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Detection of *Potato virus Y* associated with African nightshade leafy vegetable (*Solanum scabrum* miller) in Kenya

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Abstract. *The African nightshades (ANS) have been part of the food systems in sub-Saharan Africa for generations. They are particularly attractive to small scale farmers because the risks of crop losses are much lower compared to the exotic vegetables. Plant viruses are economically important pathogens affecting African nightshade production. More than 200 plant viruses are reported to infest solanaceous family crops. This study was carried out to detect Potato virus Y associated with African nightshade. Symptomatic leaf samples were obtained from four agro-ecological zones in Kenya. The viral pathogens were detected using serology and molecular techniques. Laboratory experiments were carried out at the University of Nairobi and Kenya Plant Health Inspectorate Service (KEPHIS) from February 2017 to August, 2018. Field samples had the highest mean viral percentage frequency of 44.8% compared to greenhouse samples having 29.8% using serology methods. Molecular testing revealed that greenhouse and farm field sample were positive for the three Potato virus Y strains PVY$^{N:O}$ (necrotic recombinant), PVY^{O} (ordinary) and PVY^{NTN} (necrotic). Detection of Potato virus Y in ANS suggests that it is prevalent in the sampled regions and could cause problems to other solanaceous crops.*

Keywords: molecular, necrotic, nightshades, potato virus Y, serology

Introduction

Plant viruses constrain the production of solanaceous crops especially tomato, pepper, potato and nightshade (Carusso et al., 2002; Alvarez et al., 2005). Research on viruses in solanaceous crops has tended to focus on tomatoes and potatoes and little has been done on African nightshades. The production and yield of ANS is seriously affected by the invasion of emerging and recurrent plant viruses inducing symptoms such as veinal necrosis, mosaic, mottling, yellowing, deformation, shoestring, ringspots and stunting. Potyviridae is the largest genera of viruses that attack plants. It contains six genera and about 200 virus species, most of which have a monopartite positive single strand (+) ssRNA genome. The largest genus is potyvirus which contains 128 approved species (Gray et al., 2013). All potyviruses are transmitted by aphids Jacquemond (2012). *Potato virus Y* exists as a complex of strains which can be distinguished on the basis of their biology (i.e. symptoms they elicit on indicator plants), serology and genome sequence (Kerlan et al., 2011). *Potato virus Y* strains are generally divided into the following groups: PVY^{O} (ordinary), PVY^{NTN} (necrotic recombinant), PVY^{N} (veinal necrosis) and PVY^{C} (stipple streak strain) (Kerlan et al., 2011). The general symptoms in indicator plants include mild mosaic, severe mosaic, mottle, chlorosis and necrosis (Cuevas et al., 2012). The source of African nightshade in Kenya is mainly informal and no studies have been done to detect viruses in this important leafy vegetable. Serological and molecular techniques are the most accurate methods of detecting viruses in plant tissues and were employed in this study. Testing of seed for pathogen infection is critical in order to provide adequate supplies of quality pathogen free seeds to increase crop yields. The objective of this study was to detect *Potato virus Y* associated with African nightshade leafy vegetable (*Solanum scabrum* miller) in Kenya.

Material and methods

Description of sampling sites

Seed and leaf samples were collected from four sites where African nightshade is grown in large quantities as a food and cash crop. These sites are Suneka and Ogembo in Kisii, Lurambi and Amalemba in Kakamega, Kenya. Global positioning system (GPS) was used to locate the sites (Table 1).

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Table 1. Description of the sampling regions

	Suneka	Ogembo	Amalemba	Lurambi
Location:				
-Latitude	0° 40'43.5"S	0° 50'18.8"S	0° 16'14.4"N	0° 17'42.5"N
-Longitude	34° 42' 27.7"E	34°43'47.6"E	34° 45' 14.6" E	34° 4' 47.9" E
Altitude, asl	1500-2000m	2000-2500 m	1300-1500m	1500-1900 m
	Upper midland zone AEZ (UM 2)	Lower highlands zone AEZ (LH 2)	Upper midlands zone AEZ (UM 4)	Lower midlands zone AEZ LM 2
Soil type	Well drained, dark-reddish brown	Well drained, Chromic vertisols	Well drained, dark-reddish brown	Well drained, moderately deep, dark red
Rainfall, mm	800-1000	1300-1600	1000-1600	1300-1500
T, °C	18-21	15-18	18-21	20-22

Source: FAO/UNESCO, 2000; Jaetzold et al., 2006.

