Serological, Biological and Molecular Characterization of Viruses Causing Mosaic Diseases on Cabbage (*Brassica sp L.)* in Central Ethiopia

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Abstract

The productivity of cabbage (*Brassica oleracea* var. *capitata)* in Ethiopia has been generally low due to several biotic and abiotic constraints among which are several viral diseases. There is a recent report indicating that this economically important vegetable is seriously affected in Ethiopia by cauliflower mosaic virus (CaMV) and turnip mosaic virus (TuMV). However, little information exists on the incidence and distribution of these viruses as the previous report is based on samples only from Addis Ababa. In this study, a total of 370 leaf samples were collected from 75 cabbage growing fields in Central Ethiopia in two rounds of survey. Two cabbage varieties locally known as "*Habesha gomen*" and "*Tikur gomen*" with virus-like symptoms were collected and tested with Double Antibody Sandwich Enzyme-Linked Immunosorbent Assay (DAS-ELISA) using polyclonal antibodies specific to CaMV and TuMV. Results from serological diagnosis were confirmed with PCR and Sanger sequencing. The results indicated a high incidence and wide distribution of both viruses in Central Ethiopia with an average of 29.5% infection for CaMV and 40% for TuMV. Biological inoculation tests for CaMV or TuMV or both on healthy cabbage seedlings gave similar symptoms as those observed in the field. Symptom severity was higher with co-infection of CaMV and TuMV followed by TuMV single infection. BLAST analysis showed that TuMV and CaMV isolates from Ethiopia have nucleotide identity of 95-98% and 93-98%, respectively to previously reported isolates. Phylogenetic analysis revealed that CaMV isolates from Ethiopia are closely related to isolates from USA and Italy within Group II clade whereas TuMV isolates have close similarities with isolates from World B clade including isolates from Kenya, UK, Japan and the Netherlands. The identification of the causative agents of the mosaic disease observed on cabbage in Central Ethiopia may lay the foundation for future management studies.

Key words: cauliflower mosaic virus, turnip mosaic virus, DAS-ELISA, PCR, Ethiopia

Introduction

Cabbage (*Brassica oleracea var. capitata*, genus *Brassica*, family *Brassicaceae*) is economically important vegetable plant cultivated worldwide. It is a rich source of sulfur containing amino acids, carotenes, minerals, ascorbic acid, and antioxidants [21, 16, 2]. Cabbage is an important leafy vegetable crop grown widely in Ethiopia [3]. The production of cabbage in Ethiopia has been generally low due to a number of biotic and abiotic constraints. Diseases caused by viruses such as cauliflower mosaic virus (CaMV), turnip mosaic virus (TuMV), turnip yellow mosaic virus (TYMV), beet western yellows virus (BWYV) and cucumber mosaic virus (CMV) are among the major biotic factors limiting cabbage production globally [19]. CaMV and TuMV are aphid transmitted viruses largely infecting *B. oleracea* and widespread in temperate zones of Africa, UK, Asia, New Zealand, USA and several other EU countries, wherever the crop is grown [22]. CaMV infection in cabbage results up to 25% production loss, whereas TuMV, in case of an early infection, can reduce yield and production up to 50% [2]. These viruses can also reduce the quality of cabbage by inducing different disease symptoms including leaf deformation, mosaic, mottling, necrosis, rugosity, stunting, vein banding, vein clearing and yellowing [7]. CaMV is classified in the genus *Caulimovirus* of the *Caulimoviridae* family [15] with circular dsDNA genome of about 8 kb with six open reading frames (ORFs) containing three short single-stranded regions: two in one strand, one in the other [14]. It is predominantly transmitted by an aphid species, *Brevicoryne brassicae* in a non-circulative and semi-persistent manner [15]. The host range of CaMV is predominantly in the family *Brassicassae* including cabbage, cauliflower, turnip and mustard but some strains can infect members of *Solanaceae* such as tobacco.

TuMV is a member of the genus *Potyvirus* of the *Potyviridae* family, having flexuous filamentous particles of 700–750 nm long and a genome comprised of a single-stranded positive-sense RNA approximately 10 kb [12]. It is widespread and economically important virus which has been categorized among the five most destructive vegetable viruses around the world [23]. Unlike most other potyviruses which have relatively narrow host ranges, TuMV has a wide host and infects over 318 plant species in 156 genera of 43 plant families, including many important crops and weed plants [17, 10]. TuMV is not transmitted by seed, thus, weeds are the most likely primary source of inoculum, and aphids are responsible for disseminating the virus in the field [8]. It is transmitted in a non-persistent manner by several aphid species, most notably by the green peach aphid (*Myzus persicae*) and the cabbage aphid (*Brevicoryne brassicae*). There is a recent report on the occurrence of these two viruses in cabbage fields causing severe mosaic diseases in Addis Ababa City, Ethiopia [1] and further investigation was necessary to know the incidence and distribution of these two viruses in cabbage growing areas of Central Ethiopia. In this study, therefore, the incidence and distribution of CaMV or TuMV causing mosaic disease on cabbage crop in Central Ethiopia were investigated using serological, biological, and molecular techniques and their phylogenetic relatedness was compared with previously reported isolates.

