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Detection of each of the causal agents of groundnut rosette disease in plants and vector aphids by RT-PCR☆

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Abstract

Detection of the three agents of groundnut rosette disease (groundnut rosette assistor virus, groundnut rosette virus and its satellite RNA) in plants and vector aphids by reverse transcription-polymerase chain reaction (RT-PCR) is reported. Three procedures for extraction of total RNA from groundnut were tested, of which two were found to be useful in giving RNA of sufficient quality for RT-PCR. Of these two, the total RNA extraction kit supplied by Qiagen was found to be the most versatile for extraction of all three agents from individual vector aphids (*Aphis craccivora*). Both groundnut rosette assistor virus and groundnut rosette virus could be detected from total RNA extracted from a single aphid that had been exposed to either green or chlorotic rosette-infected groundnut plants. They could be detected in aphids stored in 70% ethanol for up to 30 days at room temperature. However, satellite RNA could be amplified only when total RNA extracted from two or more aphids was used. Groundnut rosette assistor virus, groundnut rosette virus and its satellite RNA were detected by RT-PCR in aphids that had been exposed only to groundnut rosette diseased plants containing all three agents. The potential of RT-PCR in studying certain key issues of rosette disease epidemiology is discussed.

Keywords: Groundnut rosette disease; Luteovirus; Umbravirus; satellite RNA; *Aphis craccivora*; RT-PCR

1. Introduction

Rosette is the most destructive virus disease of groundnut (= peanut, *Arachis hypogaea* L.) in sub-Saharan Africa (Naidu et al., 1998) and is

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transmitted by the aphid, *Aphis craccivora* Koch. in the persistent manner (Storey and Bottomley, 1928; Watson and Okusanya, 1967). The symptoms of rosette disease in groundnut are variable; however, it occurs as two predominant symptom forms: 'chlorotic rosette' and 'green rosette' (Storey and Bottomley, 1928; Hayes, 1932; Storey and Ryland, 1957; Gibbons, 1977). The disease is caused by a complex of three agents comprising groundnut rosette assistor luteovirus (GRAV, Casper et al., 1983; Murant, 1989), groundnut rosette umbravirus (GRV, Reddy et al., 1985; Murant et al., 1995) and its satellite RNA (sat RNA, Murant et al., 1988). Murant et al. (1988) have shown that the sat RNA is primarily responsible for disease symptoms. Subsequently, it was reported that variants of sat RNA cause the different forms of rosette disease (Murant and Kumar, 1990). Although GRAV and GRV cause no obvious symptoms or at most a transient mottle in groundnut, they play crucial roles in the biology of rosette disease. GRV supports the replication of the sat RNA (Murant et al., 1988), while GRAV functions as a helper virus in vector transmission (Hull and Adams, 1968). Both GRV RNA and sat RNA are packaged in the coat protein of GRAV to form virus particles that the aphid vector can transmit. The sat RNA also plays a key role in GRV transmission by the aphid vector in that its presence in the source plants is essential for the GRAV-dependent transmission of GRV (Murant, 1990). Thus all three agents must occur together for successful transmission of the disease by the aphid vector and thereby for survival of the disease in nature. It is also noteworthy that rosette is unusual among plant virus diseases in having a complex etiology as well as causing serious losses to an economically important crop like groundnut in Africa (Murant, 1993).

