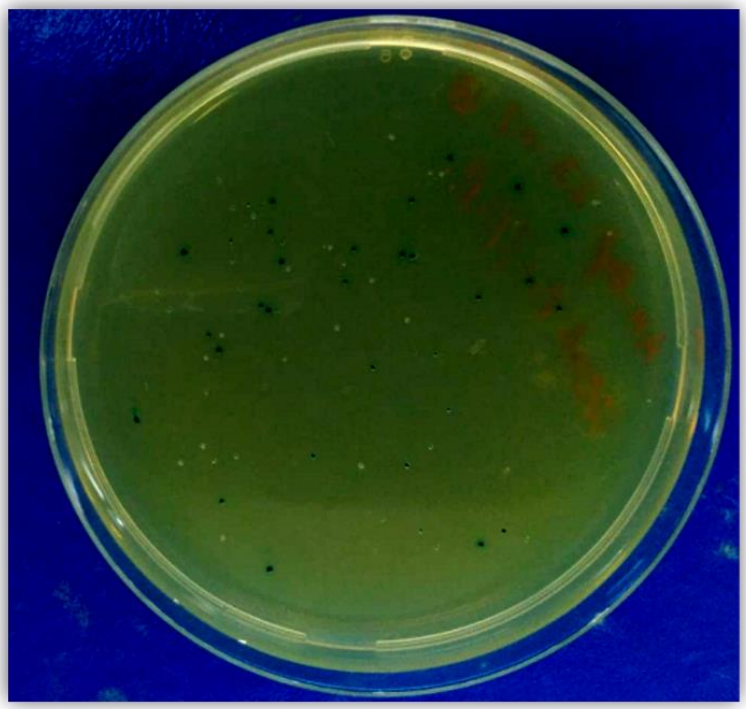


Figure 11: transformation plates showing the blue white selection

Screening of clone through plasmid isolation and Plasmid PCR

White colonies were inoculated into LB media with ampicillin for 16 hours at 37C. Next day plasmid was isolated using the alkaline lysis method. The isolated plasmid was act as template for the PCR polymerase chain reaction using the same P1F and P1R primers. Figure shows the result of PCR who has confirmed the clone. 500bp band of our gene of interest confirmed the presence of desired clone.