Materials and Methods

Sample collections

A total of 370 leaf samples of two widely grown cabbage varieties locally known as *"Habesha gomen"* and "*Tikur gomen*" with virus-like symptoms such as mosaic, leaf distortion, striking yellowing and chlorosis, stunted growth and leaf deformation were collected (Fig. 1, Table 1) in the major cabbage growing areas of Central Ethiopia (Fig. 2) including Rift Valley areas of East

Shewa, West Shewa, South west Shewa, North Shewa and Addis Ababa. Fields were visited in approximately 5 km stopping wherever cabbage is available. In the first round of survey made in November 2017, 120 symptomatic leaf samples were collected from 25 fields of different sub-cities in Addis Ababa. During the second round of survey, from January to March 2019, a total of 50 fields within approximately in 100 km radius outside of Addis Ababa were surveyed and 250 samples were collected. Samples were dried over silica gel until they are tested in laboratory for virus identification.

Identification of the causal viruses

To identify viruses associated with the symptoms and determine their prevalence, serological, biological and molecular assays were conducted.

Serological Assay

Serological assays were carried out using the standard double antibody sandwich (DAS)-ELISA [5]. All samples were analyzed using polyclonal antibodies for CaMV and TuMV. Polyclonal antibodies and corresponding conjugates were kindly provided by Dr. Stephan Winter, German Collection of Microorganisms and Cell Culture (DSMZ), Germany and tests were conducted following the standard protocols recommended by the provider.

Biological Tests

To further confirm the symptoms observed on the field and infection severity, biological tests were conducted by mechanical inoculation of greenhouse grown healthy cabbage seedlings with sap extract from infected samples collected from field as described by Spence et al [22] and Guerret et al [17]. Cabbage seedlings raised in a greenhouse were initially tested by PCR to confirm the absence of CaMV and TuMV. Sap from plants infected with TuMV, CaMV and both viruses was extracted using potassium phosphate buffer (pH 7.0), were directly rubbed on leaf surfaces of 30 seedlings (10 seedlings for each TuMV, CaMV and mixed infection) which were dusted with carborundum. Five plants (two non-inoculated and three mock inoculated) were kept as a control for each types of cabbage. The experiment was replicated twice, and all plants were inspected for virus symptoms weekly for five consecutive weeks. Leaf samples from the plants were tested for CaMV and TuMV using PCR and RT-PCR, respectively, three-week post inoculation.

DNA Extraction and PCR

For CaMV identification, total DNA was extracted using CTAB method used by Doyle and Doyle [6] with slight modification. Briefly, 250 mg of cabbage leaf sample was grinded in 800 μL of extraction buffer (2% of CTAB, 1% of PVP 40, 1.4 M NaCl, 20 mM EDTA, 100 mM Tiris pH 8, 1.5% 2-mercapthoethanol). The sample aliquots were incubated at 55 °C for 15 min in water bath followed by centrifugation at 13,000 rpm for 5 min. The supernatants were transferred to a new sterile tube and gently mixed with 250 μL of chloroform: Isomyl alcohol (24:1) followed by centrifugation at 13,000 rpm for 2 min. The upper aqueous phase was transferred to a new tube and gently mixed with 50 μL of ammonium acetate and 500 μL of ice-cold ethanol. The mixed solution was kept at -20 °C for 2 hr and DNA was pelleted by centrifugation at 13,000 rpm for 3 min. The DNA pellets were washed with 500 μL 70% ethanol, air dried, and re-suspended in 60 μL of sterile nuclease free water. DNA was visualized by gel electrophoresis on 1% agarose gel.About 720 bp of CaMV genome spanning the coat protein region was amplified using AccuPower Hot Start PCR PreMix (BioNEER Corp., Republic of Korea) according to manufacturer's instruction. CaMV specific primer pair (CM42-R: 5'TCGCTGCAACGGCTTCTAAG 3' and CM42-F 5'GGAAACAGTGCTTCATCCTC 3') was used for the amplification [9]. PCR reaction was performed in total volume of 20 µL by mixing 1 µL of template DNA, 1 µL of each primer (10 µM) and 17 µL of nuclease free water into lyophilized AccuPower Ready to use PCR PreMix and brief vortexing. The PCR conditions were set for 30 cycles of 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 45 sec preceded by 94 °C initial denaturation for 5 min and with a final extension of 72 °C for 10 min. PCR products were visualized by gel electrophoresis on 1.2% agarose gel using Lamda DNA Ladder, (Biobasic, Inc., Canada).

RNA Extraction and RT-PCR

For TuMV identification, total RNA was extracted using EZ-10 Spin Column Plant RNA extraction Mini-Preps Kit (Bio Basic Inc. Canada) following manufacturer's instruction. Extracted RNAs were visualized by gel electrophoresis through 1.5% agarose gel. Coat protein (CP) coding region of TuMV (approximately 1 kb) was amplified by one step RT-PCR using TuMV specific primers (TuMV-1: 5'GAACCAGCTCAAGAGGATCT 3' and TuMV-2: 5'GAAGCTACACTGGCTGCTT 3') [11]. The RT-PCR was carried out in a total reaction volume of 25 µL comprising 1 µL total RNA extract, 0.5 µL of each forward and reverse primers, 0.25 µL of each Taq polymerase (thermo scientific, Germany) and M-MLV Reverse transcriptase, 1.25 µL of 25 mM $MgCl_2$, 2.5 µL of 10X PCR buffer, 0.5 µL of 10 mM dNTPs mix, 0.5 µL of RNase inhibitor and 17.75 µL of nuclease free water. RT-PCR condition was set to 42 °C for 1 hr for cDNA synthesis followed by initial denaturation at 95 °C for 5 min and 35 cycles of 95 °C for 30 sec, 58 °C for 40 sec and 72 °C for 1 min and final extension at 72 °C for 10 min followed by visualizing PCR products as described above.

