

Full Length Research Paper

Molecular characterization of viruses causing maize lethal necrosis disease in South-Rift region, Kenya

Robert K. Leitch^{1*}, Jackson C. Korir¹, John O. Muoma¹, Anne Wangai², Kim Bong³, Guri Johal³ and Sue Loesch-Fries³

¹Biological Sciences Department, Masinde Muliro University of Science and Technology (MMUST), P. O. Box 190-50100 Kakamega, Kenya.

²International Maize and Wheat Improvement Centre (CIMMYT), P. O. Box 2041-00621 Nairobi, Kenya.

³Botany and Plant Pathology Department, Purdue University, 610 West Lafayette, Indiana USA.

Received 1 July, 2020; Accepted 6 August, 2020

The first report of Maize lethal necrosis (MLN) disease in Africa was in Bomet, Kenya, nine years ago. It has since spread in East and Central African (ECA) countries, causing massive yield losses. Currently, 90% of the preferred commercial maize germplasm grown by farmers in Kenya is susceptible to MLN disease. As such, the disease has continued to pose a serious challenge to food security in the ECA region. This study sought to characterize the MLN, causing viruses present in the maize leaf samples collected from the South-Rift region. Using total RNA extracted from 60 leaf samples collected from Bomet, Kericho, and Kisumu Counties, reverse transcription-polymerase chain reaction (RT-PCR) was carried out. The PCR products with the strongest bands were purified and sequenced using the Sanger sequencing technique. The results showed that samples from the three counties were positive for maize chlorotic mottle virus (MCMV) (MH645622 and MH645621) and sugarcane mosaic virus (SCMV) (MH645623, MH645624 and MH645625) and negative to wheat streak mosaic virus (WSMV). The coat protein (CP) sequences of MCMV isolates were closely related to the sequences of MCMV isolates, which had been previously reported from Eastern and Sub-Saharan Africa. For the CP sequences of SCMV isolates, only one sequence of the isolate KCO59 was similar to the sequence of a Kenyan isolate (JX286708). Sequences of isolates KCO5 and KCO24 were not identical to those of the Kenyan isolate (JX286708). Based on these results, in the surveyed counties, isolates of SCMV are genetically diverse, while those of MCMV are not. There exists a new variant of SCMV, which appears to be the main potyvirus in synergism with MCMV in causing MLN disease in Kenya.

Key words: Maize lethal necrosis (MLN), maize chlorotic mottle virus (MCMV), sugarcane mosaic virus (SCMV), wheat streak mosaic virus (WSMV).

INTRODUCTION

Maize lethal necrosis (MLN) disease has emerged as one of the major threats to food security in Sub-Saharan

Africa as maize is a staple food crop (Mbega et al., 2016). The disease has become a significant concern in

*Corresponding author. E-mail: kipta2012@gmail.com.

Table 1. Primers used in cDNA synthesis.

Virus	Sequence name	Sequence	Amplicon (bp)
MCMV	MCMV For.	TGGCATTCTCTCCGATTAC	122
	MCMV Rev.	GTTCTGTGGCATGACAATGG	122
SCMV	SCMV For.	TATGGACGGAGACGAACAAAG	186
	SCMV Rev.	TCTTGGCATGTATCGCTCTG	186
WSMV	WSMV For.	AACAGAGCACTCGGGACTTG	195
	WSMV Rev	TGAAACTGTGCGTGTCTCC	195