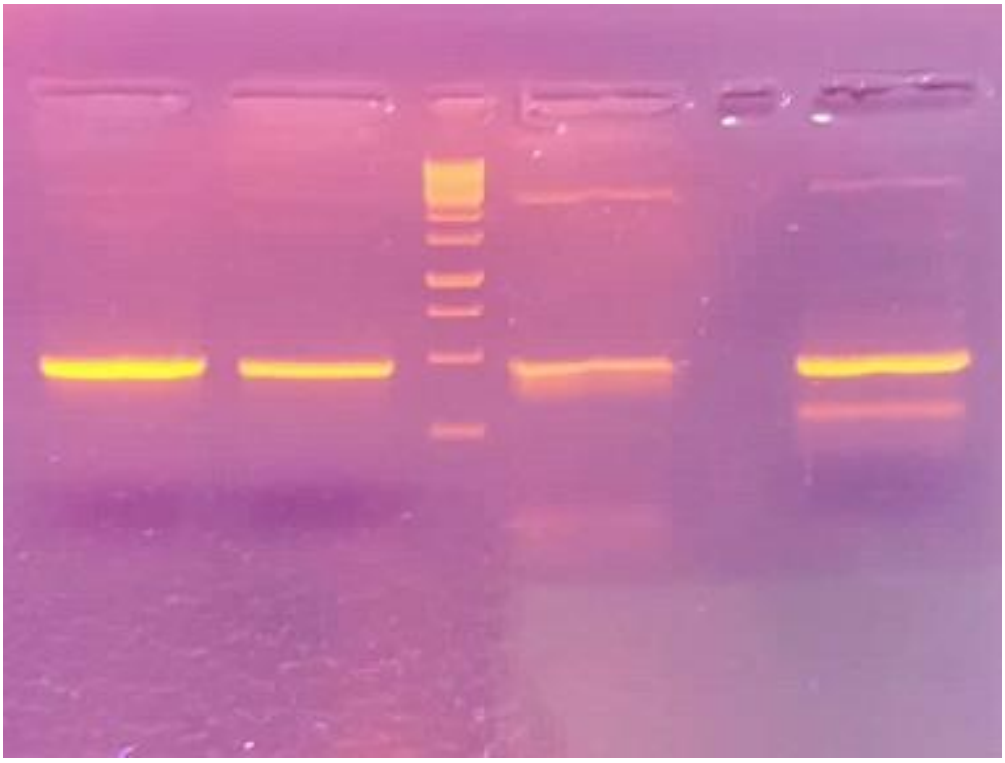


Figure 12: 1 % agarose gel of plasmid PCR showing confirmation of T/A clone

Confirmation of the clone through restriction digestion.

Specific restriction enzymes according to the sites of primers and expression vector were used for the digestion of the Plasmid (Figure 5). Clear bands of 2.8kb of the pTZ57R/T vector and approximately

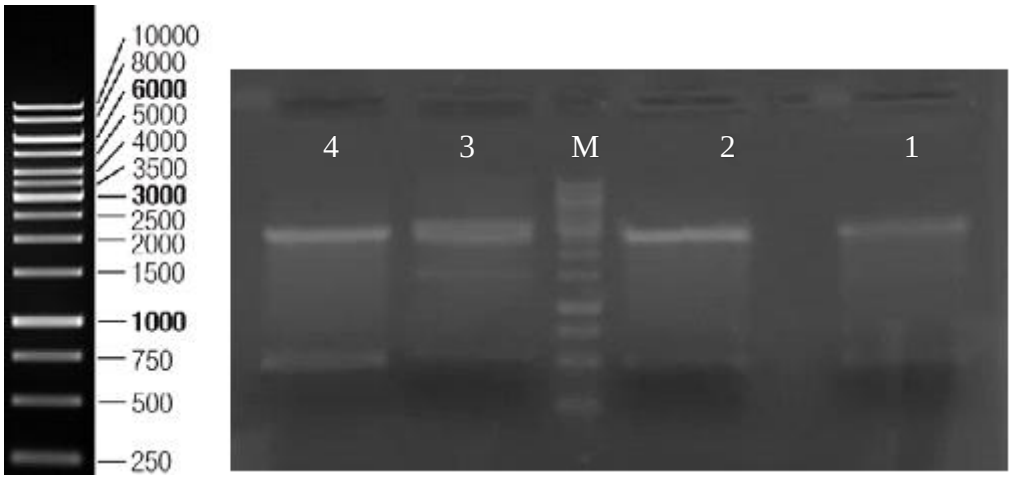


Figure 13: Representative figure of agarose gel electrophoresis showing the result of the restriction digestion. M showing the Thermo Fisher Scientific (SM 0313) 1 kb stranded DNA marker. Lane 1 and 2 shows the result of the restriction digestion with ECOR1 and HIND111 whereas lane 3 and 4 shows the result of the restriction digestion with KpN1 and Xho1

Agricultural countries like Pakistan depends on agriculture for its major economy as well as prosperity. Crops have major role in Pakistan economy that reduces productivity due to plant viruses of family *Potyvirid.* The main factor for Sugarcane yield reduction is disease of *Sugarcane mosaic virus* in Pakistan that reduces production of Sugar content leading to reduction in economy of Pakistan. Genus potyvirid are main causative agent for this lose.

The focus of present study was the identification and cloning and partial characterization of P1 gene of the *Sugarcane mosaic virus*. Samples of infected plants leaf were collected with mosaic symptoms Total genomic RNA extracted from infected samples, amplified P1 gene segment were cloned and confirmed through restriction digestion, which will be helpful in future for the analysis of the Sugarcane mosaic virus in different cultivars of the sugarcane in Pakistan.

REFERENCES

Adam, M. J., Antoniw, J. F. and Fauquet, C. M. (2005). Molecular criteria for genus and species discrimination within the family *Potyviridae*. *Archives of Virology*, 150: 459-479.

Adams, M. J., Antoniw, J. F. & Beaudoin, F. (2005a). Overview and analysis of the polyprotein cleavage sites in the family Potyviridae. Mol Plant Pathol 6, 471–487.