Sequencing and phylogenetic analysis

For further analysis, five PCR amplicons for TuMV and three for CaMV were randomly selected, gel-purified (Bio Basic Inc., Canada) and Sanger sequenced in both directions at Macrogen Inc. (South Korea). Sequence data were processed and analyzed using CLC Main Workbench v6.9.2 (QIAGEN) and Geneious R11 (Biomatters). Nucleotide sequences were further aligned together with previously reported TuMV and CaMV sequences from the NCBI and analyzed using the Clustal W multiple sequence alignment algorithm in BioEdit version 7.2 (http://www. mbio.ncsu.edu/BioEdit/bioedit.html). A total of 693 nt bases long sequences of the coat protein coding region for TuMV and 508 nt bases long sequences of the aphid transmission protein coding region for CaMV were used for the alignments and phylogenetic trees were constructed using MEGA-X [13] using the Maximum Likelihood method and the Kimura 2-parameter model with 1000 bootstrap replications. The pairwise sequence comparison (PASC) was analyzed by Sequence Demarcation Tool, SDT Version 1.2 [14].

Results

Symptoms and serological analysis

Virus-like symptoms such as leaf striping, bright mosaic and yellowing were observed in almost all of cabbage fields during the survey (Fig. 1). The incidence of plants with virus-like symptoms on field observation ranges from trace (< 1%) in some fields of Bole sub-city to 100% from fields of Nefas Silk-Lafto sub-city in Addis Ababa and varied from field to field (Table 1).

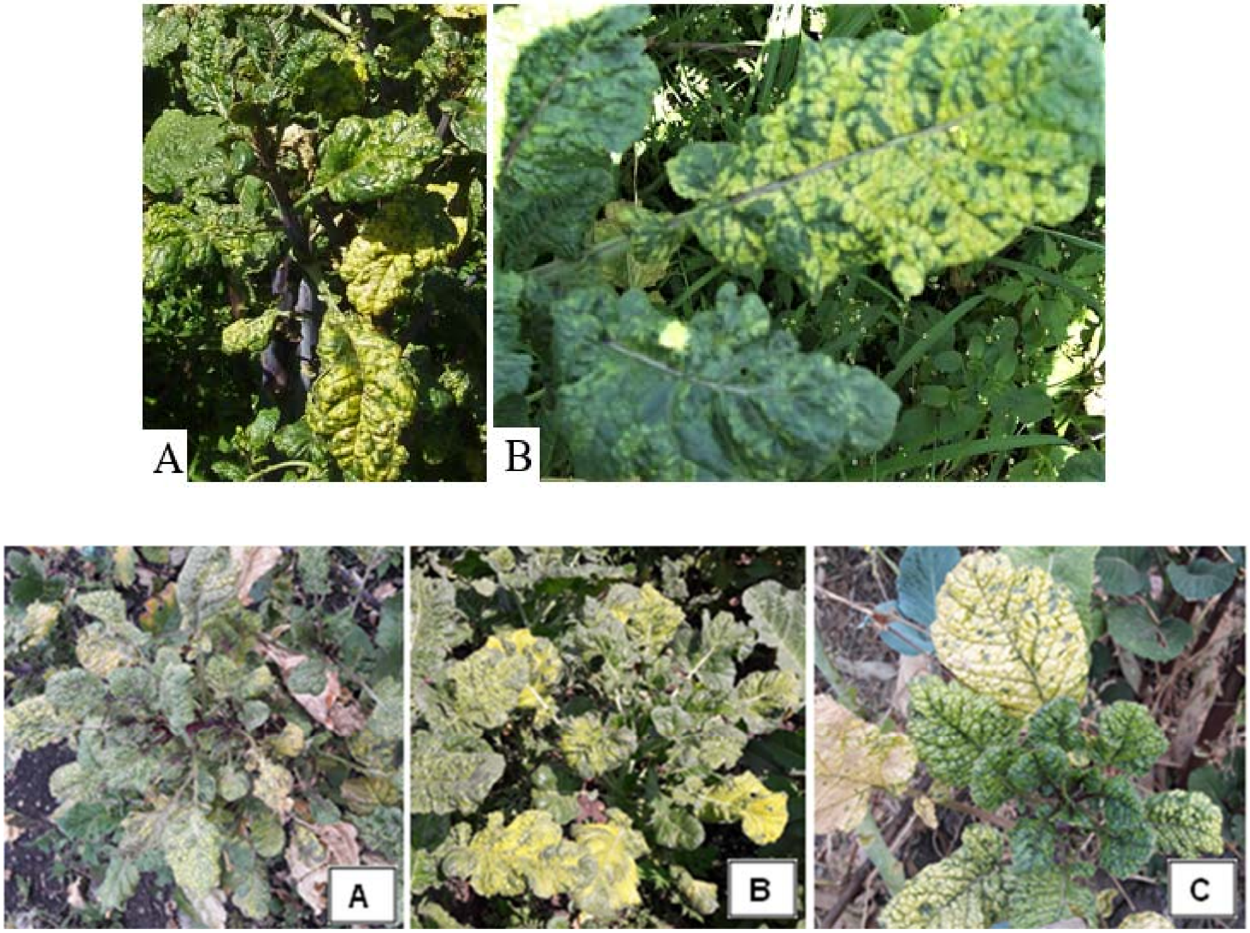

Figure 1. Symptomatic cabbages from farmers' fields of Addis Ababa infected with TuMV (A), CaMV (B), TuMV and CaMV co-infection (C).