Even though the etiology of the disease and molecular characteristics of the agents causing the disease are known, the epidemiology of rosette disease is not well understood (Naidu et al., 1998). For instance, the off-season survival of the disease, and the reasons for the large and unpredictable fluctuations in the incidence of the disease from year to year leading to sporadic epidemics in many groundnut growing countries of sub-Saharan Africa are not understood. Availability of sensitive diagnostic tools for the simultaneous detection of rosette disease agents in the aphid vector as well as in plants will help in studies addressing these issues, and in diagnostic surveys, identification of primary sources of inoculum, estimating proportion of viruliferous aphids trapped in different seasons, and resistance breeding programmes. GRAV can be detected in plants and aphids by the triple-antibody sandwich form of enzyme-linked immunosorbent assay (TAS-ELISA) using monoclonal antibodies (MAbs) raised to either potato leafroll virus or GRAV (Rajeshwari et al., 1987; Scott et al., 1996), and GRV RNA and sat RNA can be detected in plants by nucleic acid hybridization (Blok et al., 1995). Due to their cross reactions with different luteoviruses, a panel of MAbs have to be used in TAS-ELISA to verify that a luteovirus detected in groundnut or in aphids is indeed GRAV (Scott et al., 1996). Moreover, TAS-ELISA cannot provide information on whether the aphids carry particles containing either GRAV RNA and/or GRV RNA and sat RNA. Additionally, low concentrations of the rosette disease agents in vector aphids make it essential to develop a reliable and sensitive method for their detection. Recent studies have shown that viruses can be detected in individual aphid vectors using RT-PCR (Singh et al., 1995; Canning et al., 1996; Singh et al., 1996; Olmos et al., 1997; Stevens et al., 1997). The objectives of the present study were to evaluate methods for extraction of good quality RNA from groundnut leaves and aphids, and demonstrate the potential of RT-PCR for the detection of the causal agents of a complex virus disease like groundnut rosette in plants and vector aphids.

2. Materials and methods

2.1. Virus isolates

Chlorotic and green rosette diseased samples were collected in 1997 from central and northern

regions of Malawi, respectively. The two cultures were maintained separately in groundnut cv. Malimba by aphid transmission in an insect-proof glasshouse at NRI, UK (under MAFF plant health licence no. PHF 1767/1838 and 1767/1898).

2.2. Aphids

A non-viruliferous colony of *A. craccivora* derived from a single aphid was maintained on groundnut cv. Malimba and used for transmission. Adult aphids were given a 48 h acquisition access period on either chlorotic or green rosette-infected groundnut plants. They were transferred onto ten-day-old healthy groundnut seedlings for another 48 h prior to extraction of total RNA from individual aphids or groups of aphids. Adult aphids (both alate and apterous) were collected from colonies developed on chlorotic and green rosette infected plants in farmers' fields in Karonga area of northern Malawi and stored in 70% ethanol (Foottit and Richards, 1993) for different periods of time and subsequently used for extraction of total RNA.

2.3. Collection of plant samples

Leaf samples were collected from individual groundnut plants at random in six different farmers' fields in Karonga area during August 1997. They were collected from plants showing typical rosette disease (either chlorotic or green) symptoms as well as from apparently symptomless plants, adjacent to or distant from the infected plant. Samples were also collected from diseased plants from which aphids were collected. All samples were labeled seperately and tested at NRI by TAS-ELISA and RT-PCR for the presence of GRAV, GRV and sat RNA.

2.4. Enzyme-linked immunosorbent assay

Groundnut leaf samples were tested for GRAV by TAS-ELISA (Rajeshwari et al., 1987). A polyclonal antibody to GRAV was used as the first antibody and a MAb to PLRV (SCR 6) was used as the second antibody. ρ-Nitrophenyl phosphate was used as the substrate and the absorbance values at A_{405} were recorded using a Titertek Multiscan photometer (Flow Laboratories).

2.5. RNA extraction

Three methods for extraction of total RNA were initially evaluated using groundnut and *Nicotiana benthamiana* leaves: (I) extraction with phenol/chloroform followed by lithium chloride precipitation (Blok et al., 1995); (II) lithium chloride, NP-40 and sodium deoxycholate extraction without the phenol/chloroform step (Singh et al., 1995); and (III) guanidinium isothiocyanate extraction using a commercial kit (RNeasy® Plant Mini Kit, Qiagen). In all procedures, leaf tissue (100–200 mg) from groundnut or *N. benthamiana* plants infected with GRV and sat RNA from a chlorotic rosette isolate from Malawi (Murant et al., 1988), maintained at SCRI, was ground in liquid nitrogen. An appropriate volume of extraction buffer (2.5 vol in procedures I and II, 4.5 vol in procedure III) was added to the powder, and the mixture was vortexed and processed further by the appropriate method. In procedures I and II, the total RNA was collected by centrifugation, suspended in sterile distilled water and reprecipitated with ethanol at −70°C for 1 h. After centrifugation, the final pellet was washed once with 70% ethanol, dried and resuspended in 50 μl of sterile distilled water. In procedure III, the total RNA was eluted in 50 μl of sterile distilled water.

