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DEVELOPMENT OF A MULTIPLEX PCR TECHNIQUE FOR SIMULTANEOUS DETECTION OF *SWEET POTATO FEATHERY MOTTLE VIRUS* AND *SWEET POTATO CHLOROTIC STUNT VIRUS*

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SUMMARY

Virus diseases, especially those caused by mixed infections, are among the economically most devastating diseases of sweet potato. Sweet potato virus disease (SPVD), which is caused by mixed infection of *Sweet potato feathery mottle* (SPFMV) and *Sweet potato chlorotic stunt virus* (SPCSV), is the most widespread in East Africa. Lack of rapid and sensitive techniques for detecting the two viruses makes their control almost impossible. In this study, a multiplex polymerase chain reaction (mPCR) protocol was developed and subsequently evaluated for its effectiveness in simultaneous detection of SPFMV and SPCSV using 13 samples. Total RNA extracts were subjected to reverse transcription followed by PCR with two sets of virus-specific primers. DNA bands of 703 and 235 bp were obtained for SPFMV and SPCSV, respectively. No amplification products were obtained from healthy controls. Results obtained from nitrocellulose membrane enzyme-linked immunosorbent assay (NCM-ELISA) confirmed the efficiency of the mPCR protocol. We recommend the developed mPCR technique for routine molecular diagnostic purposes.

Key words: Sweet potato virus disease, survey, detection, multiplex PCR, NCM-ELISA.

INTRODUCTION

Sweet potato (*Ipomoea batatas* L.) is an important staple root crop grown in many tropical and subtropical regions in the world. In Africa, sweet potato plays a vital role in people's ability to sustain their families especially during periods of drought (Karyeija *et al*., 1998). In the last decade, the importance of sweet potato increased greatly in East and Central African regions due to adverse effects of pests and diseases on the production of alternative staple crops such as cassava, maize and banana (Thottappilly *et al.*, 1993; Otim-Nape *et al.*, 2000; Tushemereirwe *et al.*, 2004). Despite the high potential for food security, several biological, physical and socio-economic factors greatly reduce sweet potato production. Among the biological factors, virus diseases rank second to weevil in causing yield reduction (Karyeija *et al*., 1998; Gibson and Aritua, 2002). Several viruses have been reported infecting sweet potato in Africa (Geddes, 1990; Wambugu, 1991; Hahn, 1997; IsHak *et al*., 2003; Mukasa *et al*., 2003a; Ateka *et al*., 2004; Tairo *et al*., 2004). *Sweet potato feathery mottle virus* (SPFMV) is the most prevalent and widespread in many parts of the world where sweet potato is cultivated (Moyer and Salazar, 1989; Nishiguchi *et al*., 1995; Sakai *et al.*, 1997). When infecting alone, SPFMV has minor detrimental effects on sweet potato (Gibson *et al.*, 1998; Karyeija *et al.*, 2000). By contrast, the economic impact is greater when SPFMV and *Sweet potato chlorotic stunt virus* (SPCSV) occur together thus eliciting "Sweet potato virus disease" (SPVD), the most harmful disorder of the crop in Africa and elsewhere (Geddes, 1990; Gibson *et al.*, 1998; Gibson and Aritua, 2002). Although ELISA is used routinely for virus detection, this assay is sensitive only during the dry seasons of the year when the virus concentration in the plants is high (Ghosh and Aglave, 2007).

Several singlex PCR (sPCR) based methods have been reported for a number of sweet potato viruses (Nishiguchi *et al.*, 1995; Ki and Sun, 2002; Kokkinos and Clark, 2006). Since sweet potato infecting viruses in East Africa occur mostly as multiple infections (Gibson *et al*., 1998; Karyeija *et al*., 2000; Mukasa *et al*., 2003b), availability of multiplex virus detection protocols would lower the time and cost of testing. There are several multiplex PCR (mPCR) systems for the detection of two/three or even more viruses infecting different crops (Ghosh and Aglave, 2007) but none is available for sweet potato.