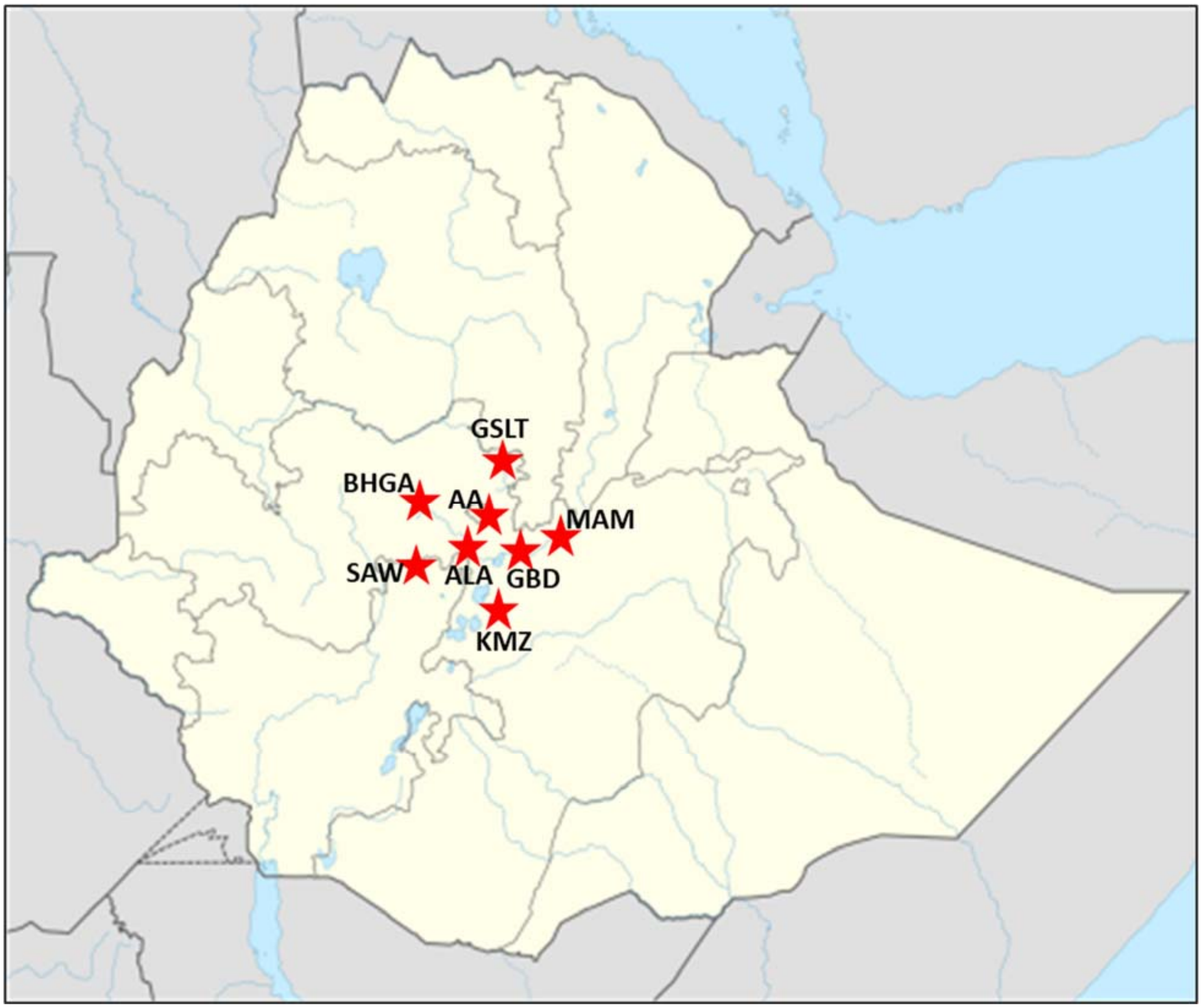

Fig. 2. Location of survey sites in Central Ethiopia (C). AA: Addis Ababa; ALA: Akaki to Lake Abasamuel; GDB: Galan, Dukem, Bishoftu; GSLT: Goro, Sululta, Lege Tafo; MAM: Modjo, Adama, Melkasa; KMZ: Koka, Meki, Ziway; SAW: Sebata, Asgori, Waliso; BHGA: Burayu, Holeta, Ginchi, Ambo.

