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Polymerase Chain Reaction for Detection of Peanut Mottle and Peanut Stripe Viruses in *Arachis hypogaea* L. Germplasm Seedlots

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Abstract: Results of peanut mottle and peanut stripe virus detection studies indicated that peanut seedlots were best handled by extracting nucleic acid from samples of seed slices pooled from rows and columns of seeds arranged in a grid. A portion of these RNA samples were then combined (plate pooled sample) and tested via RT-PCR. This approach could detect one infected seed in a 96-seed sample. The samples from the positive seedlots were then tested to determine the location of the virus-infected seeds. A similar approach with IC-RT-PCR tests of samples from rows and columns ground in buffer failed to produce consistent results. Both the RT-PCR and the IC-RT-PCR approaches were compared to the results with DAS-ELISA tests of the individual seeds. The approach of testing plate-pooled samples followed by row and column sample tests by RT-PCR resulted in faster and most reliable testing of seedlots.

Key words: Peanut, viruses, RT-PCR, IC-RT-PCR, sampling method, extracting method

INTRODUCTION

Peanut stripe virus (PStV) (Demski *et al*., 1984, 1985) and Peanut Mottle Virus (PeMV) (Kuhn, 1965) are common contaminants in peanut seed. Introduction of PStV and PeMV with germplasm in international exchange and from newly collected germplasm is a quarantine problem. Frequency of seed transmission of PstV and PeMV from naturally infected plants varies from 2 to 4%. PstV and PeMV have caused 7 and 25% reductions in yield, respectively, in research plots (Kuhn, 1965; Lynch *et al*., 1988).

Currently, there are several detection methods being used for these viruses. The double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) of slices of seeds (Pinnow *et al*., 1990) has been used successfully for large throughput seed testing. In addition, this method has been used with single-seeds or pooled samples of five seeds, but a more sensitive method utilizes the immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) (Gillaspie *et al*., 2000) which also relies on antisera, but eliminates the need for RNA extraction. This approach allows for more samples to be handled in a shorter time, but a method for even higher speed of handling with good sensitivity is needed. To accommodate screening larger numbers of seeds in a timely fashion, we developed an approach using RT-PCR in a Agrid-system. With this system as many as 96 seeds can be looked at as one pooled sample. Then, if a pooled sample tests positive, individual infected seeds can be detected by testing the 20 samples in the grid system approach.

MATERIALS AND METHODS

Sampling and sample pooling: Seeds for this test were taken from two seed lots (PI 442588 and PI 497388). Samples were arranged in a 96-well format with four rows of 12 wells each containing extract from a slice from a seed of one of the accessions. Seed slices were made as described by Pinnow *et al*. (1990). The other four rows contained slices of the other accession. Four wells in the format contained samples from slices from seeds that tested positive for one of the viruses. For DAS-ELISA, one sample was of a seed known to be free of either virus and one sample was a seed infected with the virus being tested. Samples for DAS-ELISA were macerated in seed extraction buffer (Pinnow *et al*., 1990).

Samples of leaves of the seedlings arising from tested seeds (the seeds were germinated with an ethylene gas treatment) were also arranged in the 96-well format. Leaf samples were taken by a leaf disk method utilizing a 1.5 mL microfuge tube to snap over the leaf (between the tube mouth and the lid) to remove a disk of approximately 8 mm diameter. The disks were macerated in leaf extraction buffer (Pinnow *et al*., 1990).

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Sample pooling for RT-PCR was also done on a 96-well format, i.e., seed slices or leaf disks were placed in rows (12 samples each) and columns (8 samples each) of wells. These 20 pooled samples were processed by grinding the seed slices or leaf disks in liquid nitrogen in a mortar with a pestle and RNA was extracted with a Qiagen mini prep kit (Qiagen Inc., Chatsworth, CA) in 2 mL lysis buffer with 1% mercaptoethanol. The final total RNA preparations were eluted in 100 μL of RNase-free water. Pooling of row-and column-extracts was done by taking 1.2 μL of the Arow extracted RNA and 0.8 μL of the Acolumn RNA samples and combining.

