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Full Length Research Paper

Diagnostics of viruses infecting local farmer preferred sweetpotato cultivars in Kenya

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Sweetpotato is an important starchy tuberous root crop grown in many tropical and subtropical regions of the world, with about 75% of African sweetpotato production occurring in East Africa. Its production is however, constrained by viral diseases particularly sweet potato feathery mottle virus (SPFMV), sweet potato chlorotic stunt virus (SPCSV), sweet potato mild mottle virus (SPMMV), sweet potato chlorotic fleck virus (SPCFV), and sweet potato caulimo-like virus (SPCaLV), prevalent in Kenya. This work involved the designing of primers and their validation by screening of various sweetpotato materials from various sources including field and tissue culture material. The sensitivity of the diagnostic technique was evaluated and compared with the ELISA-based techniques. It was apparent that the tool was more sensitive in diagnosing the sweetpotato viruses than the available ELISA-based tool. This tool is recommended for the virus indexing for the plant materials farmers' fields, plant quarantine and laboratories involved in sweetpotato research within the region.

Key words: Sweet potato viruses, RT-PCR, diagnostics.

INTRODUCTION

Sweetpotato is an important starchy tuberous root crop grown in many tropical and subtropical regions of the world (Tairo et al., 2007), it ranks third after potato and cassava worldwide (FAOSTAT, 2007). About 75% of African sweetpotato production occurs in East Africa (CIP, 1999), especially around Lake Victoria, and is known to be a basic subsistence crop that is mainly grown by women for household use and family income (Gibson and Aritua, 2002). The crop plays a significant role in the food security as it has a potential role in combating malnutrition and population pressure (Woolfe, 1992). Additionally, it stores well in the soil as a famine reserve crop (harvested over a long period) and performs well in marginal soils (Woolfe, 1992; Karyeija et al., 1998), considering that it is regarded as a "poor man's crop" due to low farm input required (Ndolo et al., 2001). It is noteworthy that sweetpotato provides nutritionally significant quantities of ascorbic acid, riboflavin, iron, calcium and protein and; the orange-fleshed cultivars contain high levels of β-carotene useful in combating vitamin A deficiency (Low et al., 1997), thus however, sweetpotato production has been constrained particularly by viral diseases causing reductions of 56 to 98% (Gibson et al., 1997; Ngeve 1990) and 92% in farmers' fields in Kenya (Njeru et al., 2004). The most prevalent viruses in Kenya are sweet potato feathery mottle virus (SPFMV), sweet potato chlorotic stunt virus (SPCSV), sweet potato mild mottle virus (SPMMV), sweet potato chlorotic fleck virus (SPCFV), and Sweet potato caulimo-like virus (SPCaLV) (Ateka et al., 2004). The SPFMV and SPCSV work in synergism to cause sweetpotato virus disease (SPVD) (Mukasa et al., 2003). Studies in East Africa reflect incidences of as high as 100% of susceptibility of improved sweetpotato cultivars to SPVD (Ndunguru and Kapinga, 2007). The current option is to employ biotechnology techniques such as serology and molecular-based approaches in the detection of sweet-potato viruses. This study focuses on the serological

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and molecular detection of viruses infecting local farmer-preferred sweetpotato cultivars in Kenya. The objective of this project was to design highly specific sensitive primers for SPFMV, SPMMV and SPCSV viruses and demonstrate the sensitivity of the tool than the current available tool.

METHODOLOGY

Source of virus and sampling

Virus diseased and tissue cultured sweetpotato leaf samples of nine cultivars were obtained from the greenhouse and the plant tissue culture laboratory at KARI Biotechnology center, respectively.

Virus indexing using NCM-ELISA

The sweetpotato leaf samples were serologically assayed using NCM-ELISA employing a standard kit and antisera obtained from the International Potato Center (CIP) with the provided protocol. Positive and negative reactions were determined by visual assessments with different grades of purple color indicating positive reactions, and recoded by digital camera.