Eastern Africa, having been first reported in Bomet County in the South-Rift region of Kenya in 2011 (Wangai et al., 2012). Since then, it has spread to other maize-growing areas in Central, Nyanza, Western, South, and North-Rift regions of Kenya (Davis, 2012). The disease has also been reported in other countries in East Africa where maize is an outstanding staple food. These countries include; Rwanda (Adams et al., 2014), Democratic Republic of Congo (DRC) (Lukanda et al., 2014), Tanzania (Kiruwa et al., 2016) Uganda (Kagoda et al., 2016) and more recently from South Sudan and Ethiopia (Mahuku et al., 2015). Yield losses as high as 59% or more than 300,000 tons have been reported in moist transitional zones, mostly in Western Kenya (De Groote et al., 2016) due to MLN disease. The disease has been reported to be a result of synergistic interaction between *Maize chlorotic mottle virus* (MCMV) with any potyvirus such as *Sugarcane mosaic virus* (SCMV), *Maize dwarf mosaic virus* (MDMV) or *Wheat streak mosaic virus* (WSMV) (Isabirye and Rwomushana, 2016). The synergistic interactions have been reported to be more pronounced and result in severe damage that usually kills the infected plant (Makone et al., 2014). Due to such magnitude of loss posed by the disease, it has discouraged some farmers from planting maize in the second season (Kamau and Snipes, 2014), a situation that is likely to affect maize production in the region.

Though there are commercial varieties that have already been released to farmers with appreciable tolerance to MLN, most farmers have little knowledge of how the disease spread and its control measures (Mahuku et al., 2015). Due to such inadequacies, most of the farmers practice continuous maize cropping, a practice that has been reported as one of the factors contributing to the build-up of the virus inoculum (Mezzalama, 2015). With this in mind, there is a need to characterize the virus isolates from the South-Rift region to identify the virus causing MLN disease in this region and assess their diversity. Therefore, this study sought to isolate viruses from the maize leaf samples collected from the South-Rift region in Kenya and sequence their coat protein gene.

MATERIALS AND METHODS

Sample collection

Maize leaf samples were collected between July and August 2016 in three counties of the South-Rift region of Kenya; Bomet, Kericho, and Kisumu. These counties were selected as MLN occurrence was first reported in September 2011 in Longisa in Bomet (Wangai et al., 2012), which is in Kenya's South-Rift region. Random sampling was used to select the maize farms and sampling sites. Ten farms were randomly selected from each county, and in each farm, five samples were collected. Sampling points within each maize farm were restricted to symptomatic plants, and the upper young leaf from maize plants showing MLN disease symptoms was collected. The collected leaf samples were stored in Ziploc paper storage bags in a cool box, labeled with the maize variety, where the isolate was obtained from, and the county. They were shipped to Botany and Plant pathology laboratory at Purdue University in USA for isolation of RNA and sequencing.

Extraction of RNA

From the 150 maize samples collected, 20 samples were randomly selected from each county for RNA isolation. The total RNA was extracted from individual maize leaves using the Trizol reagent (Invitrogen) as described by Chomczynski and Mackey (1995) followed by the RNeasy (QIAGEN) method as described by Adams et al. (2012) at Purdue University, Botany and Plant pathology laboratory, USA. The quantity of RNA and its purity was determined using the NanoDrop machine (2000c model) and stored at -80°C until the RT-PCR was done.

Reverse transcription (RT)-PCR amplification

RT-PCR amplification was carried out according to Jarugula et al. (2010) with modifications. It was a two-step RT-PCR reaction where cDNA was prepared from 1 µl of RNA using a Maxima First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Wilmington, DE) as per the instruction manual. The cDNA primers (Table 1) were designed using the qPCR Primer Quest tool, Integrated DNA Technologies Inc.

The specific primers for amplification of the CP gene were designed based on the reported sequence of MCMV (JX286709) and SCMV (Adams et al., 2012) (Table 2). The RT-PCR was carried out using a thermal cycler (GeneAmp PCR systems 9700, Applied Biosystems) to amplify CP-specific DNA fragments. The cycling conditions were one cycle at 95°C for 5 min to allow denaturation, followed by 35 cycles at 94°C for 30s, 58°C for 30 s,

Table 2. Primers used to amplify the CP of MCMV, SCMV, and WSMV variants.

