Prevalence of Viral Diseases in Vegetable Crops in Kathmandu Valley and their Detection by ELISA and Immunostrip Tests

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Abstract

The study was conducted from July to October, 2019. The objective of this study was to identify viral diseases in vegetable pocket areas of Kathmandu, Bhaktapur and Lalitpur districts. Sample size of 102 households of vegetable cultivated areas was selected through purposive random sampling technique. Data were collected from subsistence and commercial vegetable farms using pre- tested semi- structured questionnaires. 30 plant samples showing virus like symptoms were further diagnosed using enzyme-linked immuno-sorbent assay (ELISA) and Immunostrip detection tests. Of total 30 vegetable crops samples suspected for prevalence of virus pathogen, 21 samples were detected with viral infection. This study provided laboratory test confirmation on presence of virus diseases, like Zucchini Yellow Mosaic Virus disease in pumpkin, sponge gourd and cucumber, Cucumber Mosaic Virus disease in chilli and tomato, Tomato Mosaic Virus disease in tomato, Turnip Mosaic Virus disease in broad leaf mustard, Chilli Veinal Mottle Virus disease in chilli, Squash Mosaic Virus disease in broad leaf mustard and Bean Common Mosaic Virus disease in common bean in specific locations of Kathmandu, Bhaktapur and Lalitpur districts. This study provided first report of Squash Mosaic Virus in Broad Leaf Mustard cultivated areas of Lalitpur district. The results obtained from this study can be used in developing location specific integrated management strategies for viral diseases.

Keywords: ACP ELISA, Antibody, Antigen, Vegetable crops, Virus disease detection tests

Introduction

Agriculture sector of Nepal has prioritized vegetable farming as an integral part of both subsistence and commercial farming systems. Vegetable crops have become one of the most potential income generating sources for majority of the farmers. In 2074/75,total area under vegetable farming was 286,864 hectares and production 3,958,230 metric ton (MoALD, 2076), Vegetable farming within the cities or on the peripheries is now emerging as a major agricultural practice of peri-urban farmers in Kathmandu valley and the large tracts of land outside the central city areas are devoted to farming (Rai et al., 2019).

In vegetable cultivation areas of Kathmandu valley, farmers are focusing especially on commercial production of high value vegetable crops of family Solanaceae, Cucurbitaceae, Fabaceae, Malvaceae and Brassicaceae. Diseases come as one of the major constraints of successful vegetable cultivation. In recent years, viral diseases are appearing as the most important hindrance, which not only decline the productivity, but also the market value of the products. Viral diseases induce broad range of symptoms affecting growth, yield and quality of the produce. The major vegetable crops, like tomato, potato, chili, capsicum, cucumber, zucchini squash, beans, etc are mainly affected by viruses causing significant yield losses.

Various important vegetable crops were diagnosed with various viral diseases in farmers' fields in Nepal. Chili and Capsicum cultivated in Surkhet district were infected with Chili Veinal Mottle Virus (CVMV) (Rayapati, 2016). Timila et al. (2014) reported the prevalence of Tomato Leaf Curl Virus (TOLCV), Chili Veinal Mottle Virus (CVMV), Cucumber Mosaic Virus (CMV) in pepper (Chili and Capsicum) and Tomato Mosaic Virus (TMV), Tomato leaf curl virus, Cucumber Mosaic Virus in tomato crops cultivated in terai, inner-terai and mid hills of Nepal. The incidence of CMV ranged from 50-80% in these areas, while CVMV up to 80% in chili producing areas of Kathmandu Valley. Similarly, Tomato leaf curl New Delhi virus, Tomato leaf curl Karnataka virus, peanut bud necrosis virus, tobacco mosaic virus and cucumber mosaic virus were identified in tomato crop cultivation areas of Surkhet, Hemja, Pokhara, Kathmandu and Pokhara, respectively (Rayapati, 2016). According to Rayamajhi (2019), N.C.L 1, a popular rainy season variety of tomato in western hills of Nepal, was diagnosed with Tomato Leaf Curl Virus disease at Baglung. A large number of viruses have been reported to infect vegetable crops cultivated in Nepal belonging to family Cucurbitaceae. The identified viruses were CMV, Zucchini Yellow Mosaic Virus (ZYMV), Watermelon Mosaic Virus (WMV) 1 and 2, Squash Mosaic Virus (SqMV) and Cucumber Green Mottle Mosaic Virus (CGMMV). These viruses infected cucurbits either individually or in combination with more than one virus at the same time (Joshi et al., 2013). Also, Rayapati (2016) surveyed the farmers' fields of Surkhet, Banke, Kaski and Kathmandu districts of Nepal and identified pumpkin and squash of Surkhet district, bottle gourd, pumpkin, snake gourd and bitter gourd of Banke district and sponge gourd in Pokhara infected with ZYMV. According to Poudel & Khanal (2018), ZYMV and Cucurbit Aphid Borne Yellow Luteovirus (CABYV) were first reported in Nepal. Similarly, in broad leaf mustard, Rayapati (2016) identified Turnip mosaic virus from various locations across Nepal. Also Poudel & Khanal (2018) mentioned the report, diagnosis and identification of the mosaic disease of broad leaf mustard. Likewise, Bhindi Yellow Vein Mosaic Virus has been identified from okra cultivated farms in Nepalgunj. Beans cultivated in Lalitpur district were infected with bean yellow mosaic virus (BYMV) (Rayapati, 2016).