Primer design for RT-PCR

The publicly available sweet potato viruses sequences were downloaded from NCBI, and analyzed by a multiple alignment using the mega align software. The conserved regions were targeted for designing primers using primer select software (Lasergene software, DNASTAR), and the designed primers were obtained from Bioneer Corporation Company.

Extraction of total RNA and cDNA synthesis

The commercially available plant RNA purification kits (Qiagen and Bioneer) were used for the study with the provided protocol. Leaf tissues of 60 to 80 mg were used. cDNA was synthesized by use of the RT-Premix cyclescripts kit (Bioneer).

RT-PCR detection of greenhouse sweetpotato material

The designed primers were used to test their efficacy on the RNA obtained from positive sweetpotato leaf samples. These samples were transferred to the thermocycler with polymerase chain reaction (PCR) conditions set at 94°C for 2 min; 45 cycles of 94°C for 20 s, an annealing temperature of 53°C for 20 s and 72°C for 1 min and; 72°C for 10 min. The PCR products were ran on a 2% Agarose gel electrophoresis for 30 min at 120 volts. The bands obtained were viewed under a gel documentation system and the photos of the gels taken.

RESULTS AND DISCUSSION

Virus testing of the sweetpotato cultivars in Kenya

The sweetpotato leaf samples were serologically assayed for sweet potato feathery mottle virus (SPFMV), sweet potato chlorotic stunt virus (SPCSV), sweet potato mild mottle virus (SPMMV) and, sweet potato chlorotic fleck virus (SPCFV) using a nitrocellulose membrane enzyme linked immunosorbent assay (NCM-ELISA) employing a standard kit and antisera obtained from the International Potato Center (CIP). The kit contained polyclonal antibodies to the viruses SPFMV, SPCSV, SPMMV, SPCFV, and SPCaLV as well as alkaline phosphate-labeled goat anti-rabbit (GAR-AP). The kits also contained NCM strips pre-spotted with sap from virus-positive and negative control plants. Three leaf discs excised from leaf samples of symptomatic and asymptomatic sweetpotato material grown in the greenhouse characterized by moderate stunted growth and chlorosis (Figure 1). The discs were ground and spotted at the centre of a square made on the nitrocellulose membrane and subsequently processed according to the CIP kit manufacturer's instructions.

Results of the sweetpotato samples indexed for the four viruses namely SPFMV, SPCSV, SPMMV, and SPCFV are showed in Table 1. The cultivar Mar Ooko was infected with all viruses while, cultivar K/KA/2002/81 had no virus infections. Most (seven out of nine) cultivars tested were infected with SPFMV followed by SPCFV which was manifested in six cultivars. The results of the serological detection depicted in this work show the presence of SPFMV in majority of the cultivars thus clearly indicating that SPFMV has been the most frequent virus infecting sweetpotato wherever it is grown (Carey et al., 1998; Ateka et al., 2004). Similarly, the presence of SPFMV and SPCSV in Mar Ooko demonstrates that it is infected with SPVD suggesting that this cultivar is susceptible to the disease albeit the other cultivars could be resistant to the disease due to genetic variability (Karuri et al., 2009). The same cultivar was once more found to be positive with the virus SPCSV out of all the other cultivars, of which symptoms manifested by this cultivar confirms the presence of SPCSV (Ateka et al., 2004). This demonstrates that the virus is not common in the region as is asserted by Aritua et al. (2000) who in his study showed 44% frequency of the virus in three provinces in Kenya namely Coast, Western and Nyanza collectively. Although infection with SPCFV followed that of SPFMV in the samples tested, the virus is hardly ever heard of in Kenya and East Africa (Tairo et al., 2004; Mukasa et al., 2003; Ateka et al., 2004).