Virus	Sequence name	Sequence	Gene	Amplicon (bp)
MCMV	MCMV For.	TGGAAAACATTGCTGTTGGA	CP	1066
	MCMV Rev.	CAGGACTCTGCCAGAAGGAC	„	1066
SCMV	SCMV For.	GCACAGGGATCAAGGAAGAA	„	1192
	SCMV Rev.	TGTCCTGCAGACTGGTTCAC	„	1192
WSMV	WSMV For.	ATGCCAGATTTCAAGTTGC	„	1275
	WSMV Rev.	TGTACGCCTCTCGTGAAGAA	„	1275

72°C for 60 s; and a final extension at 72°C for 7 min. An aliquot of the RT-PCR assay product was resolved on 1.5% agarose gels, stained with ethidium bromide, and the DNA bands were visualized under a UV-transilluminator.

Sequencing of the CP gene of MCMV and SCMV variants

Polymerase chain reaction products from the samples with the strongest bands when viewed under the U.V. light were purified for sequencing using the Qiagen kit following the manufacturer's instructions (QIAGEN Inc., Valencia, CA). The eluted DNA from MCMV and SCMV was used for sequencing, while WSMV was not sequenced since all the samples tested negative. Sequencing was done at Purdue Genomics Core Facility, USA, using the Sanger sequencing technique. The resulting sequences were then compared to published sequences in the GenBank-NCBI database (Benson et al., 2013) to give the identities of the sequences using blast+ (Camacho et al., 2009).

Sequence alignment and construction of phylogenetic trees

Multiple nucleotide sequence alignments were performed using Clustal Omega version 7. Manual editing was done to obtain consensus sequences. Aligned MCMV and SCMV sequences were assessed using DnaSP version 5.1 (83) software to estimate genetic diversity, and phylogenetic trees were constructed using the MEGA program version 7 (Kumar et al., 2016).

RESULTS

Maize lethal necrosis (MLN) symptoms observed in the field

As shown in Figure 1, the MLN symptoms illustrate the extent of the damage the disease poses on maize plants, especially among the susceptible varieties viz a viz the clean, uninfected maize plant. Maize plants in picture **A** show excessive chlorotic mottling with short internodes, and necrosis on leaf margin that progresses towards the midrib; while in picture **B**, the maize plants appear dwarf with severe chlorotic mottling on the upper leaves. The lower leaves have dried up due to excessive necrosis resulting in premature aging of the plants. As for picture **C**, the maize plants show no MLN symptoms.

RT-PCR detection of viruses causing MLN disease

The DNA from the 60 samples was amplified in the RT-PCR reaction. Out of these, 20 samples were positive to MCMV, while 18 samples were positive to SCMV. As for WSMV, all the 60 samples amplified were negative (Table 3).

Coat protein (CP) gene sequence of MCMV

The CP sequence of MCMV isolates KCO 24 (accession # MH645622) and KSM 5 (accession #MH645621) were 100% identical to each other (Table 4). Also, the two isolates had 100% identity with Sub-Saharan isolate (KP851970), Ethiopia isolate (KP772217), and 100% identical to Rwanda isolate (KF744393). The sequences also had 99.86% identity to one isolate previously reported from Kenya (JX286709), 99% identity with MCMV isolates from Thailand (AM490791), and China (GU594293). Similarly, the sequences of the MCMV from the two Counties had 96.62% identity with sequences of MCMV isolates from Kansas (KS1) and Nebraska (EU358605) in the United States (Figure 2).

Coat protein (CP) gene sequence of SCMV

The KCO 5 (accession #MH645623) and KCO 24 SCMV (accession #MH645624) isolates had 99% identity with each other (Table 5), and 97% identity to KCO 59 (accession #MH645625) isolate and Kenya isolate (JX28708). Similarly, the SCMV isolate KCO 59 had 98.29% identity with the Kenya isolate (JX28708). The three SCMV isolates KCO5, KCO24, and KCO 59 had a similarity ranging from 76.97 to 81.24% with the other SCMV isolates from India, Pakistan, Australia, China, and Argentina (Figure 3).