Addy, H. S., Wahyudi, A. H. S., Sholeh, A., Anugrah, C., Iriyanto, F. E. S., Darmanto, W., & Sugiharto, B. (2017). Detection and response of sugarcane against the infection of sugarcane mosaic virus (SCMV) in Indonesia. Agronomy, 7(3), 50.

Ali, S., Nasir, I. A., Ali, A., Aslam, U., Farooq, A. M., Tariq, M., Tabassum, B., Qamar, Z., Samiullah, T. R., Rao, A. Q. and Hussnain, T. (2014). Genetic variability in coat protein gene of Sugarcane mosaic virus in Pakistan and its relationship to other strains. African Journal of Biotechnology, 13: 3950-3965

Anonymous. 2003. Sugarcane crop. Food and Agriculture Organization (FAO), New York, USA

Anonymous. 2005. Economic survey on crop situation. Federal Bureau of statistics. Government of Pakistan. Islamabad: p.10.

Anonymous. 2005. Sugar Sector. Board of Investment. Govt. of Pak. Internet file.statistics\sugar\thm.

Anwar, S. (2005). Impact of sugarcane disease on cane and sugar yield. Agricultural Biotechnology 3rd workshop. Agricultural Biotechnology Research Institute, Faisalabad, Pakistan

Apriasti, R., Widyaningrum, S., Hidayati, W. N., Sawitri, W. D., Darsono, N., Hase, T., & Sugiharto, B. (2018). Full sequence of the coat protein gene is required for the induction of pathogen-derived resistance against sugarcane mosaic virus in transgenic sugarcane. Molecular Biology Reports, 0(0), 0. https://doi.org/10.1007/s11033-018-4326-1

Arbatova, J., Lehto, K., Pehu, E., & Pehu, T. (1998). Localization of the P1 protein of potato Y potyvirus in association with cytoplasmic inclusion bodies and in the cytoplasm of infected cells. *Journal of General Virology*, *79*(10), 2319-2323

Aslam, U., Tabassum, B., Nasir, I. A., Khan, A., & Husnain, T. (2018). A virus-derived short hairpin RNA confers resistance against sugarcane mosaic virus in transgenic sugarcane. Transgenic research, 27(2), 203-210.

Balamuralikrishnan, M., Doraisamy, S., Ganapathy, T. and Viswanathan, R. (2004). Comparison of antibody and genome based diagnostic techniques for *Sugarcane mosaic virus* in sugarcane. *Phytoparasitica*, 32: 52-56.

Blanc, S., Ammar, E. D., Garcia-Lampasona, S., Dolja, V. V., Llave, C., Baker, J. and Pirone, T. P. (1998). Mutations in the *Potyvirus* helper component protein: effects on interactions with virions and aphid stylets. *Journal of General Virology*, 79: 3119-3122.

Bock, K. R. and Bailey. R. A. (1989). Streak in diseases of sugarcane. In: Major diseases, (Ricaud, C., Egan, B. T., Gillaspie, A. G. and Hughes. C. G. eds.). Elsevier Science Publishers BV, Amsterdam, The Netherlands. 323-332.

Carrington, J. C., and Dougherty, W. G. (1987). Small nuclear inclusion protein encoded by a plant potyvirus genome is a protease. J. Virol. 61, 2540–2548.

Carrington, J. C., Cary, S. M., Parks, T. D., & Dougherty, W. G. (1989). A second proteinase encoded by. The EMBO Journal, 8(2), 365–370. https://doi.org/10.1002/j.1460-2075.1989.tb03386.x

Chare, E. R., & Holmes, E. C. (2006). A phylogenetic survey of recombination frequency in plant RNA viruses. *Archives of virology*, *151*(5), 933-946.