RT-PCR diagnostic

The sweetpotato viruses sequences were down loaded from NCBI for SPFMV (AJ781787, AY459592, AY523551 and EU021067), for SPCFV (AY461421, EF990647, EU375901, EU375906, and EU375910), and for SPCSV (AJ010760, AJ010761, DQ864344, DQ864346, and DQ864354). The conserved and variable regions of the individual virus were identified by multiple alignment of

Figure 1. (A) Sweetpotato infected material and (B) leaf discs from sweetpotato leaves.

Table 1. Results obtained for the detection of sweetpotato viruses using NCM-ELISA.

Cultivar	Virus infection		
	SPFMV	SPCSV	SPMMV
K/KA/2002/81	-	-	-
Mar Ooko	+	+	+
Kalamb nyerere	+	-	-
Bungoma	+	-	+
SPK 013	-	-	-
Nyandere	+	-	-
Nyawo	+	-	-
Vindolo tamu	+	-	-
Sandak	+	-	-

the sequences, which had a homology of 95 to 98%. The diagonostic PCR primers were selected from the conserved regions, particularly on 3'end of the primers. All total of nine primers designed three from each virus were tested with the positive control and only one best from every virus was picked for screening field and tissue culture materials listed in Table 2. Primer A2/B2 produced 166 bp for SPCSV and primer A/C produced 185 bp for SPFMV PrimerA/B2 produced 268 bp for SPMMV.

Sensitivity of RT-PCR detection was tested with the serial dilution of the RNA samples. As shown in Figure 2, the RT-PCR on the serial diluted RNA resulted in amplification from undiluted to 1:1000000 dilution that while beyond this no band was observed. Therefore the detection limit of this tool is up to 10^{-6} which compares well with other findings (Abarshi et al., 2010). Polymerase chain reaction is a technology advanced assay in which a

Table 2. Sequences of primers designed specific to three sweetpotato viruses.

Parameter	'5-3` primer sequences	Expected product size
SPCSV-A2	GCAGGTTTCTACGCATCTCTATC	166 bp
SPCSV-B2	GAGCCCTGGCCCATTTCCTA	
SPFMV-A	ACTACACCTGCACGTGCTAAAGAA	185 bp
SPFMV-C	TATTGCACACCCCTCATTCCTAAG	
SPMMV-A	ACCGGGAGATGGCGATGAA	268 bp
SPMMV-B2	CACGTGATACATRGCGCTTCTTA	

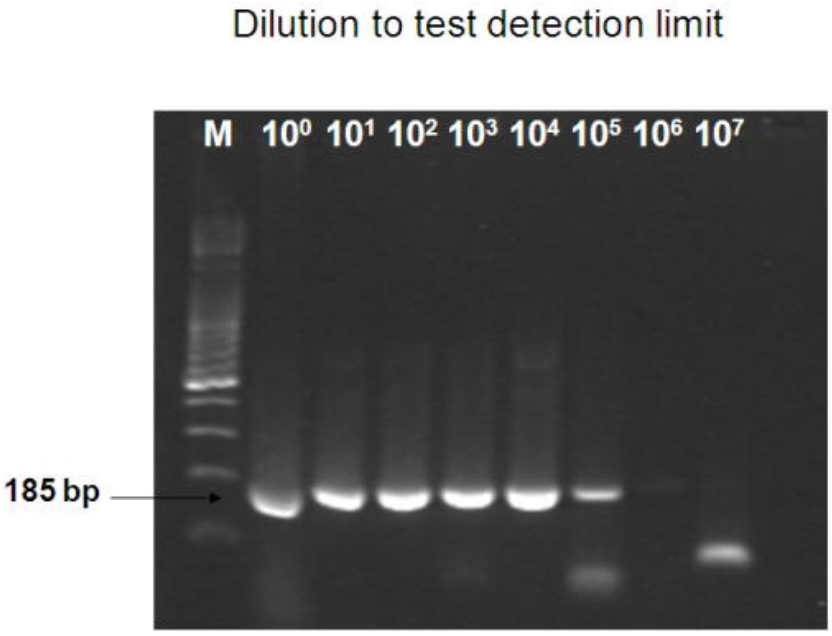

Figure 2. PCR gel showing amplifications of SPFMV primer on PCR products of diluted RNA separated on 2% Agarose gel. (M) 100 bp plus DNA ladder while lanes 1-8 are cDNA obtained from RNA serial dilutions.