DISCUSSION

The objective of the study is to characterize the viruses



Figure 1. MLN symptoms on Maize plant. **A**-Severe chlorosis; **B**-Complete necrosis on the lower leaves, **C**-Uninfected maize plant. The pictures were taken in the field during the survey.

Table 3. RT-PCR detection of MCMV, SCMV, and WSMV.

Virus	No. of samples tested	Positive (+ve)	Positive (%)
MCMV	60	20	33
SCMV	60	18	30
WSMV	60	0	0

Table 4. Percentage identities between MCMV isolates from this study and isolates from the GenBank-NCBI.

Sequence	Accession	KS	NEB	KSM	KCO	SSA	ETP	RW	KEN	TH	C.N.
Kansas	KS1	100	99.58	96.62	96.62	96.62	96.62	96.62	96.48	96.77	97.05
Nebraska	EU358605	99.58	100	96.34	96.34	96.34	96.34	96.34	96.2	96.48	97.05
Kisumu	KSM5	96.62	96.34	100	100	100	100	100	99.86	99.02	99.02
Kericho	KCO24	96.62	96.34	100	100	100	100	100	99.86	99.02	99.02
SSA	KP851970	96.62	96.34	100	100	100	100	100	99.86	99.02	99.02
Ethiopia	KP772217	96.62	96.34	100	100	100	100	100	99.86	99.02	99.02
Rwanda	KF744393	96.62	96.34	100	100	100	100	100	99.86	99.02	99.02
Kenya	JX286709	96.48	96.2	99.86	99.86	99.86	99.86	99.86	100	98.87	98.87
Thailand	AM490791	96.77	96.48	99.02	99.02	99.02	99.02	99.02	98.87	100	99.44
China	GU594293	97.05	97.05	99.02	99.02	99.02	99.02	99.02	98.87	99.44	100

KS: Kansas, **NEB:** Nebraska, **KSM:** Kisumu, **KCO:** Kericho, **SSA:** Sub-Saharan Africa, **ETP:** Ethiopia, **R.W.:** Rwanda, **KEN:** Kenya, **TH:** Thailand, **C.N.:** China

causing MLN in the South-Rift region of Kenya. From the results, only two viruses except for WSMV were detected in the samples from all the three counties studied. As

reviewed in Mbega et al. (2016), MLN disease is caused by synergistic co-infection of maize MCMV with any cereal virus in the potyvirus MDMV, WSMV, or SCMV.