Sampling for virus detection

A total of 120 samples were obtained from seeds, greenhouse and open field-raised seedlings. Forty samples from each category were used. One-hundred-milligram seeds and sections of the leaves excised from the seedlings of each seed source were crushed separately using sterile mortar and pestle in 100 ml of PBS and preserved at -20°C. To obtain leaf samples from the field, an area measuring 10 m by 5 m (quadrat) was selected to give a representative unit. In each zone ten farms growing African nightshade were selected. Top and middle leaves in ten symptomatic plants in each farm were taken at random and collected in small polythene bags (10x15 cm). Visual symptoms such as mosaic, leaf rolling, dwarfing, chlorosis, or a combination of these were assessed before sample collection and documented using a digital camera. Seeds and leaf extracts were stored at -20°C in the molecular laboratory at Kenya Plant Health Inspectorate Service (KEPHIS) laboratory awaiting serology and molecular testing for viruses

Greenhouse experiment

African nightshade seeds were planted in a greenhouse at the Department of Plant Science and Crop Protection, Field Station, University of Nairobi. African nightshade seeds were planted in 128-cell seedling trays containing commercial potting mix in an insect-proof greenhouse with temperatures ranging between 25 to 28°C day/night and relative humidity of about 85%. The experiment was laid out in a Complete Randomized Design (CRD) with seed from different sources replicated thrice. The seeds were sown on opposite sides of each cell 10 cm apart to avoid disease transmission through leaf contact. In order to reduce the risk of cross-contamination among treatments from splash dispersal of diseases, careful manual watering was done. Seedlings were examined for symptoms of seed-transmitted infections on primary or first trifoliate leaves from 7 to 14 days post planting. In order to collect samples, at least two seedlings from each pot were removed on the 14th day and trifoliate leaves excised using sterile scissors and placed in sample bags which were stored at 4°C awaiting pathological studies.

Serological detection of plant viruses

The following antibodies were purchased from the German collection of micro-organisms and cell cultures institute (DSMZ) in Germany and were used for DAS-ELISA (PVY: DAS: RT0343/PC0343). Buffers were prepared according to the manufacturer's instructions. The first antibody for detection of the virus was diluted in a coating buffer (coating buffer for 1 L of distilled water; 1.59 g Na_2CO_3; 2.93 g of $NaHCO_3$; 0.20 g of NaN_3; pH 9.6) and 100 µl were put in each well of ELISA plate. Samples were properly labeled and weighed. The plates were incubated for four hours at 37°C after which the plates were washed three times with the washing buffer and dried. An antigen extract 100 µl (0.6g plant material for 6 ml of PBS+2% of polyvinyl pirroli) were added to the first antibody and the plate was incubated at 4°C overnight.

The second antibody was diluted (1 µl in 1000 µl) to conjugate buffer and was added to the plate. After incubating for 4 hours at 37°C, the plates were washed three times with washing buffer and dried before the substrate was added for luminofluorescence detection. The plates were incubated for 30 min at room temperature and absorbance determined using a microtiter plate reader (BioTeK EI_x 800) at 405 nm.

All samples were assayed in duplicates and results scored positive if the absorbance was greater than or equal to twice the average reading of the negative (healthy) controls.

The percentage frequency (PF, %) of virus in plant samples was calculated following the formula:

PF = [(ANS samples confirmed + ve by ELISA) / (Total ANS samples tested)].100, %,

Where: ANS is African nightshades.

Molecular detection of viruses in African nightshade

Primer sequences were designed based on published sequence of genomes for each virus from the Genbank using Basic Local Alignment Search Tool (BLAST) provided online by the National Center for Biotechnology Information, NCBI (2018). Primers were synthesized by Inqaba Biotechnology and Genomics Company following the sequences for each virus (Table 2).

Table 2. Nucleotide sequences of oligonucleotide primers used for molecular testing, genomic locations and target viruses in African nightshade

Primers	Sequence (5'-3')	Product size	Target strain
2258 (F)	GTCGATCACGATGGATTTGGCGACCCCCAA	181 bp	$PVY^{N:O}$(necrotic
2439c (R)	GTTCAGGGCATGCAT		recombinant strain)
2172 (F)	CAACTATGATGGATTTGGCGACC	267 bp	PVY^{O}(ordinary strain)
2439c (R)	CCCAAGTTCAGGGCATGCAT		
5585m (F)	GGATCTCAAGTTGAAGGGGAC	452 bp	PVY^{NTN} (necrotic strain)
6032m (R)	CTTGCGGACATCACTAAAGCG		

*The full-length primer sequences for specific virus were sourced from the NCBI nucleotide database and manually aligned in a text editor. The primer design software Prifi (Fredslund et al., 2005) was used to select primers.