Only methods II and III were used for extracting RNA from aphids. Individual aphids or groups of aphids were placed in 1.5 ml Eppendorf tubes and ground with a disposable plastic pestle using an appropriate volume of extraction buffer. The samples were vortexed for 15 s before processing further to extract total RNA. In method II, 20 μg of glycogen was added as a carrier to precipitate RNA in samples extracted from single aphids and the final pellet was dissolved in 30 μl sterile distilled water. In method III, the total RNA was eluted with 30 μl of sterile distilled water. When necessary, the eluted volume was reduced to 10 μl by spinning samples in a centrifugal evaporator (DNA Speed Vac, Savant).

2.6. Oligonucleotide primers

Primers for specific amplification of nucleic acid sequences from each of the three agents of rosette disease were designed as follows. Primers GRAV-1, 5′-ATGAATACGGTCGTGGTTAGG-3′ (upstream primer) and GRAV-2, 5′-TTTGGGGTT TTGGACTTGGC-3′ (downstream primer) correspond to nucleotides 1–21 and 597–578 respectively of the GRAV coat protein gene (Scott et al., 1996; EMBL Z68894) and amplify a fragment of 597 bp. Primers GRV-1, 5′-GGAAGCCGGCG-AAAGCTACC-3′ (upstream primer) and GRV-2, 5′-GGCACCCAGTGAGGCTCGCC-3′ (downstream primer) correspond to nucleotides 2584–2603 and 3447–3428, respectively of GRV RNA (Taliansky et al., 1996; EMBL Z69910) and amplify a fragment of 863 bp. Primers for sat RNA were designed by aligning the ten sequences available (Blok et al., 1994; EMBL Z29702–Z29711). The upstream and downstream primer sequences, which represent the conserved sequences among all ten sat RNA sequences, respectively were: satRNA-1, 5′-GGTTTCAATAGGAGAGTTGC-3′ (nucleotides 3–20) and satRNA-2, 5′-AAAT-GCCTAGTTTGGGCGTG-3′ (nucleotides 891–872) and will amplify a fragment of the sat RNA of about 890 bp. A second upstream primer designated satRNA-3, 5′-AAGTGCTGAGGAACCA-GCAC-3′, corresponding to the conserved nucleotides 485–504 was also used for amplification of sat RNA sequences. Together with the primer satRNA-2, it amplifies a fragment of about 400 bp corresponding to the 3′ half of the sat RNA.

2.7. cDNA synthesis and PCR

RT-PCR reactions were set up separately for GRAV, GRV and sat RNA. A volume of 5–10 μl of total plant or aphid RNA extract were mixed with 200 ng of downstream primer GRAV-2, GRV-2 or satRNA-2 in a total volume of 12 μl, denatured at 70°C for 10 min and snap cooled in ice. After a brief spin, a total of 8 μl of reverse transcription mix (2 μl of 10 × RT buffer, 2 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTPs, 2 μl of 0.1M DTT, and 1 μl [200 U] of SuperScript™ RT [Gibco-BRL]) was added, mixed gently and incubated at 42°C for 1 h. The reaction was terminated by heating at 70°C for 15 min. Three microlitres of this product was added to tubes containing 47 μl of the PCR reaction mixture (5 μl of 10 × PCR buffer, 3 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTPs, 200 ng each of upstream and downstream primers, 0.5 U *Taq* polymerase [Boehringer-Mannheim] and appropriate volume of distilled water). In some cases, *Taq* polymerase was added to the reaction mix when the temperature reached 85°C (hot start PCR). Amplifications were carried out in a Hybaid Thermal Cycler using the following temperature regime: a denaturation phase at 94°C for 2 min followed by 35 cycles of amplification (94°C for 1 min, 55°C for 1 min, and 2 min at 72°C) and a final extension at 72°C for 10 min. Ten microlitres of PCR products were analyzed by 1.2% agarose gel electrophoresis in TBE buffer, stained with ethidium bromide and finally visualized under UV light.