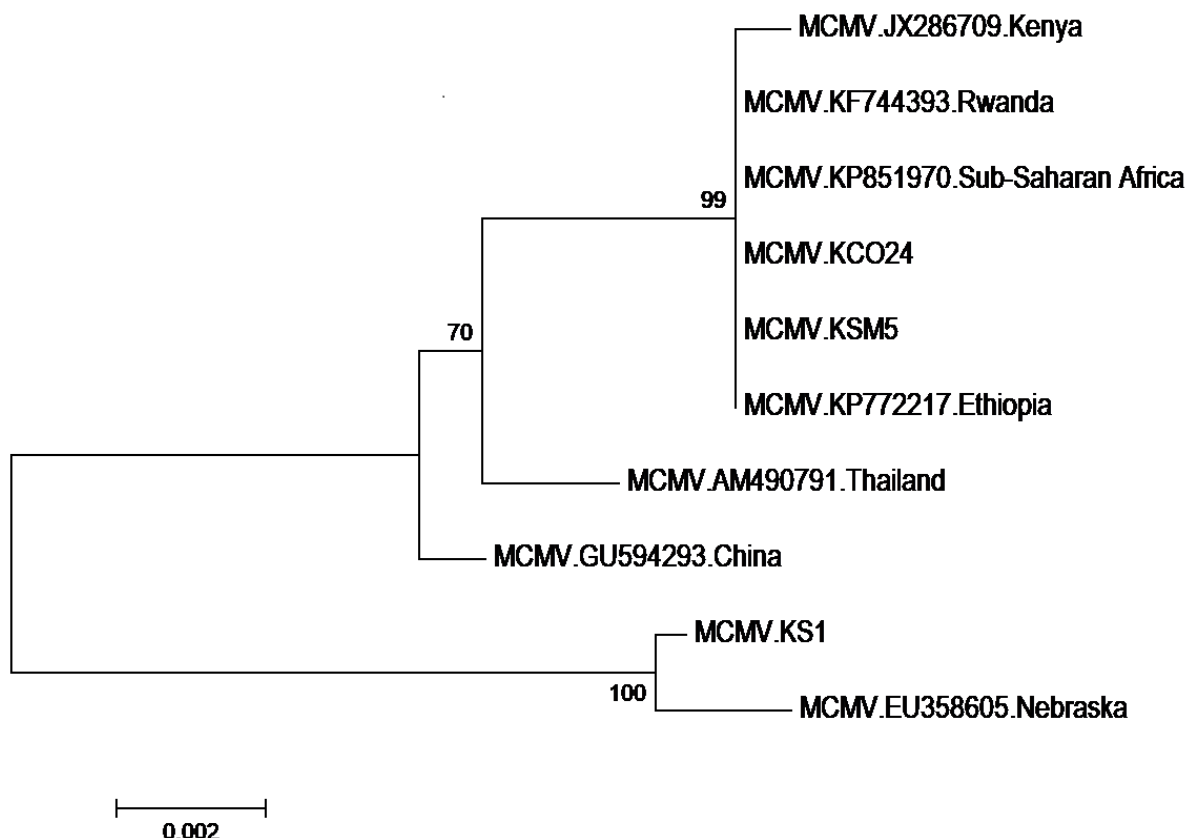


Figure 2. Phylogenetic relationships between the two isolates of MCMV coat protein sequences with other MCMV accessions from NCBI. The phylogeny was based on 711 aligned nucleotide bases. The evolutionary history with MEGA version 7, maximum likelihood method (Kumar et al., 2016) with associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Table 5. Percentage identities between SCMV isolates from this study and isolates from the GenBank-NCBI.

Sequence	Accession	IND	PAK	AUS	KCO5	KCO24	KCO59	KEN	CN	ARG
India	DQ866744	100	93.9	96.73	78.77	79.34	79.11	78.91	78.95	78
Pakistan	KU557299	93.69	100	96.3	76.97	77.3	77.08	77.33	79.08	78.2
Australia	AF006735	96.73	96.3	100	78.86	79.18	79.08	78.78	80.02	79.08
Kericho	KCO5	78.77	76.97	78.86	100	99.15	97.01	97.01	81.13	80.47
Kericho	KCO24	79.34	77.3	79.18	99.15	100	97.23	97.01	81.24	80.36
Kericho	KCO59	79.11	77.08	79.08	97.01	97.23	100	98.29	80.92	80.47
Kenya	JX286708	78.91	77.33	78.78	97.01	97.01	98.29	100	81.04	80.92
China	AY660663-1	78.95	79.08	80.02	81.13	81.24	80.92	81.04	100	93.18
Argentina	JX237958	78	78.2	79.08	80.47	80.36	80.47	80.92	93.18	100

IND: Indian, **PAK:** Pakistan, **AUS:** Australia, **KCO:** Kericho, **Ken:** Kenya, **CN:** China, **ARG:** Argentina.