Total RNA extraction

Total RNA was extracted from the leaves following the manufacturer's instructions (Qiagen): Liquid nitrogen was added to a sterile mortar containing frozen (200 mg) leaf tissues which were ground thoroughly using a sterilized pestle. The ground tissue was transferred to a round-bottom microcentrifuge Rnase-free tube that had been cooled on ice. Lysis buffer of appropriate volume was prepared with 2-mercaptoethanol and added to each sample (1.5 ml per 0.25 g of grounded tissue). The lysate was homogenized by vortexing in order to disperse the sample, which was then incubated for 3 min at room temperature. A volume of 350 µl lysate was transferred into a clean homogenization tube, and centrifuged at 13.000 rpm for 5 min. Ethanol (70%) was added to each volume of the homogenate. The sample was mixed thoroughly by vortexing in order to disperse any visible precipitate that formed after addition of ethanol. Up to 700 µl of the sample was transferred (including any remaining precipitate) to a spin cartridge (with a collection tube). The sample was centrifuged at 13.000 rpm for 15 s at room temperature, the flow-through was discarded and the spin cartridge reinserted into the same collection tube. The 700 µl wash buffer was added to the spin cartridge. The sample was again centrifuged as in the previous step, but the spin cartridge inserted into a new collection tube 500 µl of washing buffer. Ethanol was added to the spin cartridge and centrifuged at 13.000 rpm for 15 s at room temperature, and then the flow-through was discarded. This last step was repeated. The spin cartridge was centrifuged at 13.000 rpm for 2 min to dry the membrane with bound RNA. The collection tube was discarded and the spin cartridge inserted into a recovery tube. Fifty microlitres of Rnase-free water was added to the center of the spin cartridge and incubated at room temperature for 1 min. The spin cartridge was then centrifuged for 2 min at 13.000 rpm at room temperature to elute the RNA from the membrane into the recovery tube. Finally, the purified RNA was stored at -20°C. This procedure was carried out for all the samples tested.

Reverse transcription and polymerase chain reaction (PCR)

Total RNA for *Potato virus Y* (PVY) was purified after extraction. Total DNA was used for complementary DNA (cDNA) synthesis using PCR kit (GoTaq, Promega, USA). cDNA was synthesized by mixing 5 µl Dnase-treated total RNA, 2 ml dNTP mix (10mM dATP, dCTP, dGTP and dTTP); 1 µl of 20 mM reverse primer and 2.25 ml RNase-free water in 0.5 ml tube. The tube was then incubated at 65°C for 5 min, chilled once, before adding 2 ml of reverse transcriptase enzyme mix and 6ml RNase-free water. The tube was then incubated at 25°C for 10 min, 40°C for 30 min and 85°C for 5 min. The synthesized cDNA was diluted 1:4 with ddH_2O and stored at -20°C awaiting PCR. Polymerase chain reaction was used to amplify the 3' terminal genomic region of the virus using degenerate primers (Table 1). To detect potyviruses and PVY, the PCR master mix contained 2.5 µl cDNA template, 2 µl of each primer, 0.5 µl of 10mM dNTP, 10 µl of 10x Taq polymerase buffer, 0.2 µl Taq DNA polymerase (5 U/µl), 1.5µl of 25 mM $MgCl_2$ and 31.5 µl ddH_2O. The initial denaturation 2 min at 94°C, 30 cycles of 94°C for 30 s annealing at 57°C for 1 minute. Initial extension was done at 72°C for 1 min and the final extension was carried out at 72°C for 5 min.

Analysis of RT-PCR products by agarose gel electrophoresis

Amplified products of RT-PCR for each virus was examined on a 1% (w/v) agarose gel prepared in 1X-TAE buffer (Promega, USA) and stained with Ethidium bromide (1 ug/ml). The 10 µl for each product was added to 2 µl DNA loading dye (Promega, USA). The GeneRuler™ 100 bp DNA ladder (Fermantas, UK) was used. Electrophoresis was done at 100V for 90 min. DNA bands were visualized under an ultraviolet (UV) transilluminator and photographed using a gel documentary system (Uvp's GDS 5000).