small amount of DNA (very low numbers of copies of a gene or gene fragment) is amplified to make its detection feasible; it has made it become an attractive technique for the diagnosis of plant virus diseases (Henson and French, 1993; Hadidi et al., 1995; Candresse et al., 1998a). The difficulty in detecting sweetpotato viruses in sweetpotato is in some cases due to low virus titres rather than inhibitors or problems with assays (Karyeija et al., 2000b; Kokkinos and Clark 2006a). The results show that the primer is capable of detecting the virus titres upto a concentration of 10^{-6} as depicted with the weak band.

Application of the diagnostic tool

The primers designed were evaluated on samples obtained from greenhouse and tissue-cultured sweet-potato material. Figure 3 shows results of diagnostic tests carried out for three sweetpotato viruses (SPFMV, SPCSV and SPMMV) using the designed primers, on three sweetpotato cultivars namely Mar Ooko, Nyandere and Sandak (San S3) obtained from the greenhouse. The detected bands had strong, moderate and faint amplifications as depicted in Table 3. All the cultivars were found to have multiple infections with Mar Ooko infected with SPFMV (lanes 1 and 2), SPCSV on lane 1 and; and SPMMV shown on lanes 1 and 2; Nyandere was infected with SPFMV and SPCSV on lanes 2 and; Sandak was shown to be infected with SPFMV and SPCSV on lanes 2 and 1, respectively. These cultivars are evident of being infected with SPVD complex, a situation that has been noted by Gibson et al. (1997),