This study's findings have demonstrated that SCMV is the main potyvirus that causes the MLN disease in the South-Rift region. These findings concur with Adams et al. (2014), who also reported that SCMV is the most frequent potyvirus in Kenya and East Africa. Comparison of CP sequences of MCMV isolates (accession #

MH645622 & #MH645621) from this study with other CP sequences of MCMV available in the GenBank-NCBI shows that they are closely related to Sub-Saharan Africa isolate (KP851970), Ethiopia isolates (KP772217), Rwanda isolates (KF744393), and Kenya isolate (JX286709). This high degree of MCMV sequence

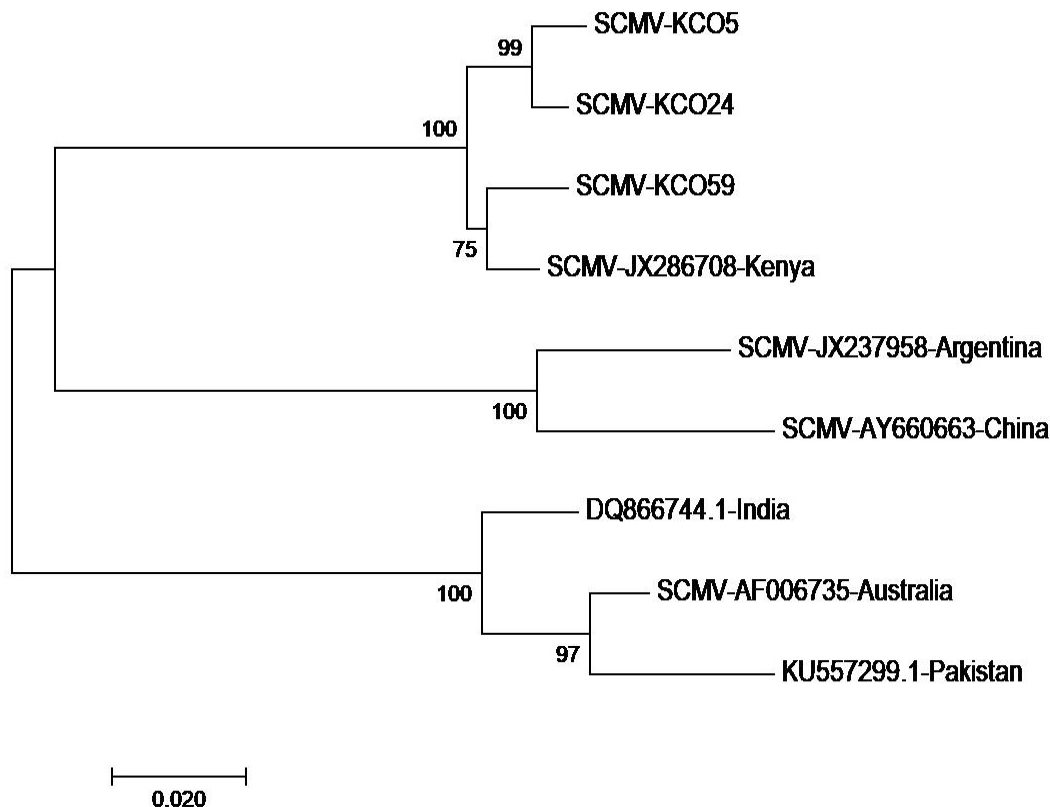


Figure 3. Phylogenetic relationships among three isolates of SCMV coat protein sequences with other SCMV accessions from NCBI. The phylogeny was based on 950 aligned nucleotide bases. The evolutionary history with MEGA version 7, maximum likelihood method (Kumar et al., 2016) with associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

similarity with those from Eastern and Sub-Saharan Africa suggests a common point of origin for the Eastern and Sub-Saharan African MCMV isolates. This is a piece of evidence that coat protein is highly conserved and an indication that MCMV virus has not undergone a lot of variation. The low variation could be attributed to the natural selection pressure which has been reported to influence the genetic variation of a virus (Roossinck and Schneider, 2006). The virus is still adapting to the new conditions and environment as a result of its newness in the area as it was first reported in September 2011 in Kenya (Wangai et al., 2012). The sequences were also similar with China isolate (GU594293), and Thailand isolates (AM490791). These findings corroborate with Adams et al. (2012) and Mahuku et al. (2015). They also reported a high degree of similarity of CP sequences of MCMV within Eastern Africa and some Asian MCMV isolates.