Chen, K. C., Chiang, C. H., Raja, J. A., Liu, F. L., Tai, C. H., and Yeh, S. D. (2008). A single amino acid of NIapro of Papaya ringspot virus determines host specificity for infection of papaya. Mol. Plant Microbe Interact. 21, 1046–1057. doi: 10.1094/MPMI-21-8-1046

Cox, M., Hogarth, M. and Smith, G. (2000). Cane breeding and improvement. Manual of Cane Growing, pp. 91–108. M. Hogarth and P. Allsop. Bureau of Sugar Experiment Stations: Indooroopilly

Cui, H., and Wang, A. (2016). Plum pox virus 6K1 protein is required for viral replication and targets the viral replication complex at the early stage of infection. J. Virol. 90, 5119–5131. doi: 10.1128/JVI.00024-16

Darsono, N., Azizah, N., Putranty, K., Astuti, N., Addy, H., Darmanto, W., & Sugiharto, B. (2018). Production of a Polyclonal Antibody against the Recombinant Coat Protein

Dolja, V. V. and Carrington, J. C. (1992). Evolution of positive strand RNA viruses. Seminars In: *Virology*, 3: 315-326.

Faoro, F., and Gozzo, F. (2015). Is modulating virus virulence by induced systemic resistance realistic? Plant Science 234, 1-13

Gemechu, A. L., Chiemsombat, P., Attathom, S., Lersrutaiyotin, R., & Reanwarakorn, K. (2004). The variations among isolates of Sugarcane mosaic virus in Thailand as determined by virus-host interaction. *Kasetsart Journal (Natural Science)*, *38*, 369-379.

Gonçalves, M. C., Pinto, L. R., Souza, S. C., & Landell, M. G. A. (2012). Virus diseases of sugarcane. A constant challenge to sugarcane breeding in Brazil. *Functional plant science and biotechnology*, *6*(2), 108-116.

Gustafson, T. J., de Leon, N., Kaeppler, S. M., & Tracy, W. F. (2018). Genetic analysis of sugarcane mosaic virus resistance in the wisconsin diversity panel of maize. *Crop Science*, *58*(5), 1853–1865. https://doi.org/10.2135/cropsci2017.11.0675

Hancock, J. M., Chaleeprom, W., Chaleeprom, W., Dale, J. and Gibbs, A. (1995). Replication slippage in the evolution of potyviruses. Journal of General Virology, 76: 3229-3232.

Handley, J. A., Smith, G. R. and Harding, R. M. (2001). Current concepts of the taxonomy and variation of sugarcane infecting potyviruses. In: Sugarcane pathology, volume II: Virus and phytoplasma diseases, (Rao, G. P., Ford, R. E., Tosic, M. and Teakle, D. S.). Enfield, NH: Sciences Publishers, Inc. 73–81.

Ivanov, K. I., Eskelin, K., Löhmus, A. and Mäkinen, K. (2014) Molecular and cellular mechanisms underlying potyvirus infection. *J. Gen. Virol.,* **95,** 1415- 1429.

J., Gao, S., Lin, Q., Wang, H., Que, Y. and Xu, L. (2015). Transgenic Sugarcane Resistant to *Sorghum mosaic virus* based on coat protein gene silencing by RNA interference. *Bio Med Research International*, doi.org/10.1155/2015/861907

Jenner, C. E., Wang, X., Tomimura, K., Ohshima, K., Ponz, F., & Walsh, J. A. (2003). The Dual Role of the Potyvirus P3 Protein of Turnip mosaic virus as a Symptom and Avirulence Determinant in Brassicas. Molecular Plant-Microbe Interactions, 16(9), 777–784. https://doi.org/10.1094/MPMI.2003.16.9.777

Jupin, I., Ayach, M., Jomat, L., Fieulaine, S., and Bressanelli, S. (2017). A mobile loop near the active site acts as a switch between the dual activities of a viral protease/deubiquitinase. PLoS Pathog. 13: e1006714. doi: 10.1371/journal.ppat. 1006714

Keizerweerd, A. T., Chandra, A. and Grisham, M. P. (2015). Development of a reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the detection of *Sugarcane mosaic virus* and *Sorghum mosaic virus* in sugarcane. *Journal of Virological Methods*, 212: 23-29.

Kendall, A., McDonald, M., Bian, W., Bowles, T., Baumgarten, S. C., Shi, J., ... & Havens, W. M. (2008). Structure of flexible filamentous plant viruses. *Journal of virology*, *82*(19), 9546-9554.