MATERIALS AND METHODS

Sweet potato cuttings, collected from symptomatic plants in the major sweet potato growing regions in

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Kenya, were transferred to Jomo Kenyatta University of Agriculture and Technology (JKUAT), Department of Horticulture, and planted in an insect-proof screenhouse. Samples were tested serologically by nitrocellulose membrane-ELISA (NCM-ELISA) (Gibb and Padovan, 1993), using disks 1 cm in diameter, taken from a leaf at the top, middle and bottom of the stem from each plant. Polyclonal antisera as well as negative and positive controls were obtained from the International Potato Center (CIP, Lima, Peru). The development of a purple color on the nitrocellulose membrane confirmed virus positive samples (Gutierrez *et al.*, 2003).

Total nucleic acid extraction and amplification. Fresh young leaf tissues (100 mg) from 13 symptomatic sweet potato samples and a healthy control were ground to fine powder in liquid nitrogen, using mortar and pestle. Total RNA was extracted with RNeasy Plant Mini Kit (Qiagen, USA) according to the manufacturer's instruction. The integrity of the extracted RNA was visually verified after electrophoresis in 1% agarose gel stained with ethidium bromide. Two sets of primers specific to SPFMV and SPCSV (Table 1) were designed and custom synthesized (Sigma, Germany). The SPFMV primers were designed from highly conserved regions of the SPFMV strains prevalent in East Africa, i.e. the East African strain (SPFMV-EA), ordinary strain (SPFMV-O), and the common strain (SPFMV-C) (Ateka, 2004; Mukasa *et al*., 2003a).

Optimization of multiplex conditions. The reactions were optimized by varying the amount of complementary DNA (cDNA) template of each target. Optimization was carried out by methodical variation of test parameters under standard PCR conditions. The cDNA template amounts tested ranged from 1.0 to 3.0 µg per PCR reaction mix of 20 µl and 40 µl for singlex PCR (sPCR) and mPCR, respectively. The annealing temperature and number of cycles were also determined experimentally.

Singlex-PCR and RT-PCR. The SPFMV- and SPCSV-specific primer sets were tested separately against RNA templates extracted from sweet potato plants showing SPVD symptoms. Reverse transcription (RT) was performed using Omniscript Reverse Transcriptase (Qiagen, USA) according to the manufacturer's protocol. Reverse primers were SPFMV-R and SPCSV-R (Table 1) specific to SPFMV and SPCSV, respectively. DNA amplification was performed using the Qiagen's Taq PCR Master Mix Kit (Qiagen, USA) following the manufacturer's instructions. PCR was carried out using the following parameters: initial denaturation at 95ºC for 5 min, followed by 35 cycles at 96ºC for 5 sec for denaturation, 63ºC for 5 sec for primer annealing and 72ºC for 30 sec for extension and a final extension at 72ºC for 1 min.

Multiplex PCR and RT-PCR., Similarly to sPCR, mPCR consisted of a two step reaction procedure which included reverse transcription followed by PCR amplification. Total RNA was subjected to cDNA synthesis in a 19.0 µl as follows: 3 µg of total RNA, 0.2 µl each of SPFMV-R and SPCSV-R primers (Table 1), 0.3 µl of random primer and 9.5 µl of RNase free H_2O were incubated at 70°C for 10 min. To the reaction mixture, 2.0 µl of 10x strand buffer, 0.3 µl of 5 mM dNTP mix, 0.5 µl of Omniscript Reverse Transcriptase (Qiagen, USA), 0.5 µl of RNase inhibitor and 2.5 µl of RNase-free H_2O were added. The resulting master mix was incubated at 42ºC for 1 h, followed by 70ºC for 10 min. The mPCR reaction mixture consisted of 1.0 to 3.0 µg of the synthesized cDNA (containing cDNA for SPFMV and SPCSV), 20 µl PCR Master Mix (Qiagen, USA), 0.3 µl of each of the two set of primers, 8.0 µl of Q-Solution (Qiagen, USA), and the final volume made up with RNase free H_2O. The mPCR was performed using the following parameters: initial denaturation at 95ºC for 5 min, followed by 30-35 cycles at 96ºC for 5 sec for denaturation; 60-63°C for 5 sec for primer annealing and 72ºC for 30 sec for extension and a final extension at 72ºC for 10 min, to determine the annealing temperature for the two primers.