Based on DAS-ELISA results, 29.5% (109) of the samples were positive for CaMV, whereas 40% (148 samples) tested positive for TuMV. Whereas, 16.7% (62) of the samples had mixed infection for CaMV and TuMV (Table 1). TuMV is the most widely distributed virus. The highest incidence of CaMV was observed in samples from Sebata, Asgori and Waliso (SAW) collections with an incidence of 41.7% (Table 1). Whereas, the highest TuMV incidence was recorded in samples from Koka, Meki, Ziway (KMZ) collections with an incidence of 50% (Table 1). On the other hand, the lowest incidence of CaMV was observed from Addis Ababa with a total of 17.5% of the samples tested positive both on DAS-ELISA and PCR. However, the lowest level of TuMV infection was recorded from Sebata, Asgori, Waliso (SAW) collections with a total of 30.7% of the samples tested positive (Table 1). A total of 27.8% of the samples from Sebata, Asgori, Waliso (SAW) found to be positive with mixed infection with both CaMV and TuMV, while samples from Addis Ababa had the lowest level of (8.3%) mixed infection (Table 1). Out of the 370 samples collected, a total of 175 samples tested negative for both viruses on DAS-ELISA and PCR.

Table 1. Number of DAS-ELISA and PCR positive samples for CaMV and TuMV from each field surveyed.

Field site	No of samples collected	Virus-like symptoms observed on field (%)	Number of DAS-ELISA positive samples			Number of PCR positive samples	
			CaMV (%)	TuMV (%)	Mixed infection (%)	CaMV	TuMV
Addis Ababa (AA)[1]	120	Trace–100%,	21 (17.5)	42 (35)	10 (8.3)	21	42
Akaki to Lake Abasamuel (ALA)[2]	36	25 –59	14 (38.9)	15 (41.7)	6 (16.7)	14	15
Galan, Dukem, Bishoftu (GDB)[2]	36	37–74	13 (36.1)	16 (44.4)	7 (19.4)	13	16
Goro, Sululta, Lege Tafo (GSLT)[2]	36	23–54	12 (33.3)	14 (38.9)	9 (25)	12	14
Modjo, Adama, Melkasa (MAM)[2]	35	44–79	11 (31.4)	16 (45.7)	5 (14.3)	11	16
Koka, Meki, Ziway (KMZ)[2]	36	51 –83	12 (33.3)	18 (50)	7 (19.4)	12	18
Sebata, Asgori, Waliso (SAW)[2]	36	28–51	15 (41.7)	11 (30.5)	10 (27.8)	15	11
Burayu, Holeta, Ginchi, Ambo (BHGA)[2]	35	31–62	11 (31.4)	16 (45.7)	8 (22.8)	11	16
Total	**370**		**109 (29.5)**	**148 (40)**	**62 (16.7)**	**109**	**148**

[1]Samples were collected in 2017. [2]Samples were collected in 2019. The percentages from single and mixed infections for each sampling locations are presented in brackets.

Back inoculation into cabbage seedlings

Clear virus-like symptom such as mosaic, leaf distortion, leaf yellowing and venal chlorosis, stunted growth and leaf deformation were observed 21 days post inoculation. Mild symptoms were induced from single infections with CaMV or TuMV (Fig. 3B and D), while mixed infection from the two viruses induced more severe symptoms (Fig. 3E and F). Symptom severity for both viruses in "*Tikur Gomen*" was higher when compared to "*Habesha Gomen*". No virus-like symptom was observed on all non-inoculated plants (Fig. 3Aand B). Leaf samples were collected from all the plants and subjected to PCR analysis as described earlier to confirm the presence of CaMV and TuMV. and amplicon of the expected sizes were obtained from samples inoculated with CaMV and TuMV while leaf samples from non-inoculated seedlings were negative.

