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BRIEF REPORT

Simultaneous detection of groundnut rosette assistor virus (GRAV), groundnut rosette virus (GRV) and satellite RNA (satRNA) in groundnuts using multiplex RT-PCR

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Abstract Groundnut rosette disease (GRD) is the most devastating disease of groundnuts in sub-Saharan Africa. The disease is caused by synergistic interactions between viruses and virus-like pathogens: groundnut rosette assistor virus (GRAV), groundnut rosette virus (GRV) and a satellite RNA (satRNA). The multi-pathogenic nature of GRD requires efficient diagnostic systems for plant breeding and pathology work. Currently, TAS-ELISA and RT-PCR are used to detect all three pathogens. This approach is time-consuming, expensive and not easily amenable to high throughput. A multiplex PCR-based approach was developed to detect all three pathogens at once, reducing diagnostics costs and time by two thirds. The technique is highly robust and amenable to high throughput, with sensitivity and specificity values of 88 % and 100 %, respectively. The positive predictive value for the technique is 100 %, and the negative predictive value is 90.6 %.

Keywords Groundnut rosette disease · Two-step multiplex RT-PCR · Two-step RT-PCR

Groundnut rosette disease (GRD) of groundnuts (*Arachis hypogea* L.) is exclusively endemic to Sub-Saharan Africa (SSA), causing an estimated annual loss of US$156 million every year [1]. The disease is caused by synergistic interactions between two viruses – the luteovirus groundnut rosette assistor virus (GRAV) and the umbravirus groundnut rosette virus (GRV) – and a satellite RNA (satRNA) of GRV [1–4]. All agents of GRD are persistently transmitted by aphids (*Aphis craccivora* Koch) [5–8], and so far there is no evidence of seed transmission. Deployment of host resistance is the most cost-effective way to manage epidemics given that groundnuts are produced by subsistent smallholder farmers. Breeding of resistant genotypes and their deployment is most effective when supported by efficient pathogen diagnostic systems, even in the absence of symptoms. Diagnostic methods for the three pathogens may be applied singly and or in combination. GRAV can be detected by either TAS-ELISA or RT-PCR, while detection of GRV and satRNA is only done by RT-PCR [9, 10]. There is no information on the availability of antibodies to detect GRAV by TAS-ELISA, and antisera produced for chickpea luteovirus (CPLV) or potato leaf roll virus-1 (PLRV-1) cross-react with GRAV [1]. Therefore, there is a need for a more sensitive and specific method to detect GRAV. The other method available for detection of the GRD-associated satRNA is dot blot hybridization [11]. This method nevertheless is less common due to its complexity. In general, all these methods can detect GRAV, GRV and the satRNA in plants and aphids, but the reactions are performed individually, are expensive, and are not amenable to the high throughput commonly required in breeding programs. To overcome the inherent disadvantage of cost, as well as to improve diagnostic capacity, multiplex PCR, a PCR variant in which more than one target sequence is amplified using more than one pair of primers, is an interesting alternative [12]. Multiplex PCR supports screening for individual or multiple viruses and disease development studies [13, 14]. The objective of this study was to develop a multiplex PCR approach for the detection of all three GRD pathogens and

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Fig. 1 Rosette-disease-affected groundnut in a farmer's field in Malawi. 1a Chlorotic rosette symptoms on groundnut

to study their interactions in pathogenesis and epidemiology.

To isolate total RNA, plants of the GRD-susceptible variety JL-24 [15] were inoculated with viruliferous aphids that had been reared on infected plants showing disease symptoms (Fig. 1). Uninfected plants were grown under protection in a separate greenhouse to prevent infection from extraneous sources by viruliferous aphids. Total RNA was isolated from 150-200 mg of young infected and uninfected JL-24 leaves using a plant RNA miniprep kit (Zymo Research, Irvine, CA, USA). Leaves from an equal number (27) of infected and healthy plants were used to extract RNA that was subsequently used to synthesize cDNA. The purity and quantity of the extracted RNA was assessed using an spectrophotometer). First-strand cDNA synthesis was performed on approximately 3-5 µg of total RNA using a RevertAid Premium First-Strand Synthesis Kit (Thermo Scientific, Waltham, MA, USA), using 200 ng of GRAV-2, GRV-2 or satRNA-2 downstream primers [16].