Pooled plate samples were also prepared by combining slices of 94 seeds from each of three seed lots (cultivars Georgia Green, MDR 98 and DP-1) in a 96-well grid. One pooled sample of each lot was tested by DAS-ELISA and one was extracted and tested by RT-PCR. Again, the samples for DAS-ELISA were macerated in seed extraction buffer. The slices in the pooled sample for RT-PCR were cut into fourths and a pooled sample of only one fourth of each slice was ground in 15 mL Qiagen lysis buffer. One milliliter of this preparation was extracted using the Qiagen mini prep method. A slice from each seed was also tested singly by DAS-ELISA. In addition, the seeds were sampled with a 1 mm drill bit on a Dewalt model DW990 14.4 V handheld cordless drill (Dewalt Industrial Tool Co., Hampstead, MD) by drilling the cotyledons of each seed in similar fashion as the procedure used by (Von Post *et al*., 2003) for extracting DNA from barley seeds. These samples were extracted using the Qiagen mini kit as earlier.

Sample testing: Serological studies were all done using the DAS-ELISA (Pinnow *et al*., 1990). Assay samples were considered positive when the mean value of the absorbance reading was twice the mean value for healthy controls plus 2.5 times the standard deviation among wells containing these controls per plate.

Primers used were those previously published for use in IC-RT-PCR (Gillaspie *et al*., 2000). For first strand c-DNA synthesis, an RT mix (20 μL, consisting of 4 μL of 5X first strand RT buffer [Invitrogen, Chicago, IL], 2 μL each of 0.1 M dithiothreitol and 10 mM deoxynucleoside triphosphate [dNTP], 0.25 μL of SUPERSCRIPT RT RNase H-Reverse Transcriptase [Invitrogen], 0.1 μL RNasin RNase inhibitor [Promega Corp., Madison, WI], 9.15 μL nuclease-free water and 0.5 μL of the reverse primer (primers at 100 ρmol μL^{-1}), was added to 2 μL of total RNA from test tissue. The reaction mix was then incubated at 37°C for 1 h followed by a treatment at 94°C for 2 min to inactivate the enzyme.

Viral cDNA was amplified in 25 μL of PCR mix containing 2.5 μL of 10X PCR buffer (Promega Corp.), 3.5 μL of 25 mM $MgCl_2$, 2.5 μL of 2 mM dNTP, 0.2 μL each of upstream and downstream primers, 0.2 μL of *Taq* DNA polymerase (Promega), 12.9 μL of nuclease-free water and 3 μL of RT product as follows: 94°C, 2 min; 35 cycles of 94°C, 30 sec; 50°C, 30 sec; 72°C, 60 sec and one cycle of 72°C, 10 min. The amplification products were assessed by electrophoresis in 2% agarose gels in TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.3) and stained in ethidium bromide. The expected product size for PeMV was 339 bp and for PStV 611 bp.

IC-RT-PCR (Gillaspie *et al*., 2000) was done by the published method. Some tests were run with samples of seed slices ground in buffer and others were run with slices ground in liquid nitrogen followed by the addition of buffer.

Symptoms: Seeds sampled by taking slices from the distal end of the cotyledon still germinate and produce seedlings. The seeds sampled for this test were grown to the seedling stage and examined for the presence of viral symptoms.

RESULTS AND DISCUSSION

The results of the tests in which peanut seeds and leaves from their resultant seedlings were sampled and tested individually by DAS-ELISA and sampled (pooled) by rows and columns for testing by RT-PCR are compared in Table 1 and 2. The gels of the RT-PCR products are shown in Fig. 1 and 2. The RT-PCR results for PStV (Table 1) in the row at the top and the column at the left correlate with the positive ELISA results for seed and leaf in R5/C8. This plant had strong virus symptoms. The positive seed at R2/C3 is apparently a mistake made in setting up the ELISA. The seed sample at R8/C12 was taken from a seed that had tested positive for PStV previously, but it was not positive in this test.

The RT-PCR results for PeMV (Table 2) for R6/C3 correlates with the positive seed and leaf sample for ELISA. This plant had viral symptoms. And R2/C9 as well as R8/C12 match with positive ELISA results at R2/C9 and R8/C12 although these seeds did not produce seedlings. The positive seeds R4 and C9 for RT-PCR did not yield a positive ELISA results at R4/C9 which may simply be the result of the greater sensitivity of PCR.