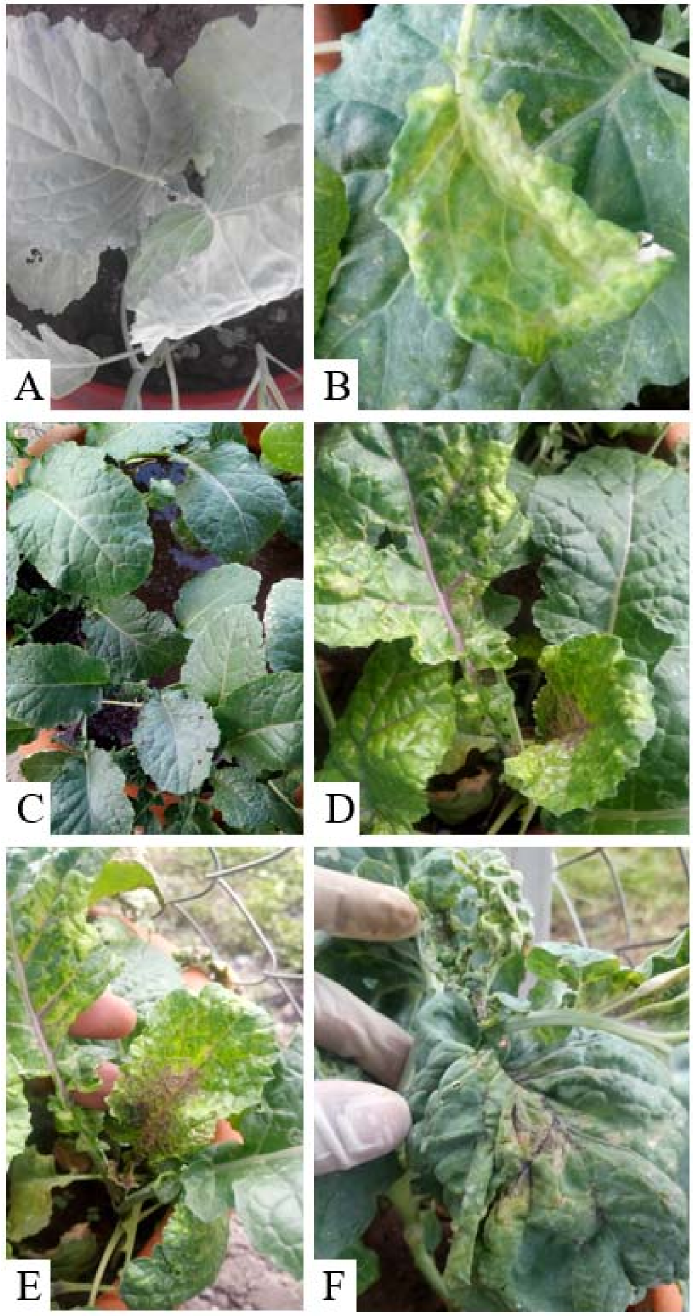

Fig. 3 Biological infection of *Brassica oleracea var.capitata* ("*Habesha Gomen*" and "*Tikur Gomen*") with CaMV and TuMV in greenhouse., Biological infection "*Habesha Gomen"* with CaMV; non-innoculated (A), and 21 days post innoculation (B). Biological infection of "*Tikur Gomen"* with TuMV; non-inoculated (C), 21 days post inoculation (D)., Mixed infection with both CaMV and TuMV *Tikur gomen* (E), *Habesha gomen* (F).

PCR and sequence analysis

The entire 370 samples were subjected to PCR screening using virus specific primers targeting CaMV or TuMV. The PCR results showed a total of 109 (29.5%) samples tested positive for CaMV. Whereas, 148 (40%) samples were positive for TuMV. The PCR results were consistent with DAS-ELISA findings in which all the samples which tested positive for CaMV or TuMV on DAS-ELISA also tested positive by PCR (Table 1).

Pairwise Sequence Comparison (PASC) and phylogenetic analysis

BLASTn analysis using Sanger sequenced data showed that TuMV or CaMV isolates from Ethiopia shared high nucleotide identity with their respective isolates reported from elsewhere. TuMV isolates from this study showed 94.5-97% nucleotide identity with each other and 94-97% with TuMV isolates from the GenBank such as UK1 (AB194797), Au1 (AB989628) and Ken1 (AB093605). CaMV isolates from Ethiopia showed 94-98% nucleotide identity among themselves and 93-98% nucleotide identity with previously reported CaMV isolates including GRC92D (AB863202), GRC97E (AB863197) and IRNWKB15 (KF357598) from the GenBank. Based on BLASTp results, the putative amino acid sequences of the coding regions of TuMV and CaMV isolates from Ethiopia shared identity scores of 90-99% and 91-100% with previously reported isolates, respectively.

Based on phylogenetic analysis the three CaMV isolates from Ethiopia clustered within group II clade together with other CaMV isolates from North America and Italy forming a terminal branch (Fig. 4). Similarly, the TuMV isolates from Ethiopia clustered together forming a terminal branch within the World-B clade of TuMV (Fig. 5). The TuMV isolates from Ethiopia appear to be closely related to each other and to other TuMV isolates reported from UK, Netherlands, Japan and Kenya within the World-B clade (Fig. 5).