This study was carried out with a general objective of identifying the viral diseases of high value, seasonal vegetable crops prevailing in Kathmandu valley by ELISA and Immunostrip tests.

Materials and Methods

This study was carried out from July 15 to October 15, 2019, as a field survey and in lab. Field survey was carried out to observe the prevalence of virus-like symptoms on vegetable crops and their sample collection, while lab works were concentrated to diagnose the viral pathogens in the samples via ELISA and Immunostrip tests.

Field survey and plant sample collection:

The survey sites for virus disease sample

collection in vegetable crops were from Kathmandu (Kageshwori-Manohara, Tarkeshwor and Chandragiri municipalities), Bhaktapur (Changunarayan and Surya Binayak municipalities) and Lalitpur (Godawari and Mahalaxmi municipalities and Lalitpur Metropolitan city) districts. A total of 102 households were selected (34 households from each district) using purposive random sampling technique, including both subsistence and commercial farms. Primary data were collected from the selected households using pre-tested semi-structured questionnaire. Secondary data were obtained from reports, newsletters, relevant articles from websites and libraries (National Plant Pathology Research Centre / NARC and Himalayan College of Agricultural Sciences and Technology).

Collection of plant samples

Diseased plant samples with virus-like symptoms were collected and put into zip-lock bags, airtight jars, envelopes or newspapers, wherever suited, and labelled well. A "Virus disease handbook" with pictures of diseased plants or plant parts with specific viruses was used to assist tentative identification.

Diseased leaf samples with some healthy parts were cut with a sterilized scissor folded along with a newspaper wrap and put into different zip-lock bags. The zip-lock bags were placed individually into large sized envelopes. Information of the samples were written on a small piece of paper, placed inside the envelopes and sealed. The envelopes were also labelled outside with full information and taken to virology lab at NARC for immediate diagnosis, or preserved into a refrigerator until use.

Laboratory study

Final, detective analysis of diseased plant samples were carried out with ELISA (with use of Antigen-coated plate: ACP) and Immunostrip tests in virology laboratory at Nepal Agricultural Research Council (NARC), Khumaltar.

Enzyme linked immuno-sorbent assay (ACP ELISA)

Extraction of antigen from leaf samples:

Working solution of indirect sample extraction buffer was prepared by mixing 1 ml of indirect sample extraction buffer concentrate with 9 ml of distilled water. One gram each of the diseased leaf samples was mashed with 9 ml of working solution of sample extraction buffer added few drops at a time as required in separate mortars and pestles. The 10 times diluted sample suspension were filtered using filter paper. The filtrate was further diluted 10 times by mixing 1 ml of the suspension into 9 ml of the working solution to have a 100 times final dilution, and placed into the labelled beakers.

Testing procedure

Placing of antigen extract into microtiter plates: Solid microtiter plates (agdia) containing 96 wells were labeled 1:10, 1:100, replicated 3 times, and with a positive and negative control. 100 µl of 10 and 100 times diluted sample extracts were poured into the labeled, respective wells with a multi-channel pipette. Same amounts of the positive and negative controls were also pipetted into each well. Positive control was received along with ELISA kit (Patho Screen Kit, agdia, USA) and for negative control; working solution of indirect sample extraction buffer was used. The microtiter plates were wrapped with plastic film and incubated inside a refrigerator at 5 degrees Celsius for 2 hours, to let antigen bind to the bottom of the microtiter plates.