Results

Visual viral symptoms in African nightshade leaves

The leaf samples obtained from the field had a variety of visible symptoms that were associated with virus infection. Some of these symptoms included yellow-green mosaic, stunting, rugosity, vein clearing, yellowing, leaf curling, wilting (Figure 1).

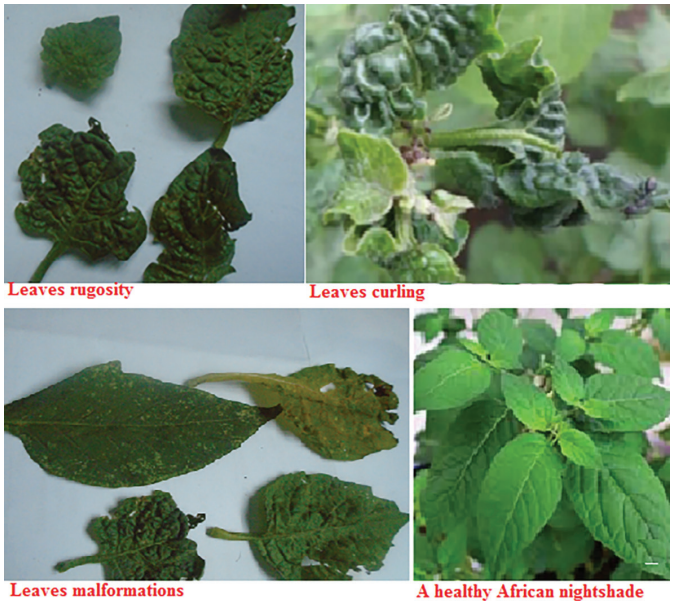

Figure 1. Leaf viral-like symptoms in African nightshade observed in the field

Serological test

Field and greenhouse samples reacted positively for viruses with ELISA test and had significant p≤0.05 differences comparing the four agro ecological zones. Field samples had the highest mean viral percentage frequency of 44.8% compared to greenhouse samples having 29.8%. UM4 had the highest mean virus frequency of 42% and 46% for greenhouse and field samples, respectively, while LH2 had the lowest at 21% and 38% in greenhouse and field samples, respectively (Figure 2).

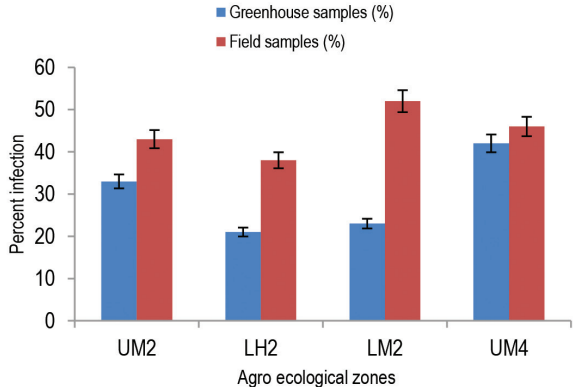

Figure 2. *Potato virus Y* frequency percentage in plant samples on ELISA

Detection of Potato virus Y in African nightshade

Greenhouse and farm field sample were positive for the three virus strains ($PVY^{N:O}$ PVY^{O} and PVY^{NTN}). The positive samples were PVY 1A, 2A, 2B, 3A, 4A and 4B. The negative samples were 1B and 3B. UM2 and UM4 AEZs had one sample each positive for PVY, while the other sample was negative whereas all the greenhouse samples were positive. In addition, all samples from AEZ LH2 and LM2 were positive for PVY. The virus strain with the highest frequency of occurrence was $PVY^{N:O}$ (necrotic recombinant) occurring in 75% of the samples followed by PVY^{O} (ordinary) occurring in 62.5% of the samples and the lowest was PVY^{NTN} (necrotic) occurring in 12.5% of the samples (Figure 3).