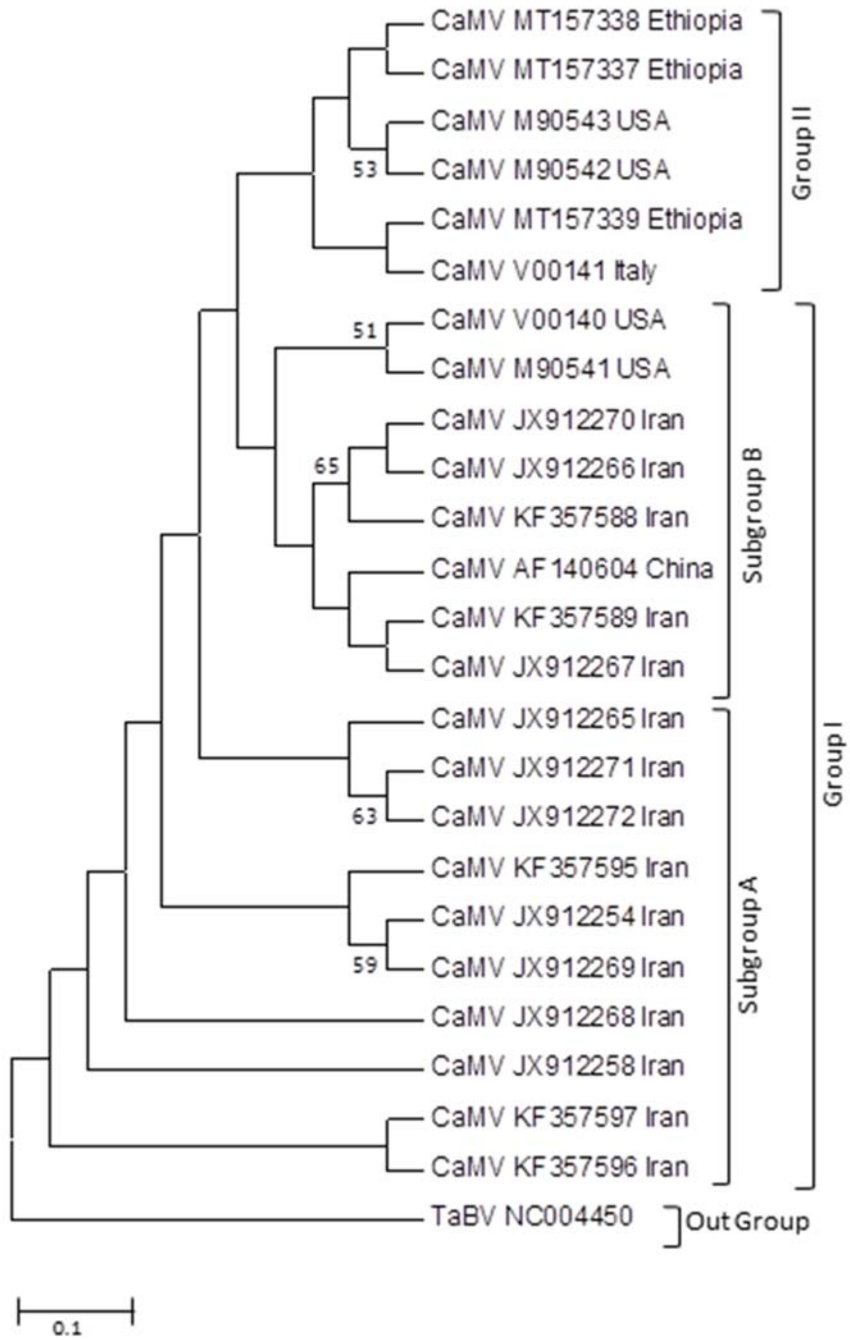

Fig. 4 Phylogenic tree showing evolutionary relationships of CaMV (cauliflower mosaic virus) isolates identified from Ethiopia with previously characterized isolates using nucleic acid sequences. Bootstrap values greater than 50% are shown, and the scale bar indicates substitutions per site. TaBV (taro bacilliform virus) was used as an out group.