3. Results

3.1. Evaluation of total RNA extraction procedures for RT-PCR

Three extraction procedures were used to obtain total RNA from groundnut and *N. benthamiana* leaves infected with GRV and its sat RNA. cDNA made from the total RNA preparations was subsequently used in PCR to amplify GRV and sat RNA-specific sequences using primers GRV-1 and GRV-2, and satRNA-1 and satRNA-2, respectively. A 860 bp fragment was amplified from total RNA extracted from *N. benthamiana* with all three extraction procedures (Fig. 1, lanes 2, 4 and 6). However, a fragment of similar size was amplified from total RNA preparation of groundnut extracted with methods II and III (lanes 3, 7), but not with method I (lane 5). PCR products with similar intensities were obtained from groundnut and *N. benthamiana* regardless of whether method II or III was used for extraction of total RNA. Extracts made by method III yielded the fewest bands additional to the major

GRV-specific 860 bp product, when amplified with GRV-1 and GRV-2 primers. Similar results were obtained when a 900 base pair fragment specific to sat RNA was amplified with satRNA-1 and satRNA-2 primers from the same RNA extracts (data not shown). No PCR product was obtained from RNA preparations extracted by any of the methods from uninfected tissue of either groundnut or *N. benthamiana*.

3.2. RT-PCR detection of GRAV RNA, GRV RNA and sat RNA in groundnut plants

A total of 150 samples collected from individual groundnut plants showing rosette symptoms in farmers' fields were initially tested for GRAV by TAS-ELISA. About 20% of plants tested gave negative results in this assay even though they showed characteristic disease symptoms. This was observed with either chlorotic or green rosette infected plants. Total RNA extracted from these plants using either procedure II or III was then used to amplify GRAV, GRV and sat RNA specific sequences by RT-PCR. As shown in Fig. 2, a 550 bp fragment was amplified with primers GRAV-1 and GRAV-2 only from TAS-ELISA positive plants (lane 2) but not from either ELISA-negative (lane 3) or uninfected samples (lane 4). On the other hand, a 860 bp fragment specific for GRV and a 900 bp fragment specific

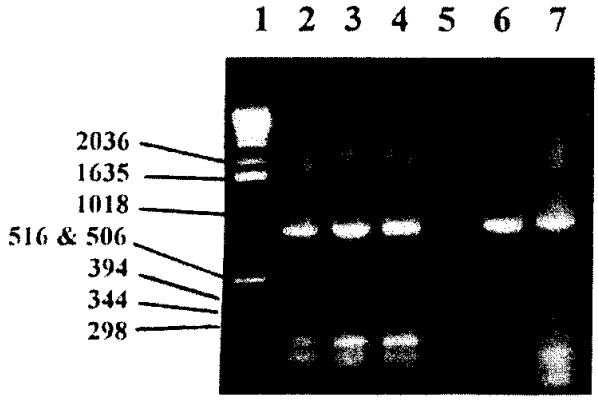

Fig. 1. Agarose gel electrophoresis of RT-PCR products specific to GRV from RNA extracted with procedure I (lanes 4, 5), II (lanes 2, 3) or III (lanes 6, 7). Lanes 2, 4 and 6 represent products from total RNA extracted from *N. benthamiana* and lanes 3, 5 and 7 represent products from total RNA from groundnut leaves. Lane 1, DNA size markers (1 kb ladder, Gibco BRL).