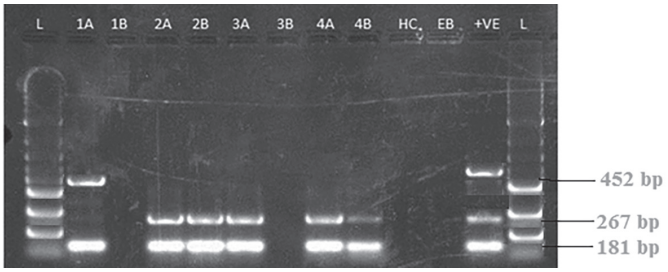

Figure 3. Detection of *Potato virus Y* strains in samples of African nightshade from different agro ecological zones using RT-PCR. L-DNA ladder, samples 1A & 1B (UM2), samples 2A & 2B (LH2), samples 3A & 3B (UM4) and sample 4A (LM2). Sample 4B (Greenhouse sample), HC-Healthy Control, EB-Extraction Buffer (blank) and +VE positive control. 181bp=$PVY^{N:O}$ (necrotic recombinant), 267bp=PVY^{O} (ordinary) & 452 bp=PVY^{NTN} (necrotic).

Discussion

This study has demonstrated that African nightshade is a host to *Potato virus Y* (PVY). The virus was detected in symptomatic African nightshade leaves obtained from greenhouse and farm fields in Nyanza and Western Kenya using serology and molecular techniques. Although data on the pathogenic potential of the viruses is not readily available, intensive cultivation of the crop might lead to build up of inoculum to levels beyond the economic threshold. The detection of this virus concurs with studies done on other solanaceous crops like potato, tomato and tobacco Coutts and Jones (2015). Scanty work has been done on viruses infecting African nightshade and this study compared the findings with those reported by Kaur (2010), Lee et al. (2011), Cuevas et al. (2012), Hamin et al. (2014) and Groves et al. (2016) on detection of pathogens in tomato and potato.The use of symptoms was not adequate in virus detection as demonstrated by use of serology and molecular methods. The use of symptomatology as a traditional method has its shortcomings since some symptoms may be as a result of environmental factors and also not all symptomless plants are negative for viruses due to latent infections (Naidu and Hughes, 2003). Apart from confirming virus presence in the field samples, this study demonstrated seed transmission of viruses. Studies have shown that seed transmission is the source of primary inoculum in the field but it depends on the survival of embryo during seed maturation. According to Robert et al. (2003) seed transmission rates of potyviruses in potato and tomatoes ranged from 2.6 to 30.6%. The viruses invade the cells during the early stages of seed development through transient vesicles present at the suspensor in the micropylar region, then enter the seed embryo (Kaur, 2010). In addition, transmission of potyviruses from plant to plant is mainly by aphids as vectors (Lee et al., 2011). More than 32 species of aphids transmit PVY in a non-persistent manner (Cuevas et al., 2012). PVY is responsible for decreases in yield and quality, and just like in potatoes, there is need for strict tolerance limits in seed certification (Abbas et al., 2014). Promoting quality production of African nightshades seeds will not only lead to increased yields but also reduce the risk of transmitting the virus by acting as alternative hosts.

This study found serology and molecular techniques to be effective diagnostic tools for detection of pathogenic viruses.

Serological methods (DAS/TAS ELISA) employed in the current study were relatively simple to use, less costly, sensitive, reliable and suitable for testing on a large scale as reported by Abbas (2014). Today, serology testing can be done on ungerminated seeds as was the case in the present study to determine the incidence of virus transmission through seed to seedlings as reported by Sastry (2013). Reverse transcriptase polymerase chain reaction (RT-PCR) was employed in the current investigation. It was more effective in detecting viruses in the samples because of its specificity, sensitivity and robustness. According to Hull (2009), PCR has the ability to detect low levels of target pathogens and isolate several of them simultaneously. It also enables easy quantification of pathogens on seeds and interpretation of results due to its ability to distinguish between closely related organisms. However, use of serology in assessment of sanitary status is faster, simple and inexpensive. Pathogen incidence in the four agro ecological zones varied and this could be due to different levels of inocula in the seeds in different farmlands. Infection is favored by the climatic conditions prevailing, handling and presence of other host plants which increases the pathogen inoculum.

Conclusion

The African nightshade is highly infected with *Potato virus Y* and the three main serotypes included PVY^{NTN}, PVY^{O} and $PVY^{N:O}$. The PVY^{NTN} necrotic serotype was the predominant type. The PVY was prevalent in all the agro ecological zones (UM2, LH2, UM4 and LM2) sampled, which indicates the economic importance of the virus in Kenya and the need for its surveillance.

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Conflict of interest

The authors declare no conflict of interest.

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