Konvalinka, J., Krausslich, H. G., and Muller, B. (2015). Retroviral proteases and their roles in virion maturation. Virology 479–480, 403–417. doi: 10.1016/j.virol. 2015.03.021

Lakshmanan, P., Geijskes, R. J., Aitken, K. S., Grof, C. L. P., Bonnett, G. D., & Smith, G. R. (2005). Sugarcane biotechnology: The challenges and opportunities. In Vitro Cellular & Developmental Biology - Plant, 41(4), 345–363. https://doi.org/10.1079/IVP2005643

Liu, Y. P., Peremyslov, V. V., Medina, V., and Dolja, V. V. (2009). Tandem leader proteases of Grapevine leafroll-associated virus-2: host-specific functions in the infection cycle. Virology 383, 291–299. doi: 10.1016/j.virol.2008. 09.035

Mbanzibwa, D. R., Tian, Y., Mukasa, S. B. and Valkonen, J. P. T. (2009). *Cassava Brown Streak Virus* (*Potyviridae*) Encodes a Putative Maf/HAM1 Pyrophosphatase Implicated in Reduction of Mutations and a P1 Proteinase That Suppresses RNA Silencing but Contains No HC-Pro. *Journal of Virology*, 83: 6934–6940.

Miras, M., Miller, W. A., Truniger, V., and Aranda, M. A. (2017). Non-canonical translation in plant RNA viruses. Front. Plant Sci. 8:494. doi: 10.3389/fpls.2017. 00494

Mohammadi, M. R. and Hajieghrari, B. (2009). *Sugarcane mosaic virus*: The causal agent of mosaic disease on sorghum (*Sorghum bicolor* L.) in Tehran province of Iran. *African Journal of Biotechnology*, 8: 5271-5274.

Mollov, D., Tahir, M. N., Kaye, C., Lockhart, B., Comstock, J. C. and Rott, P. (2016). First report of *Sugarcane mosaic virus* infecting Columbus Grass (*Sorghum almum*) in the United States. *Plant Disease,* 100: 1510.

Moradi, Z., Mehrvar, M., Nazifi, E., Zakiaghl, M., 2016. The complete genome sequences of two naturally occurring recombinant isolates of *Sugarcane mosaic virus* from Iran. Virus Genes 52, 270–280.

Oertel, U., Schubert, J., & Fuchs, E. (1997). Sequence comparison of the 3'-terminal parts of the RNA of German isolates of sugarcane mosaic potyvirus (SCMV). Archives of Virology, 142(4), 675–687. https://doi.org/10.1007/s007050050110

of the Sugarcane Mosaic Virus and Its Application in the Immunodiagnostic of Sugarcane. Agronomy, 8(6), 93

Pan, Q. (2016). Molecular Characterization of the Potyviral First Protein (P1 Protein). Thèse,

Pasin, F., Bedoya, L. C., Bernabe-Orts, J. M., Gallo, A., Simón-Mateo, C., Orzaez, D., *et al.* (2017) Multiple T-DNA delivery to plants using novel mini binary vectors with compatible replication origins. *ACS Synth Biol,* **6,** 1962- 1968.

Pasin, F., Simón-Mateo, C. and García, J. A. (2014) The hypervariable amino terminus of P1 protease modulates potyviral replication and host defence responses. *PLoS Pathog,* **10,** e1003985

Peng, D., Zheng, G., Zheng, Z., Tong, Q., & Ming, Y. (2018). High variability in the N terminus of coat protein among potyviruses and its advantage in producing a specific antibody. *European Journal of Plant Pathology*, 1-9.

Putra, L. K., Astono, T. H., Rasminah, S., Syamsidi1, C. and Djauhari1, S. (2015). Investigation on transmission modes and host range of Sugarcane streak mosaic virus in sugarcane (Saccharum officinarum L.) in Indonesia. Journal of Agricultural and Crop Research, 3: 59-66

Qureshi, M. A. and S. A. (2005). Sugarcane cultivation in Pakistan, (August 2005), 11

Rein, P. (2007). Prospects for the conversion of a sugar mill into a biorefinery. *International Society of Sugar Cane Technologists.* M. Hogarth. Durban, South Africa. 26:44–59.