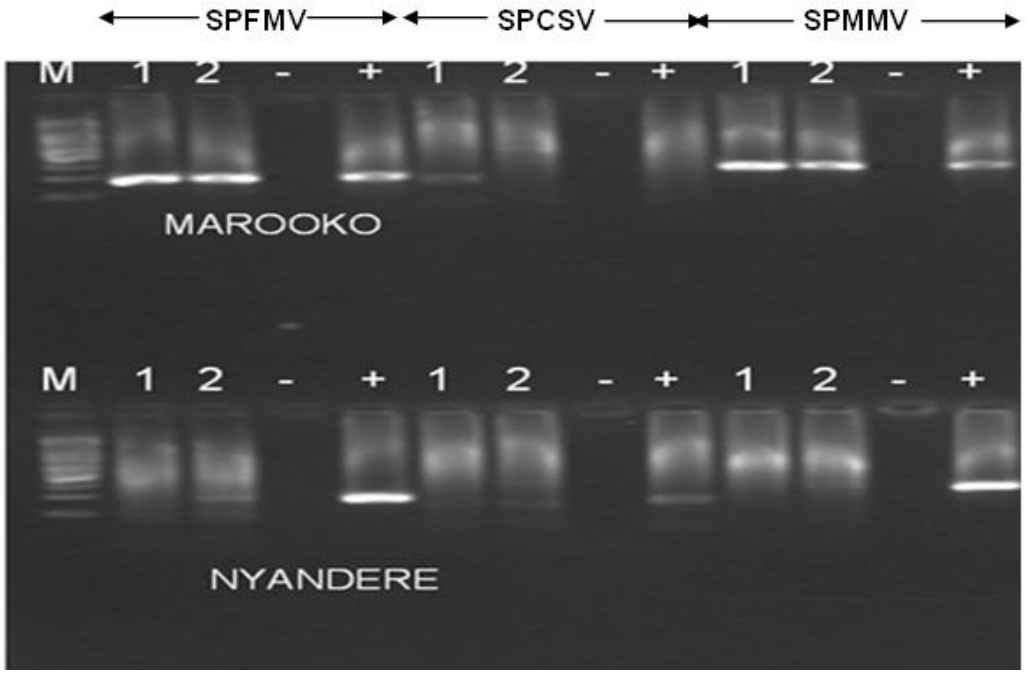

Figure 3. PCR gel showing amplifications of primers specific to three viruses namely SPFMV, SPCSV and SPMMV on three sweetpotato cultivars namely Mar Ooko and Nyandere. Lanes: (M) 100 bp plus DNA ladder; lanes 1-2 are duplicate samples of each cultivar while lanes 3 and 4 are negative and positive control samples, respectively.

Table 3. Results of the diagnostic PCR on 3 cultivars for 3 viruses.

Cultivar	SPFMV	SPCSV	SPMMV
MAROOKO	+++	+	+++
NYANDERE	++	++	-

Key: +++ Strong band; ++ moderate band; + weak band; - no band.

Tairo et al. (2007) and Ateka et al. (2004), and is a confirmation of the diagnostic test previously carried out in this study using NCM-ELISA technique, although, Nyandere was found to be negative as previously assayed using serological methods.

Both diagnostic techniques were concurrently employed on greenhouse material to test their efficacies. The NCM-ELISA technique was employed using the CIP kit. The samples spotted on the membrane were developed and Table 1 shows ELISA results which compared well with RT-PCR however, ELISA recorded negative result for SPCSV on Nyandere cultivar while it was confirmed positive by RT-PCR as observed on Figure 3. In another exceptional observation RT-PCR detected one sample positive while Elisa tested negative for SPFMV in Mar Ooko cultivar as showed in Figure 4. This indicates that molecular diagnostics of viruses, such as PCR, is more sensitive than protein-based techniques (Souto, 2003; Abad and Moyer, 1992) and hence this tool could be used as a complimentary confirmatory diagnostic test.

The effectiveness of the diagnostic tool was tested on tissue-cultured material multiplied using *in vitro* micropropagation techniques. The purpose of multiplying was to obtain adequate material through tissue culture for use as samples for testing the efficacy of the diagnostic tool. The multiplied material was found to grow vigorously and in good health within 3 to 4 weeks of incubation (Figures 5 and 6) and was used for the diagnosis of the viruses SPFMV. We also included the tissue culture materials cleaned up and appeared as virus free for RT-PCR diagnostics. From the 17 samples tested, we have identified six tissue culture samples were positive including four samples from the previously cleaned materials (lane 9, 11, 13 and 15). Through this study, we confirmed the importance of the sensitive virus diagnostic protocol like RT-PCR in virus indexing of the tissue culture materials.

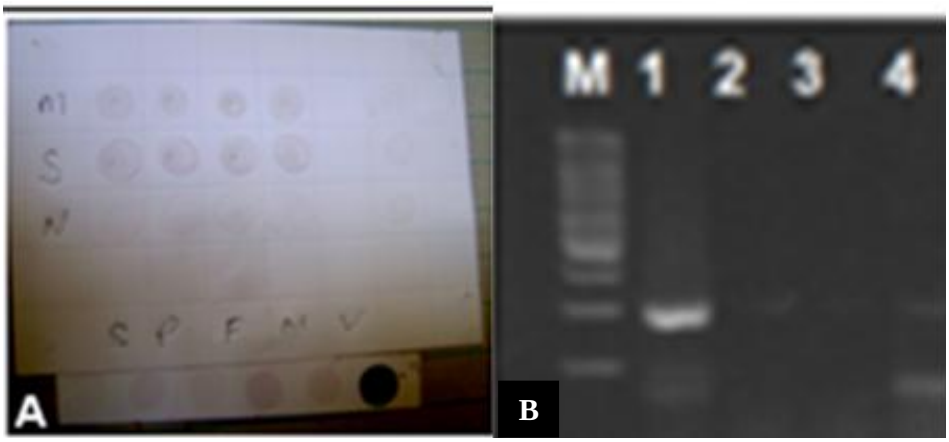

Figure 4. Results for SPFMV showing (A) negative results on NCM-ELISA and (B) positive results on RT-PCR tested on the cultivar Mar Ooko.