Primers for specific amplification of nucleotide sequences from each of the three agents of GRD were designed (16) as indicated in Table 1. The primers for GRAV amplify a 597-bp fragment; for GRV, a 863-bp fragment; and for satRNA, a 890-bp fragment (satRNA-1/satRNA-2) or a 400-bp fragment corresponding to the 3′ half of the satRNA (satRNA-2/satRNA-3). The multiplex PCR reaction conditions were optimized by using different Mg^{2+} concentrations, annealing temperatures, primer concentrations, extension times and cycle numbers to minimize nonspecific priming. The multiplex PCR reaction was set up in one tube as a 50-µl mixture containing 3 µl of each cDNA, 5 µl of 10 × PCR buffer, 1 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTPs, 2 µl of each primer combination (2 µM) and 0.5 U of Hot Start Taq DNA Polymerase (Fermentas). The PCR was performed in a PTC-100 thermal cycler (MJ Research). The PCR protocol consisted of the following: a hot start at 94 °C for 2 min followed by 35 cycles of amplification (94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min) and a final extension at 72 °C for 10 min. As a control, the PCR was performed separately for each of the tested pathogens as described previously [16]. The PCR amplicons were assayed for appropriate product size using 2 % agarose gel electrophoresis in TBE buffer.

The efficiency of multiplex PCR to detect all three pathogens was assessed using positive predictive value, a statistical tool that assesses the proportion of positive test results that are true positives, and negative predictive value, which assesses the proportion of negative results that are true negatives [17]. Results from the multiplex PCR were also compared against simplex PCR with a 95 % confidence interval using exact binomial tests [18]. All statistical analyses were performed using Genstat 15th Edition (www.vsni.co.uk).

Good-quality RNA was obtained from leaf samples and subsequently used to synthesize cDNA that was used in both multiplex and simplex PCR assays. PCR amplicons of 863 bp for GRV, 597 bp for GRAV, and 400 bp for satRNA were generated by the multiplex PCR (Fig. 2). No amplification was detected among the negative samples.

The result showed that there is no difference between simplex PCR and multiplex PCR, with both processes generating the targeted product amplicons. The two processes were, however, distinct when the number of reactions and volume of reagents used were compared. Multiplex PCR used one third of reagents used in the simplex PCR. In terms of number of reactions, 288 singleplex reactions are needed to detect each of the three viruses compared to 96 multiplex reactions. One inclusive simplex PCR assay for GRV, GRAV and the satRNA costs about 2.4 USD compared to 0.8 USD for the multiplex PCR.

Two samples from a total of 27 leaf samples obtained from inoculated plants were negative for all three pathogens. Twenty-three samples were positive for GRV and satRNA when assayed using both multiplex and simplex PCR, and the remaining four were negative for the two pathogens. Overall, multiplex PCR detected GRAV in 22 samples, whereas simplex PCR detected GRAV in 25 samples (Table 2). The sensitivity of multiplex PCR was 88 % at the 95 % confidence level and the specificity was 100 %. The positive predictive value was 100 % and the negative predictive value was 90.6 % (Table 3).

Rapid and efficient detection of any pathogen is critical for the development and deployment of disease management strategies. In this study, we developed and tested the simultaneous detection of three viral agents that cause groundnut rosette disease, the most devastating disease of groundnuts in sub-Saharan Africa. The process involved the simultaneous amplification of GRV, GRAV and

Table 1 Oligonucleotide primer pairs for the two-step multiple RT-PCR detection of GRD agents

Virus name	Primer	Size (bases)	Sequence (5′ to 3′)	Size of the amplified product (base pairs)	Accession number
GRV	GRV1	20	GGAAGCCGGCGAAAGCTACC	863	EMBLZ69910 [21]
	GRV2	20	GGCACCCAGTGAGGCTCGCC		
GRAV	GRAV1	21	ATGAATACGGTCGTGGTTAGG	597	EMBLZ68894 [22]
	GRAV2	19	TTTGGGTTTTGGACTTGGC		
Sat- RNA	Sat- RNA1	23	GGTTTCAATAGGAGGAGAGTTGC	890	EMBLZ29702-Z29711* [23]
	Sat- RNA2	20	AAATGCCTAGTTTGGGCGTG		
	Sat- RNA3	20	AAGTGCTGAGGAACCAGCAC	400	