Revers, F., and García, J. A. (2015). Molecular biology of potyviruses. Adv. Virus Res. 92, 101–199. doi: 10.1016/bs.aivir.2014.11.006

Rodamilans B, Shan H, Pasin F and García JA (2018) Plant Viral Proteases: Beyond the Role of Peptide Cutters. *Front. Plant Sci.* 9:666. doi: 10.3389/fpls.2018.00666

Rodamilans, B., Valli, A. and García, J. A. (2013) Mechanistic divergence between P1 proteases of the family *Potyviridae*. *J. Gen. Virol.,* **94,** 1407- 1414.

Rohožková, J., & Navrátil, M. (2011). P1 peptidase–a mysterious protein of family Potyviridae. *Journal of biosciences*, *36*(1), 189-200.

Rojas, M. R., Zerbini, F. M., Allison, R. F., Gilbertson, R. L. and Lucas, W. J. (1997). Capsid protein and helper component-proteinase function as *Potyvirus* cell-to-cell movement proteins. *Virology*, 237: 283–295.

Sankaranarayanan, R., Palani, S. N., & Tennyson, J. (2018). Hubei poty-like virus 1 is likely an interspecies recombinant of sugarcane mosaic virus and putative bean yellow mosaic virus. *Archives of virology*, 1-5.

Sasaya, T., Torrance, L., Cowan, G. and Ziegler, A. (2000). Aphid transmission studies using helper component proteins of *Potato Virus* Y expressed from a vector derived from *Potato Virus* X. *Journal of General Virology*, 81: 1115–1119.

Shan, H., Pasin, F., Tzanetakis, I. E., Simón-Mateo, C., García, J. A., & Rodamilans, B. (2018). Truncation of a P1 leader proteinase facilitates potyvirus replication in a non-permissive host. *Molecular plant pathology*, 19(6), 1504-151

Tang, W., Yan, Z. Y., Zhu, T. S., Xu, X. J., Li, X. D., & Tian, Y. P. (2018). The complete genomic sequence of Sugarcane mosaic virus from Canna spp. in China. Virology journal, 15(1), 147.

Taupier, L. O. G. and Bugallo, S. R., eds. (2000). Handbook of Sugarcane Derivatives.Cuban Research Institute of Sugar Cane By-Products: Havana, Cuba.

Thorat, A. S., Pal, R. K., Shingote, P. R., Kharte, S. B., Nalavade, V. M., Dhumale, D. R., B.H. Pawar, B. H. and Babu, K. H. (2015). Detection of Sugarcane Mosaic Virus

in Diseased Sugarcane using ELISA and RT-PCR Technique. *Journal of Pure and Applied Microbiology*, 9.

Tran, P. T., Widyasari, K., Park, J. Y., and Kim, K. H. (2017). Engineering an autoactivated R protein that is in vivo activated by a viral protease. *Virology* 510, 242–247. doi: 10.1016/j.virol.2017.07.020

Ullah, Z., Chai, B., Hammar, S., Raccah, B., Gal-On, A. and Grumet, R. (2003). Effect of substitution of the amino termini of coat proteins of distinct *Potyvirus* species on viral infectivity and host specificity. *Physiological and Molecular Plant Pathology*, 63: 129-139.

Valli, A. A., Gallo, A., Rodamilans, B., López-Moya, J. J., & García, J. A. (2018). The HCPro from the Potyviridae family: an enviable multitasking Helper Component that every virus would like to have. *Molecular plant pathology*, 19(3), 744-763

Valli, A., Dujovny, G., & García, J. A. (2008). Protease activity, self-interaction, and small interfering RNA binding of the silencing suppressor P1b from Cucumber vein yellowing ipomovirus. *Journal of virology*, *82*(2), 974-986.

Verchot, J. and J. C. Carrington. 1995(a). Debilitation of plant *Potyvirus* infectivity by P1 proteinase-inactivating mutations and restoration by second-site modifications. *Journal of Virology*, 69:1582–1590.

Verchot, J., & Carrington, J. C. (1995). Evidence that the potyvirus P1 proteinase functions in trans as an accessory factor for genome amplification. *Journal of virology*, *69*(6), 3668-3674.

Viswanathan, R., Karuppaiah, R., & Balamuralikrishnan, M. (2010). Detection of three major RNA viruses infecting sugarcane by multiplex reverse transcription-polymerase chain reaction (multiplex-RT-PCR). *Australasian Plant Pathology*, *39*(1), 79-84.