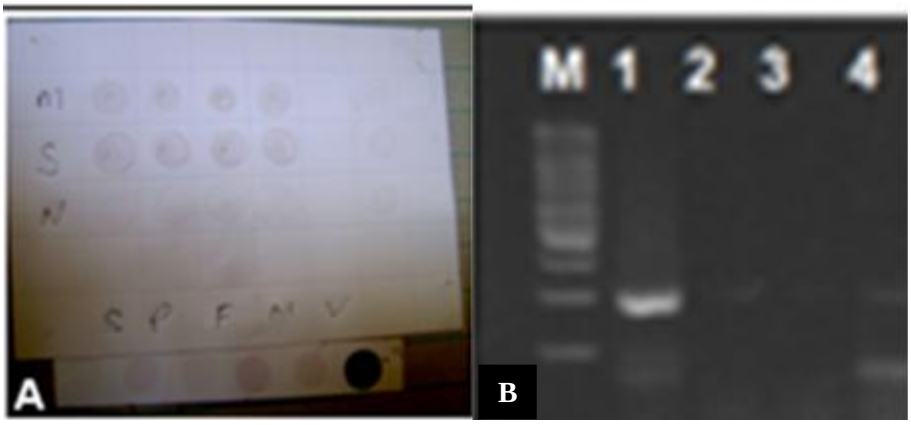

Figure 4. Results for SPFMV showing (A) negative results on NCM-ELISA and (B) positive results on RT-PCR tested on the cultivar Mar Ooko.

Figure 5. *In vitro* tissue culture sweetpotato material.

Conclusion

This diagnostic tool is a useful resource in establishing clean sweetpotato planting material as compared to ELISA-based techniques, due to the detection of viruses in low concentrations. Its application is useful in various

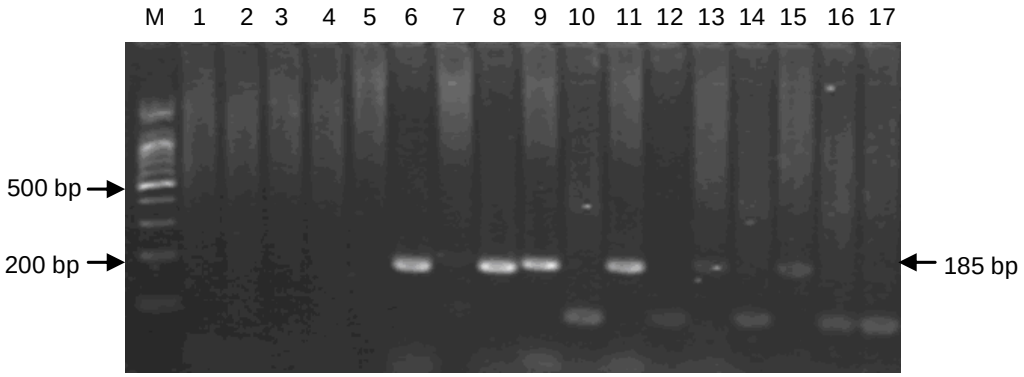

Figure 6. RT-PCR diagnostics of SPFMV primer A and C (185 bp) tested on sweetpotato tissue culture 2% Agarose gel. Lanes 1~17 have tissue culture materials from various sources, and laneM for 100bp DNA size marker.

research and plant quarantine laboratories and sweet-potato seed production systems, as depicted by the diagnostics of both field and tissue-cultured material. Additionally, both diagnostic techniques would be good tools to use, however, the RT-PCR technique is considered to be more sensitive.

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