Tests for the viruses in seeds of different peanut seed lots with sampling done as above but using IC-RT-PCR failed to work consistently although there were several infected seeds in each seedlot tested as determined by DAS-ELISA. There were six attempts to get the grid procedure to work with IC-RT-PCR with either no success or with positives only with some samples. Tests

Table 1: RT-PCR-pooling screening method correlation with DAS-ELISA results for peanut stripe virus

		C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
		S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S+,L+	S-,L-	S+,L-	S-,L-	S-,L-
R1	S-, L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-
R2	S-, L-	S-,L-	S-,L-	S+,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-
R3	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-
R4	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-
R5	S+,L+	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S+,L+	S-,N	S-,L-	S-,L-	S-,L-
R6	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-
R7	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,N	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-
R8	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,N	S-,L-	S-,L-	S-,N

S = Seed, L = Leaf, N = No plant, Column on left and row at top are RT-PCR results and other rows and columns are DAS-ELISA results

Table 2: RT-PCR-Pooling Screening Method Correlation with DAS-ELISA results for peanut mottle virus

		C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
		S-,L-	S-,L-	S+,L+	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S+,L-	S-,L-	S-,L-	S+,L-
R1	S-, L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-
R2	S+, L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S+,N	S-,L-	S-,L-	S-,L-
R3	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-
R4	S+,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-
R5	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,N	S-,L-	S-,L-	S-,L-
R6	S+,L+	S-,L-	S-,L-	S+,L+	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-
R7	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,N	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-
R8	S+,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,L-	S-,N	S-,L-	S-,L-	S+,N

S = Seed, L = Leaf, N = No plant, Column on left and row at top are RT-PCR results and other rows and columns are DAS-ELISA results

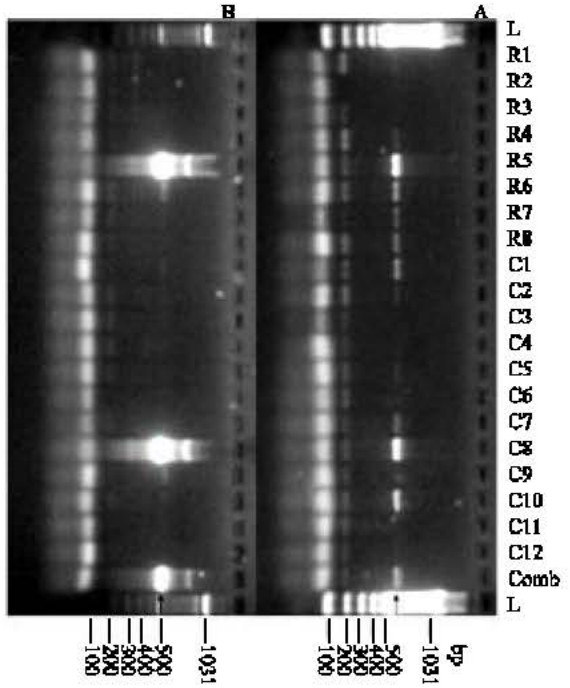

Fig. 1: RT-PCR products with Peanut stripe virus primers for pooled rows and pooled columns with A. Peanut seed samples and B. Peanut leaf samples. Lanes 1 and 23 (L) are 100 bp ladders; lanes 2 to 9 are rows 1 to 8, respectively; lanes 10 to 21 are columns 1 to 12, respectively; and lane 23 is pooled rows and columns combined (Comb). Arrows denote the location of the expected amplicon fragments