In the phylogenetic tree containing the SCMV sequences (Figure 3), two isolates KCO5 (accession #MH645623) and KCO24 (accession #MH645624) shared a high degree of identity. In contrast, one isolate, KCO59 (accession #MH645625), had high similarity with

the Kenyan isolate (JX286708), which had been previously reported. These results indicate the presence of two groups of SCMV sequences in the region. It suggests they may be new variants of SCMV in the South-Rift region. These findings concur with Gao et al. (2011) and Perera et al. (2009), who found out that new strains of SCMV continue to be reported in many countries. Therefore, these results reveal that SCMV is more diverse than MCMV in the region. The diverse nature of SCMV could be attributed to the fact that SCMV had been reported in Kenya as early as the 1970s (Louie, 1980) and in Eastern Africa in the mid-1930s (Kulkarni, 1972) and therefore it may have undergone some variations with time. Also, the heavy maize losses as high as 50% reported by De Groote et al. (2016) in areas between Kisii and Kericho could be attributed to the new variants of SCMV in the region. Besides, the diverse nature of SCMV in the region could also be attributed to the extensive-scale cultivation of SCMV susceptible crops in Poaceae family (maize, sorghum, sugarcane, napier grass, pasture grasses). This has been reported by Wu et al. (2012) as facilitating build-up of SCMV in fields and dissemination of the virus over large

areas in many countries worldwide.

Conclusion

This research has shown that SCMV is the main potyvirus in synergism with MCMV in causing MLN disease in Kenya. It has also demonstrated that MCMV isolates (accessions #MH645621 and #MH645621) from this study share a common origin with the MCMV isolates from Eastern and Sub-Saharan Africa. Further, the research has revealed that SCMV is more diverse than MCMV in the South-Rift region due to the existence of new variants of SCMV.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGMENTS

The authors sincerely appreciate the USDA-Norman E. Borlaug Fellowship for funding this study, Botany and Plant Department of Purdue University, for hosting him during the fellowship, Lonni Kucik, for the logistics support, Longfei Wang for the advice and graduate students in Johal's Lab, Tesfaye and Woloshuk Lab.