*Primers for sat RNA were designed by aligning the ten sequences available

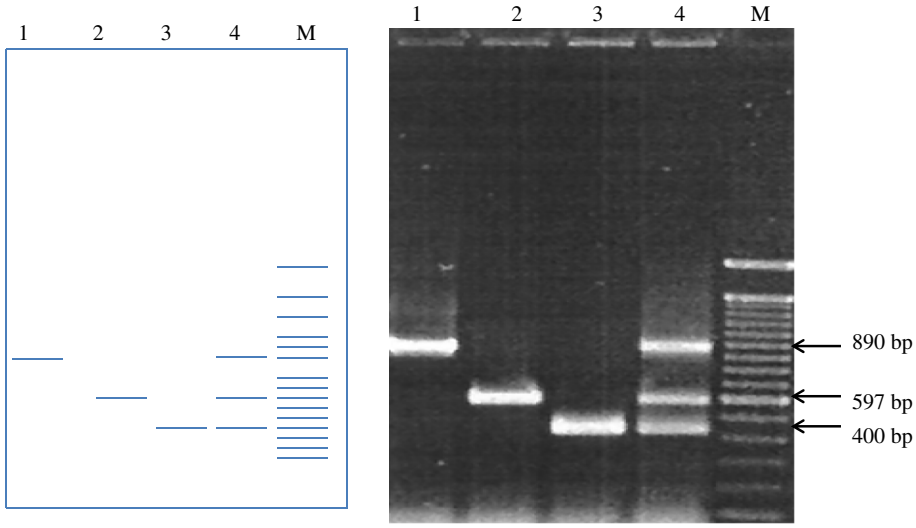

Fig. 2 Representative ethidium-bromide-stained 2 % percent agarose gel containing PCR products of GRV, GRAV and satRNA. Lane 1 (890 bp), lane 2 (597 bp), and lane 3 (400 bp) show individual PCR products of GRV, GRAV and SatRNA, and lane 4 shows multiplex PCR products of GRV, GRAV and SatRNA. Lane M shows a 100-bp DNA ladder (Fermentas)

Table 2 Results obtained by simplex PCR and multiplex PCR

Virus	Number of samples with the indicated result	
	Simplex PCR multiplex PCR no. of positives/total number	No. of positives/ total number
GRAV	25/27	22/27
GRV	22/27	22/27
Sat RNA	22/27	22/27
Control	-/27	-/27

Table 3 Sensitivity and specificity of multiplex PCR when compared to simplex PCR for detecting GRV, GRAV and satRNA*

Multiplex PCR	Number and % of samples in simplex PCR		
	Positive	Negative	Total
Positive	22 (40.74 %)	0 (0.00 %)	22 (40.74 %)
Negative	3 (5.56 %)	29 (53.7 %)	32 (59.26 %)
Total	25 (46.3 %)	29 (53.7 %)	54 (100.00 %)

The figures in parentheses were computed based on visual scoring of PCR amplicons for both simplex and multiplex. *The sensitivity of multiplex PCR is 88 %, with a 95 % confidence interval of 68-97%; the specificity is 100 %. The positive predictive value (PPV) is 100 %; the negative predictive value (NPV) is 90.6 %

satRNA in one PCR reaction mixture. A comparison with individually run PCR reactions showed that the multiplex PCR assay had a sensitivity of 88 %, and 100 % specificity. The positive predictive value of 100 % and the negative predictive value of 91 % show that the technique is thus highly robust, detecting either of the pathogens accurately.

The simplex PCR system includes only two primers, while the multiplex PCR system has varying hybridization kinetics of multiple primer pairs. A primer that binds with high efficiency could consume a greater amount of the PCR reaction components and thus reduce the yield of the other amplicons. This may result in unamplified DNA sequences or absence/poor intensity of one or more of the expected PCR products. In our case, the 88 % sensitivity in multiplex PCR shows that mostly this kind of problem is rare,

and out of 25 GRAV-positive samples in simplex PCR, 22 were also positive in multiplex PCR. This is far better than the serological cross-reactions, which often result in false positives or false negatives. The ability of this technique to detect all three pathogens in one run will also support pathogenicity studies of GRD, a disease whose epidemiology is invariably influenced by the synergistic interaction between GRV, GRAV and the satRNA. The type of GRD symptom developed (chlorotic, green and mosaic) is dependent on the satRNA and its variants, and not on GRV or GRAV [19, 20]. The multiplex PCR technique, which is capable of detecting all three pathogens, expands the scope for resistance screening. It will provide a simplified tool for understanding resistance in plants to chlorotic GRD.

In this study, we have developed a multiplex PCR approach for detection of all three viral pathogens associated with groundnut rosette disease. Currently, each individual pathogen is detected separately by singleplex PCR. The two-step multiplex RT-PCR developed was consistently reproducible and is a robust, specific and relatively inexpensive tool compared to simplex PCR approaches available for detection of GRD pathogens.

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