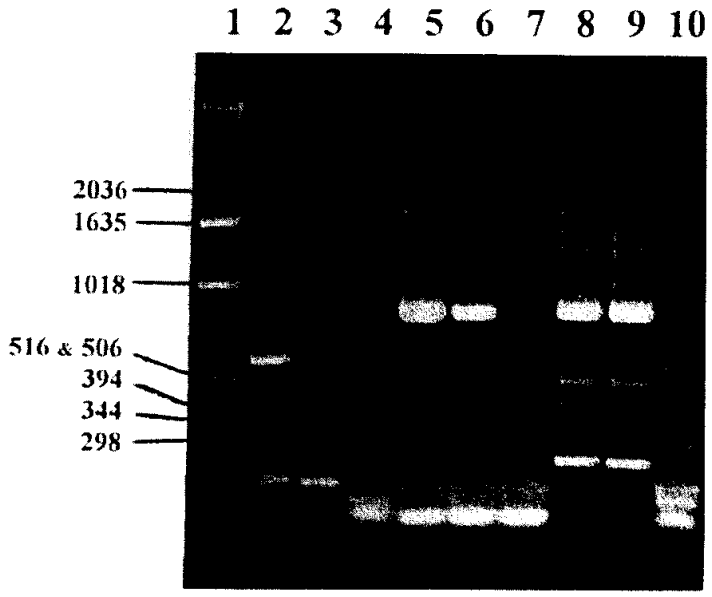

Fig. 2. Agarose gel electrophoresis of RT-PCR products specific to GRAV (lanes 2–4), GRV (5–7) and sat RNA (8–10) from rosette diseased groundnut leaves. Lane 1 shows 1 kb DNA ladder (Gibco BRL). Lanes 2, 5 and 8 represent total RNA from diseased leaves positive for GRAV in TAS-ELISA and lanes 3, 6 and 9 represent total RNA from those negative for GRAV. Lanes 4, 7 and 10 represent total RNA from uninfected leaves.

for sat RNA were amplified from all diseased samples (Fig. 2, lanes 5, 6, 8, 9). Similar results were obtained with plants showing either chlorotic or green rosette symptoms. No fragments corresponding to GRAV, GRV or sat RNA were amplified from uninfected control plants (Fig. 2, lanes 4, 7, 10).

Out of 75 apparently healthy groundnut plants collected from the field, about 10% were found to contain GRAV, as judged by TAS-ELISA. A 550 bp fragment specific for GRAV, but neither GRV nor sat RNA specific fragments, was amplified from such plants. Analysis of 20 leaf samples from each category (i.e. chlorotic and green rosette with or without GRAV, symptomless plants positive or negative for GRAV) showed good correlation between TAS-ELISA and RT-PCR for detection of GRAV, and symptoms and presence of GRV and its sat RNA (Table 1). These results show that TAS-ELISA using SCR6 MAb can be employed, without recourse to RT-PCR except under special circumstances, for routine detection of GRAV in groundnut leaf samples.

Table 1
Correlation between rosette disease symptoms and detection of GRAV, GRV and sat RNA by TAS-ELISA and RT-PCR

Symptom	TAS-ELISA[a]	RT-PCR[b]		
		GRAV	GRV	sat RNA
Chlorotic rosette	1.291 ± 0.062	+	+	+
Chlorotic rosette	0.027 ± 0.002	−	+	+
Green rosette	1.344 ± 0.071	+	+	+
Green rosette	0.028 ± 0.016	−	+	+
No symptom	1.323 ± 0.076	+	−	−
No symptom	0.025 ± 0.001	−	−	−

[a] Mean A_{405} values from ten different samples ± S.E., buffer control, 0.021, healthy leaf, 0.029.

[b] (+) Indicates amplification and (−) indicates no amplification of GRAV-, GRV-, and sat RNA-specific fragments.

3.3. RT-PCR detection of GRAV RNA, GRV RNA and sat RNA in A. craccivora

A total of 40 aphids consisting of equal numbers of alate and apterous adults exposed to either green or chlorotic rosette were tested for the presence of GRAV RNA, GRV RNA and sat RNA. Initial experiments were aimed at detecting all three agents from a single aphid. Therefore, one-third of the total RNA extract from each aphid was used separately for RT-PCR amplification of sequences specific to GRAV, GRV and sat RNA. With primers GRAV-1 and GRAV-2, a 550 bp fragment was amplified only from viruliferous aphids exposed to either green or chlorotic rosette infected plants (Fig. 3A, lanes 3–6). A second band of about 450 bp was also amplified but it was present in both viruliferous and non-viruliferous aphids suggesting that it was not a virus-specific product. This band was consistently amplified even at higher annealing temperatures of 58°C or 60°C. A single band of about 860 bp was amplified from viruliferous aphids with primers GRV-1 and GRV-2 (Fig. 3B, lanes 3–6). In contrast, difficulties were encountered in amplifying sat RNA-specific bands. Primers satRNA-1 and satRNA-2 failed to amplify the expected 900 bp fragment when one third or even the entire total RNA extract from a single aphid was used in RT-PCR. A 500 bp fragment could be amplified from the same cDNA with primers satRNA-2 and satRNA-3; however, it was not consistently amplified from all ten viruliferous aphid samples tested. In addition, a faint band corresponding to the 500 bp sat RNA fragment