Viswanathan, R., Karuppaiah, R., Malathi, P., Kumar, V. G., & Chinnaraja, C. (2009). Diagnosis of Sugarcane yellow leaf virus in asymptomatic sugarcane by RT-PCR. *Sugar Tech*, *11*(4), 368-372.

Wylie, S. J., Adams, M., Chalam, C., Kreuze, J., López-Moya, J. J., Ohshima, K., *et al.* (2017) ICTV Virus Taxonomy Profile: *Potyviridae*. *J. Gen. Virol.*, **98,** 352-354

Xia, Z., Zhao, Z., Li, M., Chen, L., Jiao, Z., Wu, Y., ... & Fan, Z. (2018). Identification of miRNAs and their targets in maize in response to Sugarcane mosaic virus infection. *Plant Physiology and Biochemistry*, *125*, 143-152.

Xie, X., Chen, W., Fu, Q., Zhang, P., An, T., Cui, A. and An, D. (2016). Molecular variability and distribution of *Sugarcane mosaic virus* in Shanxi, China. *PLoS One,* 11(3): e0151549

Xu, T., Lei, L., Shi, J., Wang, X., Chen, J., Xue, M., ... & Zhou, T. (2018). Characterization of maize translational responses to sugarcane mosaic virus infection. Virus Research.

Yasmin, T., Iqbal, S., Farooq, A., Zubair, M. and Riaz, A. (2011). Prevalence, distribution and incidence of major sugarcane infecting viruses in NWFP and Punjab. Pakistan *Journal of Phytopathology,* 23: 24-3

Thesis 11 dec 2018 900am

ORIGINALITY REPORT

11%	5%	8%	4%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Submitted to Higher Education Commission Pakistan Student Paper	1%
2	Virus and Virus-like Diseases of Major Crops in Developing Countries, 2003. Publication	1%
3	Rasappa Viswanathan, Balasubramanian Parameswari, Kathirvel Nithya. "Molecular Characterization of Sugarcane Viruses and Their Diagnostics", Elsevier BV, 2018 Publication	1%
4	Nurmalasari Darsono, Novita Azizah, Kiky Putranty, Natalia Astuti, Hardian Addy, Win Darmanto, Bambang Sugiharto. "Production of a Polyclonal Antibody against the Recombinant Coat Protein of the Sugarcane Mosaic Virus and Its Application in the Immunodiagnostic of Sugarcane", Agronomy, 2018 Publication	<1%
5	www.alice.cnptia.embrapa.br Internet Source	<1%

6	krishikosh.egranth.ac.in Internet Source	<1%
7	Archives of Virology, 1992. Publication	<1%
8	Submitted to Columbia College of Missouri Student Paper	<1%
9	Yongwang Zhong. "Identification of a Naturally Occurring Recombinant Isolate of Sugarcane Mosaic Virus Causing Maize Dwarf Mosaic Disease", Virus Genes, 01/2005 Publication	<1%
10	Submitted to University of Newcastle Student Paper	<1%
11	Submitted to The University of Manchester Student Paper	<1%
12	opus.uni-hohenheim.de Internet Source	<1%
13	d-nb.info Internet Source	<1%
14	Submitted to University of Exeter Student Paper	<1%
15	Molecular Virology, 2013. Publication	<1%

16	Mukhopadhyay, . "Appendix III", Plant Virus Vector, 2010. Publication	<1%
17	Submitted to University of Leeds Student Paper	<1%
18	Dangora, D. Balarabe, Adama Yahaya, Azmat U U Khan, and M. Aisha Zangoma. "Identification of virus isolates inducing mosaic of sugarcane in Makarfi Local Government Area of Kaduna State, Nigeria", AFRICAN JOURNAL OF BIOTECHNOLOGY, 2014. Publication	<1%
19	Ramamoorthy Sankaranarayanan, Sankara Naynar Palani, Jebasingh Tennyson. "Hubei poty-like virus 1 is likely an interspecies recombinant of sugarcane mosaic virus and putative bean yellow mosaic virus", Archives of Virology, 2018 Publication	<1%
20	repository.cimmyt.org Internet Source	<1%
21	riunet.upv.es Internet Source	<1%
22	shareok.org Internet Source	<1%