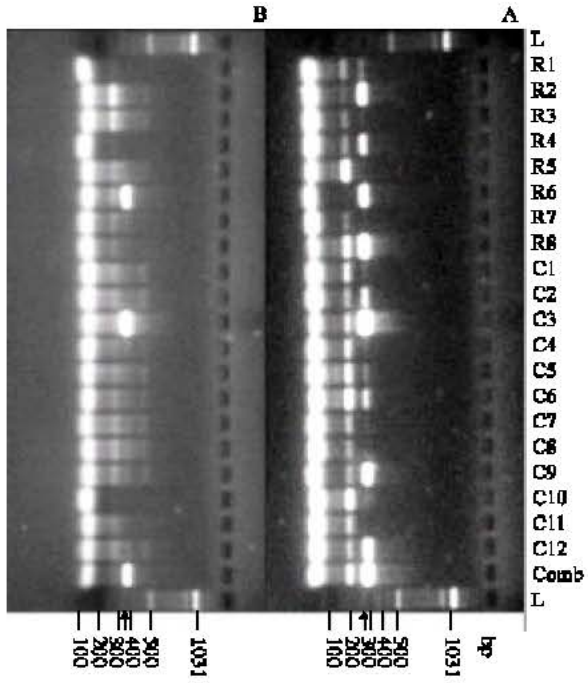

Fig. 2: RT-PCR products with Peanut mottle virus primers for pooled rows and pooled columns with A. Peanut seed samples and B. Peanut leaf samples. Lanes 1 and 23 (L) are 100 bp ladders; lanes 2 to 9 are rows 1 to 8, respectively; lanes 10 to 21 are columns 1 to 12, respectively; and lane 23 is pooled rows and columns combined (Comb). Arrows denote the location of the excepted amplicon fragments

done using IC-RT-PCR with single seeds produced the expected results, but the lipid present in the larger samples apparently kept the samples from reacting as expected at all times.

Pooled samples in which seed extracts from rows and columns were combined and where each individual slice was pooled and extracted as one sample were tested by DAS-ELISA. The serological test produced positives only with samples which had strong positives when done individually. Those pooled samples with lower virus titers failed to test positive and the use of ELISA for testing pooled samples could not be used (Pinnow *et al*., 1990). Similar pooling of RNA from samples by row and column demonstrated that the sensitivity of the RT-PCR is adequate to detect virus in these samples. Again, when RNA was extracted from pooled samples of each seed in the plate (~96), the results by RT-PCR were similar to those by DAS-ELISA-the problems with the extraction of total RNA from the large numbers of slices in these pooled samples apparently reduce the sensitivity of the test. The large amounts of lipids present in the seeds may be involved. When the sample size from each seed was reduced by using only one-fourth of a slice, the pooled sample still did not show the necessary sensitivity. The samples taken by drilling had less volume and were therefore somewhat less difficult to extract, but they were no better for detecting low titers of virus in the seed lots (data not shown for either sampling test).

Earlier attempts to detect both viruses in a multiplex PCR with primers from PeMV and PStV failed (Eun and Wong, 2000). In the present study, different primers are designed with similar annealing temperatures and still found that there was mispriming and misamplification (data not shown). Therefore, all testing was performed separately with the primers for the two viruses. There were still some non-specific bands in some tests (data not shown).

The results detail a method that allows the original objective to be met. The RNA extracts of the pooled seed samples coupled with RT-PCR can be used for detection of these two viruses in peanut seed lots. The procedure for handling large numbers of seeds and seed lots is a RT-PCR test of pooled samples from the total RNAs extracted from the rows. If a pooled sample of the seeds from a seedlot is positive, the extracts from the pooled rows and columns of the positive seed lot would then be tested so that individual infected seeds can be determined. This approach is the significant finding of the research and should improve handling of the peanut seedlots being tested for possible exotic viruses or virus strains.

Improvements are still necessary for the maximum throughput for detection of viruses in seed lots. Improved methods for collection of samples from seeds and for RNA extraction are needed. The use of IC-RT-PCR eliminates RNA extraction problems, but it necessitates the use of good antisera and good extracts with little lipid contaminate. Problems arise when errors are made in the IC-RT-PCR testing since there are no RNA samples to retest. An extraction method has been published for handling leaves by heating leaf disks at 95°C for 10 min in a buffer with SDS and PVP before RT-PCR (Roberts *et al*., 2000). A similar approach for seeds would be an improvement. Also, a sampling method must be developed which allows a uniform portion of the seed cotyledon to be removed without destroying of the ability of seed to germinate and grow. In the future, the use of labeled primers (Roberts *et al*., 2000) and of the molecular beacon technique (Eun and Wong, 2000) may improve sensitivity enough for multiple viruses to be detected in one sample in one tube.

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