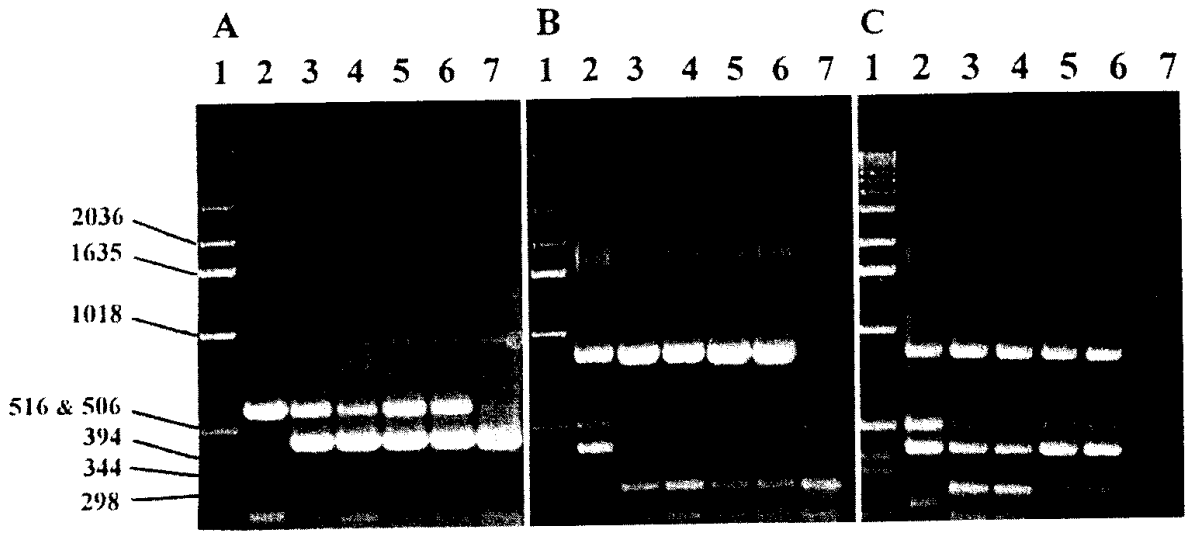

Fig. 3. Amplification from RNA prepared from aphids of cDNA for (A) GRAV, (B) GRV and (C) sat RNA. Lane 1, DNA size markers (1 kb ladder, Gibco BRL); lane 2, RNA from chlorotic rosette-infected leaf; lanes 3, and 4, RNA from alate and apterous aphids, respectively that had been exposed to a chlorotic rosette-infected plant; lanes 5, and 6, RNA from alate and apterous aphids, respectively, that had been exposed to green rosette-infected plant; lane 7, RNA from a non-viruliferous aphid. One third of total RNA from the same aphid was used separately for preparing cDNA to GRAV and GRV, whereas total RNA from a group of 5 aphids was used for preparing cDNA to sat RNA.