Washing of microtiter plates: Wash buffer was prepared by diluting one 20X pouch of wash buffer with 950 ml of distilled water. Antigen sample extracts and controls were washed from the microtiter plates with washing buffer using quick flipping motion without mixing the contents when the sample incubation was complete. The papers were tapped 3 times on paper cushion to remove any unbound antigen. This process was repeated three times and special care was

given not to leave any air bubble, wash buffer or visible antigen extract into washed microtiter wells.

Placing of primary antibody in microtiter plates:Working solution of antibody buffer (ECI buffer) was prepared by mixing 2 ml of 5X ECI buffer concentrate with 8 ml of distilled water. Then, 10 µl of concentrated primary antibody was added to the prepared 10 ml of working ECI buffer solution. For antigens suspected with Potyvirus infection, Potyvirus antibody were used and for samples suspected with BCMV infection BCMV antibody were used. The diluted antibody suspension, 100 µl/well, was added to the washed wells of the microtiter plates using a multichannel pipette. The microtiter plates were wrapped with plastic film and incubated overnight inside a refrigerator overnight at 5 degrees Celsius.

Washing of microtiter plates: Unbounded primary antibody were washed from the microtiter plates with washing buffer using quick flipping motion without mixing the contents when the incubation was complete. The papers were tapped 3 times on paper cushion. This process was repeated three times and special care was given not to leave any air bubble or/and wash buffer into washed microtiter wells.

Placing of secondary antibody in microtiter plates:Working solution of antibody buffer (ECI buffer) was prepared. Then, 10 µl of concentrated secondary antibody was added to the prepared 10 ml of working ECI buffer solution. The diluted secondary antibody suspension, 100 µl/well, was added to the washed wells of the microtiter plates using a multichannel pipette. The microtiter plates were wrapped with plastic film and incubated for two hours in working bench at room temperature.

Washing of microtiter plates: Unbounded secondary antibody were washed from the microtiter plates with washing buffer using quick flipping motion without mixing the contents when the incubation was complete. The papers were tapped 3 times on paper cushion. This process was repeated three times and special care was given not to leave any air bubble or/and wash buffer into washed microtiter wells.

Preparation of PNP substrate solution: Working solution of PNP (p-nitrophenyl phosphate) substrate solution was prepared by adding one tablet of PNP per 5 ml of PNP working buffer solution(10 ml of working PNP substrate buffer was prepared by mixing 2 ml of 5X PNP buffer concentrate with 8 ml of distilled water) in a beaker and the beaker was immediately wrapped with aluminum foil to avoid reaction of the substrate with light. The container was shaken for a few minutes to dissolve the tablets into the solution.

Placing of substrate solution in microtiter plates:The PNP substrate solution was added into the washed wells, 100 µl/well, of the microtiter plates using a multichannel pipette. The plates were wrapped with aluminum foil and rested on working bench for 30 minutes.

Visual interpretation of the result was done based on the colour development in wells of the microtiter plates. Wells in which colour developed indicated positive results. Wells in which there was no significant colour development indicated negative result. Test results were considered valid when positive control wells gave a positive result and buffer wells remain colourless.

Immunostrip test

Diseased leaf samples were cut into approximate sample size of 2.5 cm2 with scissors and were placed inside sample extraction pouch. The samples were ground with a pestle from outside the pouch until the considerable amount of cell sap for test was released inside the pouch. The resulting solution was allowed to settle for 3 minutes before inserting the Immunostrip. One immunostrip specific to the suspected virus was placed inside the pouch carefully from the edge of the pouch. Care was taken to dip the strips

into the extract only up to the demarcation line on the strip. Incubation of the strips was done for 10-20 minutes until the development of bands of pink colour on the Immunostrips.

Test strip(s) were removed from extract and interpretation of results were done. If the control line was visible and the test line was also present at any intensity of pink/purple, this indicated a positive result. If only the control line was visible, this indicated a negative result. The control line assured that the test is working properly. If the control line did not appear, the test was considered invalid.

The collected data from laboratory research and household survey were entered and analysed using MS-Excel version 10.