Evaluation of the developed mRT-PCR protocol. As mentioned, the mRT-PCR protocol for the simultaneous detection SPFMV and SPCSV was applied to 13 symptomatic and one healthy sweet potato samples were tested for infection. Two µg of cDNA template were used in the PCR under the following parameters: one cycle at 95ºC for 5 min followed by 35 cycles at 96ºC for 5 sec for denaturation, 63ºC for 5 sec for primer annealing and 72ºC for 30 sec for extension and a final extension at 72ºC for 10 min.

Table 1. Virus-specific primer pairs used to amplify *Sweet potato feathery mottle virus* (SPFMV) and *Sweet potato chlorotic stunt virus* (SPCSV).

Virus	Primer name	Sequence	Expected product size (bp)
SPFMV	SPFMV-F	5'-GGACGAGACACTAGCAA-3'	703
	SPFMV-R	5'-TTCTTCTTGCGTGGAGACGT-3'	
SPCSV	SPCSV-F	5'-ACGTTGGTTGGCGTTGA-3'	235
	SPCSV-R	5'-ATCTGCGGGAACTGACACG-3'	

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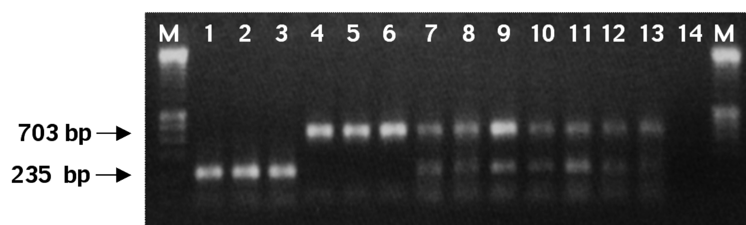

Fig. 1. Electrophoresis profile of DNA amplified products from total RNA obtained from 13 symptomatic and 1 healthy sweet potato samples by singlex and multiplex PCR. Lane M: DNA size marker. Lanes 1-3, singlex PCR using primers specific to SPFMV; lanes 4-6, singlex PCR using primers specific to SPCSV; lanes 7-13, multiplex PCR with mixed viral cDNAs using two primer pairs specific to SPFMV and SPCSM; lane 14, healthy control.

RESULTS AND DISCUSSION

Different amplification assays with varying amounts of template (1.0-3.0 µg), varying annealing temperatures (60-63°C) and varying number of cycles (30-35) were performed to optimize the multiplex reaction conditions. The best amplification was obtained with 2.0 µg of cDNA template with 35 cycles compared to 1.0 µg and 3.0 µg (results not shown). There was no significant difference between 60ºC and 63°C for the annealing temperature. Products of the size expected for SPFMV and SPCSV (703 and 235 bp, respectively) were amplified from double-infected samples by both sPCR and mPCR (Fig.1) while no amplicons were obtained from healthy plants. All the samples tested by NCM-ELISA, including the positive control, reacted positively with antisera to both SPFMV and SPCSV, thus confirming PCR results. By contrast, no colour reactions was given by the healthy sweet potato samples used as negative controls.

In East Africa, the presence of a high incidence of virus diseases in sweet potato is attributed to the widespread use of infected planting materials. The vegetative propagation to establish a new crop, usually done using cuttings from a previous crop (Karyeija *et al.*, 1998), increases the risk of virus build up. To reduce the chances of virus dissemination through infected cuttings, the use of virus-tested planting materials is necessary. Results from this study show the successful use of a simplified mPCR as a rapid assay for the simultaneous detection of SPFMV and SPCSV leading to improved turnaround time and reduced costs of virus detection. Since the primers used for SPFMV amplification were broad-based, this increases the chances of detection, making them appropriate for routine assays. We therefore recommend the developed mPCR protocol for employment in screening sweet potato germplasm and cultivars for the presence of viruses both in field crops and in tissue culture laboratories.

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