REFERENCES

- Adams IP, Harju VA, Hodges T, Hany U, Skelton A, Rai S, Boonham N (2014). First report of maize lethal necrosis disease in Rwanda. *New Disease Reports* 29(22).
- Adams IP, Miano DW, Kinyua ZM, Wangai A, Kimani E, Phiri N, Mumford R (2012). Use of next-generation sequencing for the identification and characterization of Maize chlorotic mottle virus and Sugarcane mosaic virus causing maize lethal necrosis in Kenya. *Plant Pathology* 10:1-9.
- Benson DA, Cavanaugh M, Clark K, Karsch-mizrachi I, Lipman DJ, Ostell J, Sayers EW (2013). GenBank. *Nucleic Acids Research* 41(November 2012):36-42.
- Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL (2009). BLAST+: architecture and applications. *BMC Bioinformatics* 9:1-9.
- Chomzynski P, Mackey K (1995). Substitution of chloroform by bromochloropropane in the single-step method of RNA isolation. *Analytical Biochemistry* 225(1):163-164.
- Davis ERM (2012). First Report of Maize chlorotic mottle virus and Maize Lethal Necrosis in Kenya. *Plant Disease* 96(10):11-12.
- Gao B, Cui XW, Li XD, Zhang CQ, Miao HQ (2011). Complete genomic sequence analysis of a highly virulent isolate revealed a novel strain of Sugarcane mosaic virus. *Virus Genes* 43(3):390-397.
- De Groot H, Oloo F, Tongruksawattana S, Das B (2016). Community-survey based assessment of the geographic distribution and impact of maize lethal necrosis (MLN) disease in Kenya. *Crop Protection* 82:30-35.
- Isabirye BE, Rwomushana I (2016). Current and future potential distribution of maize chlorotic mottle virus and risk of maize lethal necrosis disease in Africa. *Crop Breeding and Applied Biotechnology* 5(2):215-228.
- Jarugula S, Alabi OJ, Martin RR, Naidu RA (2010). Genetic Variability of Natural Populations of Grapevine leafroll-associated virus 2 in Pacific Northwest Vineyards. *Virology* (21).
- Kagoda F, Gidoi, R, Isabirye BE (2016). Status of maize lethal necrosis in eastern Uganda. *African Journal of Agricultural Research* 11(8):652-660.
- Kamau CN, Snipes K (2014). Kenya Grain and Feed Annual.1-5.
- Kiruwa FH, Feyissa T, Ndakidemi PA (2016). Insights of maize lethal necrotic disease: A major constraint to maize production in East Africa 10(9):271-279.
- Kulkarni HY (1972). Survey of viruses affecting east african major food crops. *East African Agricultural and Forestry Journal* 39(2):158-164.
- Kumar S, Stecher G, Tamura K (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets Brief communication. *Molecular Biology and Evolution* 33(7):1870-1874.
- Louie R (1980). Sugarcane Mosaic Virus in Kenya. *Plant Disease* 64(10):944-947.
- Lukanda M, Owati A, Ogunsanya P, Valimunzigha K, Katsongo K, Ndemere H, Kumar PL (2014). First Report of *Maize chlorotic mottle virus* Infecting Maize in the Democratic Republic of the Congo. *Plant Disease* 98(10):1448-1448.
- Mahuku G, Lockhart BE, Wanjala B, Jones MW, Kimunya JN, Stewart LR, Cassone BJ, Sevgan S, Nyasani JO, Kusia E, Lava Kumar P, Niblett CL, Kiggundu A, Asea G, Pappu HR, Wangai A, Prasanna BM, Redinbaugh MG (2015). Maize Lethal Necrosis (MLN), an Emerging Threat to Maize-Based Food Security in Sub-Saharan Africa. *Ecology and Epidemiology* 105(7):956-963.
- Makone SM, Menge D, Basweti E (2014). Impact of Maize Lethal Necrosis Disease on maize yield: A case of Kisii, Kenya. *International Journal of Agricultural Extension* 02(03):211-218.
- Mbega ER, Ndakidemi PA, Mamiro DP, Mushongi AA, Kitenge KM, Ndomba OA (2016). Role of Potyviruses in Synergistic Interaction Leading to Maize Lethal Necrotic Disease on Maize. *International Journal of Current Microbiology and Applied Sciences* 5(6):85-96.
- Mezzalama M (2015). MLN Pathogen Diagnosis, MLN-free Seed Production and Safe Exchange to Non-Endemic Countries. CIMMYT Institutional Multimedia Publications Repository.
- Perera MF, Filippone MP, Ramallo CJ, Cuenya MI, García ML, Ploper LD, Castagnaro AP (2009). Genetic diversity among viruses associated with sugarcane mosaic disease in Tucumán, Argentina. *Phytopathology* 99(1):38-49.
- Roossinck MJ, Schneider WL (2006). Genetic Diversity in RNA Virus Quasispecies Is Controlled by Host-Virus Interactions Genetic Diversity in RNA Virus Quasispecies Is Controlled by Host-Virus Interactions. *Journal of Virology* 299(14):337-348.
- Wangai AW, Redinbaugh MG, Kinyua ZM, Miano DW, Leley PK, Kasina M, Mahuku G, Scheets K, Jeffers D (2012). *Zea mays*. *Plant Disease* 96(10):1582.
- Wu L, Zu X, Wang S, Chen Y (2012). Sugarcane mosaic virus e Long history but still a threat to industry. *Crop Protection* 42:74-78.