Results

Vegetable Crops Grown in Respondent's Field

The vegetable crops cultivated in study sites during the survey period (July- October) were of family Solanaceae, Brassicaceae, Cucurbitaceae and Leguminosae. Tomato, BLM and Capsicum were cultivated in plastic tunnels by 12%, 14% and 9% of the total respondent farmers respectively and while Cucumber, Chilli, Pumpkin, Sponge gourd, Chayote and Vegetable beans were grown in open field by 17%,16%,15%,8%,4% and 5% of the total respondent farmers respectively.

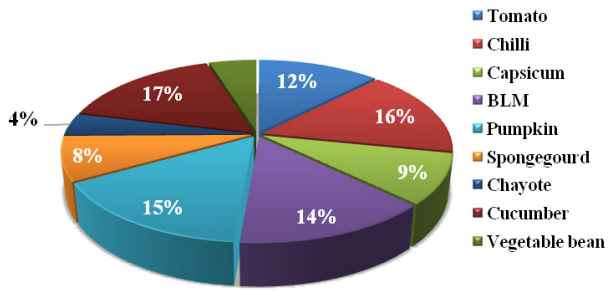

Figure 1: Vegetable crops cultivated in study sites of Kathmandu valley during study period.

Source: Field Survey, 2019

Prevalence of virus like symptoms in vegetable fields of study sites

The study revealed that, of 102 vegetable fields visited in study sites, 30 vegetable fields (29%) in study sites of Kathmandu valley were suspected with virus infection in various vegetable crops (Table 1).

Table 1: Vegetable fields of respondent farmers with prevalence of virus like symptoms

Prevalence of virus like symptoms in vegetable fields	Frequency
Yes	30(29)
No	72(71)

Figure in parentheses indicate percentage

Source: Field Survey, 2019.

Prevalence of viral diseases in the suspected vegetable crop samples at study sites

Of 30 suspected samples of the vegetables, 21 (70%) samples were diagnosed with virus diseases (Table 2) with ACP ELISA and Immunostrip test in the lab. A total of 7 different viruses belonging to family Potyviridae (ZYMV, BCMV, TuMV, ChiMV), Bromoviridae (CMV), Secoviridae (SqMV) or Virgoviridae (ToMV) were identified in 7 different vegetables (Pumpkin, Cucumber, Chill, BLM, Sponge gourd, Tomato, Common bean) of Kathmandu valley by ELISA and Immunostrip tests (Table 3). Out of suspected samples, ZYMV disease was detected at highest number from all three districts through both ELISA and Immunostrip tests.

Table 2: Viral disease detection in samples of vegetable crops in lab

Virus diseases detected in suspected samples	Frequency
Positive result (Virus detected)	21(70)
Negative result (Virus not detected)	9(30)

Figure in parentheses indicate percentage

Source: Field Survey, 2019

Table 3: Detection of viruses in vegetable crops from Kathmandu valley

Virus	Crop	Detection test	Location
Zucchini Yellow Mosaic Virus (ZYMV)	Pumpkin Sponge gourd Cucumber	ELISA	Kathmandu
	Pumpkin	Immunostrip Test	
	Cucumber Pumpkin	ELISA	Bhaktapur
	Cucumber Pumpkin	Immunostrip Test	
	Cucumber Pumpkin	Immunostrip Test	Lalitpur
ChilliVeinal Mottle Virus (ChiVMV)	Chilli	ELISA	Bhaktapur
Bean Common Mosaic Virus (BCMV)	Common bean	ELISA	
Cucumber Mosaic Virus (CMV)	Chilli	Immunostrip	
ChilliVeinal Mottle Virus (ChiVMV)	Chilli	Immunostrip test	Lalitpur
Cucumber Mosaic Virus (CMV)	Tomato		
Tomato Mosaic Virus (ToMV)			
Turnip Mosaic Virus (TuMV)	Broad Leaf Mustard		
Squash Mosaic Virus (SqMV)			

Virus infected leaf samples of various vegetables from fields of Kathmandu, Bhaktapur and Lalitpur districts are presented in Figures 2, 3 and 4.

Figure 2: Virus infected leaf samples from Kathmandu district

2.1: Cucumber (Cucumis sativus) leaf infected with ZYMV at Kageshwori Manohara-7.

2.2: Sponge gourd (Luffa cylindrica) leaf infected with ZYMV at Tarkeshwor-2.