23	"Abstracts Submitted for Presentation at the 2010 APS Annual Meeting", Phytopathology, 2010. Publication	<1%
24	helda.helsinki.fi Internet Source	<1%
25	Submitted to Mahidol University Student Paper	<1%
26	Submitted to Jawaharlal Nehru Technological University Student Paper	<1%
27	Submitted to Oxford Brookes University Student Paper	<1%
28	M. Hema. "Development of recombinant coat protein antibody based IC-RT-PCR for detection and discrimination of sugarcane streak mosaic virus isolates from Southern India", Archives of Virology, 05/01/2003 Publication	<1%
29	Submitted to Hastings College Student Paper	<1%
30	www.ajol.info Internet Source	<1%
31	www.cabi.org Internet Source	<1%

32	R. Viswanathan. "Duplex — reverse transcription — polymerase chain reaction (D-RT-PCR)-a technique for the simultaneous detection of viruses causing sugarcane mosaic", Sugar Tech, 03/2008 Publication	<1 %
33	search.cosmobio.co.jp Internet Source	<1 %
34	Wen-Feng Li, Hong-Li Shan, Rong-Yue Zhang, Xiao-Yan Wang et al. "Identification of resistance to Sugarcane streak mosaic virus (SCSMV) and Sorghum mosaic virus (SrMV) in new elite sugarcane varieties/clones in China", Crop Protection, 2018 Publication	<1 %
35	www.nvri.gov.ng Internet Source	<1 %
36	ccsenet.org Internet Source	<1 %
37	Soumaya Yakoubi. "Algerian watermelon mosaic virus (AWMV): a new potyvirus species in the PRSV cluster", Virus Genes, 08/2008 Publication	<1 %
38	Hardian Addy, Nurmalasari, Agus Wahyudi, Ahmil Sholeh, Cahya Anugrah, Febrian Iriyanto, Win Darmanto, Bambang Sugiharto.	<1 %

	"Detection and Response of Sugarcane against the Infection of Sugarcane Mosaic Virus (SCMV) in Indonesia", Agronomy, 2017 Publication	
39	stud.epsilon.slu.se Internet Source	<1%
40	Cholo, Moloko C. van Rensburg, Elizabeth. "Expression of the genes encoding the Trk and Kdp potassium transport systems of Mycobacterium tuberc", BioMed Research International, Annual 2015 Issue Publication	<1%
41	W. A. Monger. "The complete genome sequence of Canna yellow streak virus", Archives of Virology, 05/22/2010 Publication	<1%
42	www.diss.fu-berlin.de Internet Source	<1%
43	Plant Virus and Viroid Diseases in the Tropics, 2014. Publication	<1%
44	pdfs.semanticscholar.org Internet Source	<1%
45	Bhat. "Characterization of a potyvirus from eggplant (Solanum melongena) as a strain of potato virus Y by N-terminal serology and	<1%

	sequence relationships", Plant Pathology, 10/1999 Publication	
46	Sean Chapman, Graham Hills, John Watts, David Baulcombe. "Mutational analysis of the coat protein gene of potato virus X: Effects on virion morphology and viral pathogenicity", Virology, 1992 Publication	<1 %
47	Mališa TOŠIC. "Cross protection among some strains of sugarcane mosaic virus and maize dwarf mosaic virus", Agronomie, 1981 Publication	<1 %
48	Stewart, L. R., R. Teplier, J. C. Todd, M. W. Jones, B. J. Cassone, S. Wijeratne, A. Wijeratne, and M. G. Redinbaugh. "Viruses in Maize and Johnsongrass in Southern Ohio", Phytopathology, 2014. Publication	<1 %
49	P. Narayanasamy. "Detection of Virus and Viroid Pathogens in Plants", Microbial Plant Pathogens-Detection and Disease Diagnosis, 2011 Publication	<1 %
50	Atlas, . "Media, Alphabetical", Handbook of Microbiological Media Third Edition, 2004. Publication	<1 %

51	Plant Virus and Viroid Diseases in the Tropics, 2013. Publication	<1%

Exclude quotes	On	Exclude matches	< 3 words
Exclude bibliography	On		