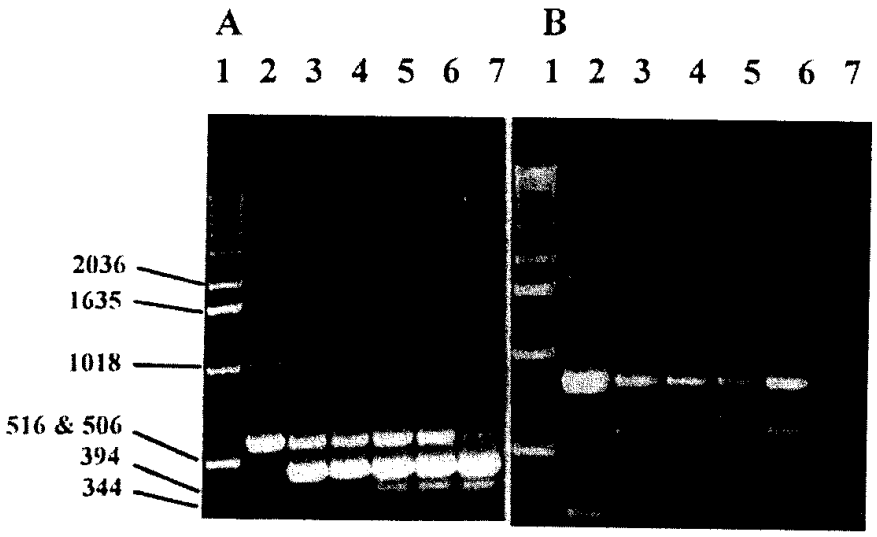

Fig. 4. Amplification from RNA prepared from aphids stored in 70% ethanol of cDNA for (A) GRAV and (B) GRV. Lane 1, DNA size markers (1 kb ladder, Gibco BRL); lane 2, RNA from chlorotic rosette-infected leaf; lanes 3, 4, 5 and 6, RNA from single aphids stored for 5, 10, 15 and 30 days, respectively; lane 7, RNA from a non-viruliferous aphid stored for 30 days.

was also amplified from non-viruliferous aphids suggesting that primers satRNA-2 and satRNA-3 can not be reliably used for sat RNA amplification from aphid vectors. However, the 900 bp sat RNA fragment could be successfully amplified with primers satRNA-1 and satRNA-2 when total RNA extracted from groups of two or more aphids was used in RT-PCR (Fig. 3C, lanes 3–6). No fragments corresponding to GRAV, GRV or sat RNA were amplified from nonviruliferous aphids (Fig. 3A, B and C, lane 7).

Aphids collected from GRAV-positive groundnut plants showing either chlorotic or green rosette symptoms in the farmers' fields were stored in 70% ethanol at room temperature and ten aphids tested individually each time at different intervals for the presence of GRAV RNA and GRV RNA. As shown in Fig. 4, both GRAV (A, lanes 3–6) and GRV (B, lanes 3–6) specific bands could be amplified from all aphids stored up to 30 days. No virus-specific bands were detected in the product from non-viruliferous aphids (Fig. 4, A and B, lane 7).

3.4. Detection of GRAV RNA, GRV RNA and sat RNA in aphids exposed to rosette plants with or without GRAV

Aphids were exposed for 48 h to groundnut plants with chlorotic rosette symptoms that were either positive or negative for GRAV by TAS-ELISA. PCR fragments specific to all three agents were amplified from rosette-affected GRAV-positive plants (Fig. 5, lanes 2, 6, 10), whereas only GRV RNA and sat RNA were detected in rosette-affected GRAV-negative plants (Fig. 5, lanes 3, 7, 11). In two separate experiments, total RNA was extracted from groups of five aphids exposed to these two types of plants and analyzed by RT-PCR for GRAV RNA, GRV RNA and sat RNA. As shown in Fig. 5, PCR fragments specific to all three agents were detected only in aphids that were fed on the GRAV-positive plants (lanes 4, 8, 12). No bands were obtained from aphids exposed to diseased plants that did not contain GRAV (lanes 5, 9, 13).

4. Discussion

In this report, we have shown that an RT-PCR protocol can be used for the simultaneous detection of the three groundnut rosette disease agents in plants and vector aphids. Of the three RNA extraction procedures tested, the procedures described by Singh et al. (1995) and by Qiagen, were found to be useful in providing good quality RNA from groundnut for subsequent use in RT-PCR. The RNA preparation obtained with procedure I, but not procedures II or III, always looked