2.3: Pumpkin (Cucurbita pepo) leaves (left mature and right young) infected with ZYMV at Kageshwori Manohara-7

Figure 3: Virus infected leaf samples at Bhaktapur district

3.1: Cucumber (Cucumis sativus) leaves infected with ZYMV at Changunarayan-2

3.2: Cucumber (Cucumis sativus) leaves infected with ZYMV at Changunarayan-2.

3.3: Pumpkin (Cucurbita pepo) leaves infected with ZYMV showing mosaic symptom atChangunarayan-2.

3.4: Pumpkin (Cucurbita pepo) leaves infected with ZYMV showing mosaic and leaf narrowing symptoms at Changunarayan-2

3.5: Chilli (Capsicum annuum) leaves infected with CMV at Changunarayan-2.

3.6: Common bean (Phaseolus vulgaris) leaves infected with BCMV at Changunarayan-2.

Figure 4: Virus infected leaf samples from Lalitpur district

4.1.: Tomato (Solanum lycopersicum) leaves infected with ToMV at Mahalaxmi-8 (left) and Godawari-14 (right).

4.2. Tomato (Solanum lycopersicum) leaves infected with CMV at Mahalaxmi-8 (left) and Godawari-14 (right)

4.3. Pumpkin (left) and cucumber (right) leaves infected with ZYMV at Mahalaxmi-8 (left) and Lalitpur metropolitan city- 28 (right).

4.4 BLM (Brassica sp.) leaf infected with TuMV and SqMV together at Godawari- 14.

4.5 BLM (Brassica sp.) leaf infected with TuMV at Godawari-14.

4.6. Chilli (*Capsicum annuum*) leaves infected with at Mahalaxmi- 8

Discussion

Of the ZYMV screened by ELISA and Immunostrip tests in cucurbit samples from Kathmandu, the disease was detected in pumpkin, sponge gourd and in cucumber crops. Similarly, from Bhaktapur the disease was detected in cucumber and pumpkin crops. Likewise, from Lalitpur, the disease was detected from cucumber and pumpkin leaf samples. Of the ChiVMV screened by ELISA and immunostrip tests in pepper samples from Bhaktapur and Lalitpur, the disease was detected from Chilli crop at both locations. BCMV disease was detected in bean crop from Bhaktapur. Of the CMV screened by Immunostrip test in pepper sample, CMV was detected from Chilli sample at Bhaktapur. CMV and ToMV diseases were detected from tomato crop in Lalitpur district. Of these two virus diseases screened by Immunostrip test, TuMV and SqMV were detected in BLM crop in Lalitpur. These surveys in various locations in three districts of Kathmandu valley provided the first report of SqMV in two locations of BLM cultivated farms of Lalitpur district. Most notably, this study provided the laboratory test confirmation on the presence of various viral diseases, like ZYMV, CMV, ToMV, TuMVand ChiVMV in specific locations of three districts in Kathmandu Valley. The results obtained through detection tests complemented the findings of other studies of prevalence of virus like ZYMV in cucurbits; ChiVMV and CMV in Chilli; BCMV in Common bean; ToMV and CMV in tomato and TuMV in BLM in mid- hills of Nepal. But in contrary, this study states the prevalence of these virus diseases in specific locations of Kathmandu, Bhaktapur and Lalitpur districts.

Conclusion

The study focused on identification of the virus diseases of high value vegetable crops cultivated in vegetable cultivation areas of Kathmandu, Bhaktapur and Lalitpur districts. The viral diseases are causing subsequent losses in yield of different vegetable crops but the studies and research works are limited in Nepal. In various vegetable cultivation areas in Kathmandu valley, ZYMV infection was highly detected from pumpkin crop than other cucurbitaceous crops. CMV and ChiVMV diseases were detected from chilli crop. ToMV and CMV infections were detected from tomato samples at two locations in Laliitpur. In BLM samples, TuMV was detected from one location and SqMV was detected from two locations in Lalitpur district. The study also reports the first incidence of SqMV in Broad

Leaf Mustard cultivated in Kathmandu valley. As the loss caused by virus diseases in vegetable crops are being reported frequently, the research works on virus diseases should now be focused on the molecular level (with use of different detection and diagnosis tests) as well as on finding the location specific integrated management practices for these virus diseases.

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Declaration of conflict of interest and authors' contribution

All the authors have read the manuscript before submitting to the journal and declare that there is no any type of competing interest regarding the current manuscript.

The current article does not include any human participants or animals by the authors and has taken prior approval if applicable.

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