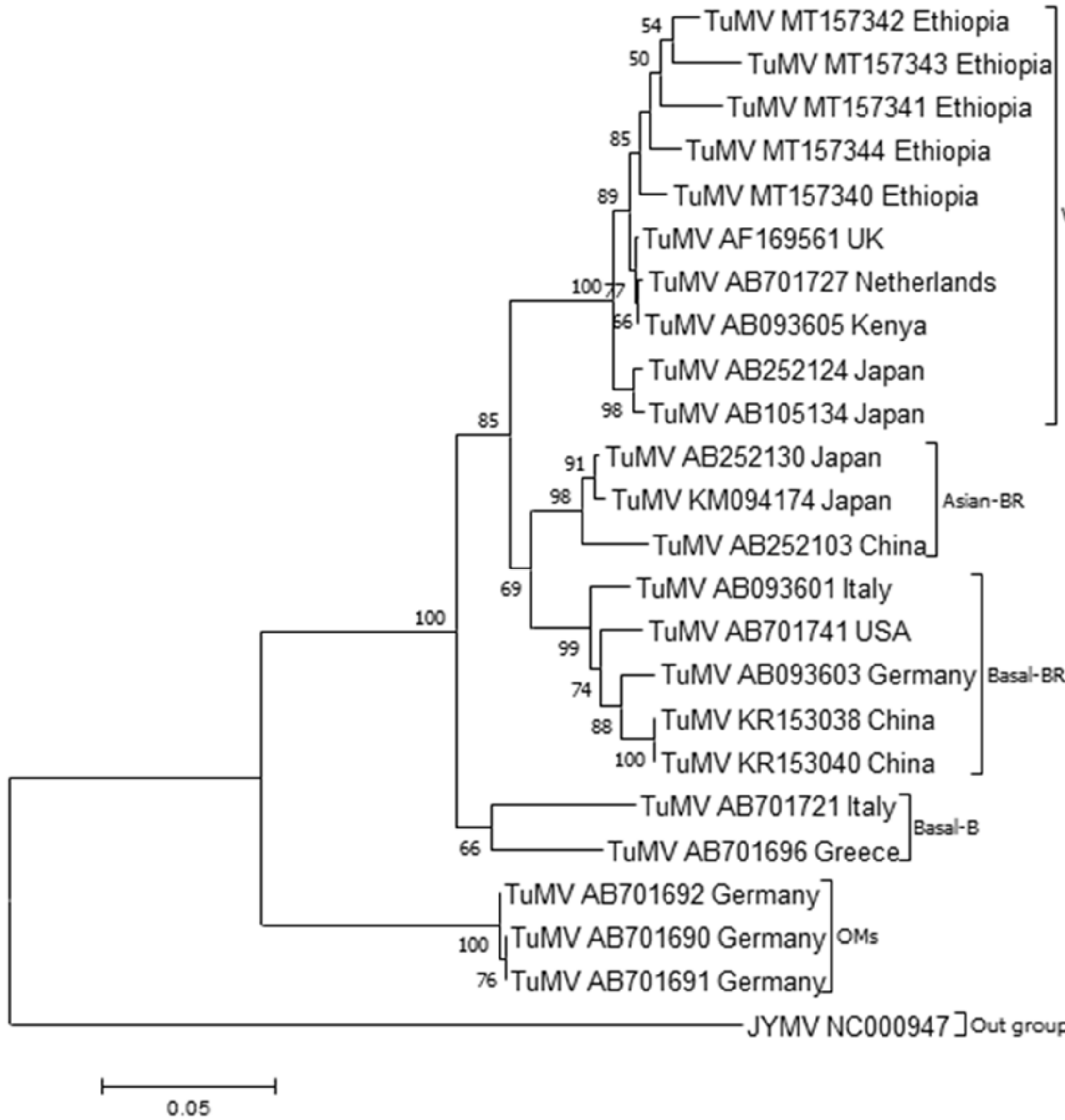

Fig. 5 Phylogenetic tree showing evolutionary relationships of TuMV (turnip mosaic virus) isolates identified from Ethiopia with previoulsy identified isolates using nucelic acid sequences. Bootstrap values greater than 50% are shown, and the scale bar indicates substitutions per site. JYMV (japanese yam mosaic virus) was used as an out group.

Discussion

In this study, cabbage samples with mosaic disease from Central Ethiopia were subjected to serological, biological, and molecular analyses to identify viruses associated with the disease and characterize their coat protein gene sequences. This is the first comprehensive report to characterize the CaMV and TuMV viruses associated with cabbage crops in Ethiopia. Even though both CaMV and TuMV are viruses with widespread distribution in *Brassica* spp worldwide, they were reported only recently from only few samples in Addis Ababa area, Ethiopia [1]. Hence, in this study we screened samples from the major cabbage growing regions in Central Ethiopia. Furthermore, biological studies by sap inoculation of healthy cabbage seedlings using CaMV, TuMV or both viruses were carried out to confirm the symptoms observed in the field are from the viruses.

Despite the difference in their incidence, both TuMV and CaMV were identified in all surveyed areas. The highest virus incidence was recorded at Sabeta to Woliso field sites for CaMV (42%) and Koka to Ziway for TuMV (50%), whereas the highest mixed infection was recorded at Sebeta to Woliso sites (27%). Incidence of the two viruses was high in the Rift Valley areas such as Meki, Ziway, Koka and Awash-Melkasa. Unlike Rift Valley, the incidence of the viruses in highland areas like Addis Ababa was relatively low with only 18% CaMV and 35% TuMV infections. This may be due to the warmer weather condition around the Rift Valley area which may favors the population buildup of aphids that transmit the viruses. TuMV was more prevalent in all surveyed fields. Other studies also reported TuMV as one of the widespread viruses of brassica crops worldwide [8, 20, 18]. All the samples collected had virus like symptoms however, a total of 175 samples tested negative both on DAS-ELISA and PCR. This could be either the samples are infected with viruses other than CaMV and TuMV or the symptoms observed may be from abiotic factors such as nutrient deficiency or other environmental factors.

Upon mechanical inoculation, chlorotic, necrotic local lesions, systemic vein clearing and veinal flecking developing into severe mosaic symptoms were observed with both TuMV and CaMV on cabbage plants within 21 days post inoculation. These symptoms were similar to those observed on infected cabbage plants in the field. Symptoms severity on "*Tikur Gomen*" were stronger compared to symptoms observed on "*Habesha Gomen*" both in the field as well as greenhouse experiments. The overall high incidence of viral diseases observed in cabbage fields can be attributed to the susceptibility of cultivated varieties, availability of source inoculum, conditions favoring the high population of aphids, and environmental conditions [2]. Disease severities were high with co-infection of CaMV and TuMV which is also reported earlier [4] followed by TuMV single infection which is in line with previous reports in Kenya [22].

PASC and phylogentic analysis revealed that CaMV isolates from Ethiopia are geneticaly similar at the CP-coding region with more than 94% identity at the nucleotide level. Simialrly, TuMV isolates from Ethiopia have more than 94% nucleotide identity with each other on PASC. Based on our analysis all TuMV isolates from Ethiopia grouped with TuMV islates from World-B group. Further studies by including several sequences and full-length genome sequenes is needed in the future to identify if there are several TuMV groups in Ethiopia. Both PASC and phylogentic analyses for CaMV and TuMV suggested that the two viruses might have been introduced into Ethiopia from a single origin. However, a survey including samples from other alternative hosts as well as cabbage samples grown in different region should be conducted at a national level to clearly understand the genetic diversity of CaMV and TuMV in Ethiopia.

In conclusion, TuMV and CaMV were found to be widespread viruses infecting cabbage crop in Central Ethiopia and causing severe mosaic disease affecting the production of the crop in Ethiopia. From this survey and field observation, TuMV causes more serious damage to cabbage while CaMV has relatively low incidence and disease severity. Since TuMV and CaMV are not transmitted by seeds, alternative hosts may be the most likely overwintering hosts and serve as primary source of inoculum and aphid vectors may disseminate the virus in the field. To our knowledge, this is the first serological, biological and molecular study to identify viruses associated with cabbage crops in Central Ethiopia. Therefore, incidence and distribution of the two viruses in other cabbage growing areas as well as their occurrence in other brassica crops such as rapeseed should also be assessed in the future to obtain complete picture of the importance of these viruses in the country.

Conflicts of interest

The authors declare no conflict of interest.

Ethical approval

This article does not contain any work conducted on animals or human participants.

Authors contribution

AA: conceptualize the project idea, supevision, critical review and edit of the manuscript; MK and DG: conduct the experiment, data anlaysis, write the original draft; DG and DK: data anlaysis and validation, critical review and edit of the manuscript.

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