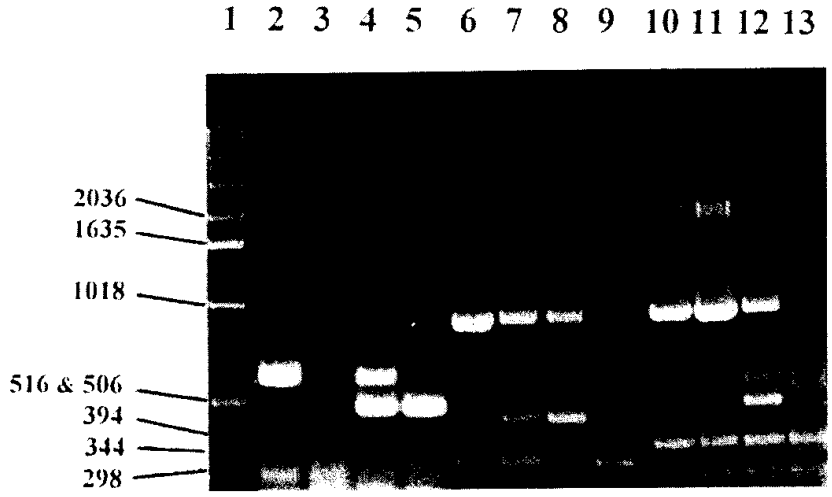

Fig. 5. Amplification of cDNA for GRAV (lanes 2–5), GRV (lanes 6–9) and sat RNA (lanes 10–13) from RNA from chlorotic rosette-infected plants that were tested either positive (lanes 2, 6 and 10) or negative (lanes 3, 7 and 11) for GRAV in TAS-ELISA, and from RNA from aphids exposed to GRAV-positive (lanes 4, 8 and 12) or GRAV-negative (lanes 5, 9 and 13) rosette-affected plants.

brown in colour. Since groundnut is known to be rich in polyphenolic compounds, this discolouration of RNA preparations was probably due to intimate association of polyphenols with RNA, which might be a contributing factor in inhibiting amplification of viral sequences by RT-PCR. In addition, other inhibitors like polysaccharides (Demeke and Adams, 1992) that coprecipitate with total RNA might also be contributing to this problem because several plant species are known to be rich in inhibitors of the RT or PCR reactions (Candresse et al., 1998). Since procedures II and III avoid using phenol/chloroform extraction, both protocols can be used in laboratories with minimum facilities for handling hazardous reagents. However, in our experience the protocol given by Qiagen was found to be the most satisfactory for a number of reasons: it utilizes a silica gel-based membrane which binds selectively the total RNA thereby eliminating inhibitors that interfere with subsequent RT-PCR, it involves a smaller number of steps in RNA extraction and hence minimizes losses of viral RNA during extraction, especially from aphids, and a large number of aphid samples can be processed in a relatively short period of time. We also observed that, unlike the protocol described by Singh et al. (1995), the total RNA extracted with the Qiagen method in different locations could be carried or sent by mail in ethanol to a central location for analysis, without obvious RNA degradation. This is especially important when testing plant and aphid samples collected in different African countries and/or remote areas for the presence of rosette disease agents.

The inability to amplify sat RNA from a single aphid might be due to its being present in low concentration. Alternatively, some property of the sat RNA, such as secondary structure, may make reverse transcription inefficient. However, this scarcely limits the usefulness of the RT-PCR test. Indeed, our tests suggest that aphids that contain GRV RNA also contain sat RNA; this would not be unexpected, because the presence of sat RNA is required for aphid transmission of GRV (Murant, 1990).

Our results also provide unequivocal evidence that not all plants in the farmers fields that show rosette symptoms contain GRAV (Fig. 2). *A. craccivora* exposed to rosette diseased plants lacking GRAV did not acquire GRV RNA and sat RNA (Fig. 5). Consequently, such plants can not serve as sources of inoculum and thus remain 'dead ends' of the disease with no epidemiological significance. These observations also stress the need to use a specific test, such as RT-PCR, when assessing the relationship between disease incidence, proportion of infective aphids in a given population at a given time, and field spread of the disease. It is equally interesting to note that some

of the apparently symptomless plants in the field contained GRAV. These results together with other data (Naidu et al., unpublished results) suggest that GRAV can be separated from GRV and its sat RNA in time and space. How this happens requires further study and also raises the fundamental question of whether a single aphid can transmit both particles containing GRAV RNA and those containing GRV RNA and sat RNA. TAS-ELISA, which detects only GRAV coat protein, cannot answer such questions. By using RT-PCR, it is now possible to address these issues and understand their implications in rosette disease epidemics.

The RT-PCR protocol described here will be a valuable tool in addressing several aspects of the ecology and epidemiology of rosette disease. The ability to detect rosette disease agents in aphids preserved in 70% ethanol will enable samples of *A. craccivora*, collected at different times during the season and at different sites, to be tested at a central location and will provide information, for example, on the proportion of immigrating aphids that are viruliferous at a given time. This is not necessarily the same as the proportion of disease transmitters in a given aphid population (Watson and Okusanya, 1967), but the technique will be invaluable in future studies to (a) correlate the presence of the three agents in the aphid with the ability to transmit the disease, (b) study interactions between the aphid vector and the three agents of rosette disease, (c) distinguish strains/variants of the agents carried by aphids, and (d) facilitate the development of an infectivity-based disease forecasting system.

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