International Aspects of Groundnut Virus Research

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Several virus diseases of groundnut occur in the Semi-Arid Tropics (RAT) (Chohan 1974; Feakin 1973; lizuka et al. 1979; McDonald and Raheja 1980) and some are economically important (Gibbons 1977; lizuka et al. 1979). Peanut mottle virus (PMV) is the most widespread (Reddy et al. 1978) and can cause considerable yield losses (Kuhn and Demski 1975). Other economically important virus diseases have more restricted distributions. For instance, groundnut rosette is important in Africa, south of the Sahara (Gibbons 1977; Gillier 1978; Rossel 1977; Yayock et al. 1976); peanut clump (PCV) in West Africa (Trochain 1931; Bouhot 1967; Germani et al. 1975) and in India (Reddy et al. 1979); bud necrosis (caused by tomato spotted wilt virus-TSWV) in India (Ghanekar et al. 1979); and witches' broom (a disease associated with mycoplasma-like organisms) in Southeast Asia (lizuka, personal communication).

Applied research on plant virus diseases differs from that on fungal and bacterial diseases because of the special nature of viruses. Some important prerequisites to the eventual control of virus diseases are characterization of the causal virus and elucidation of its mode of transmission. Precise virus characterization involves complicated techniques which are constantly being improved as a result of rapid technological advances and increasing interest in the mode of replication of plant viruses.

For effective management of plant virus diseases it is essential that their ecology is understood. The distribution of each disease should be ascertained and yield losses assessed. High priority should be given to screening for host plant resistance and production of resistant cultivars and this depends on close cooperation with scientists in other disciplines. To enable these aims to be achieved it is necessary that simple and effective techniques should be developed for the detection and identification of viruses.

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Problems of Virus Research in the Semi-Arid Tropics (SAT)

Most reports on the occurrence of groundnut virus diseases in the SAT have been based largely upon visual symptoms. However, it is well known that external symptoms can be greatly influenced by such factors as genotype, plant age, environment, and strain of virus present. On the basis of symptoms alone it appears that bud necrosis in India (Ghanekar et al. 1979) has been described under six different names; each being regarded as a new disease by the authors. Again, on the basis of external symptoms, rosette has been reported from India, the Philippines, Indonesia, Australia, Russia and Argentina (Rossel 1977).

For most areas of the SAT, data on the incidence and distribution of groundnut virus diseases are either incomplete or lacking. Causal viruses, with very few exceptions (Bock 1973; Germani et al. 1975; Dubern and Dollet 1978 and 1979) have not been fully characterized. This is true even for groundnut rosette virus which has been under investigation in Africa for almost half a century. Reports on limited characterization of this virus (Okusanya and Watson 1966; Hull and Adams 1968) are yet to be confirmed.

Losses due to diseases have been reliably assessed for only few groundnut virus diseases, including those which have been characterized.

Methods for screening groundnut germplasm for resistance to viruses (and to their vectors) have been developed for only a few diseases, and only in the case of groundnut rosette has there been successful development of resistant cultivars (Gibbons 1977; Gillier 1978; Harkness 1977).

The most important objectives of the ICRISAT program are to characterize the economically important virus diseases in the SAT and to present reliable data on their distribution and interrelationships with similar viruses occurring in other countries.

In order to provide a basis for the control of virus diseases, research should be pursued into: (1) screening for disease resistance in *Arachis hypogaea* and in wild *Arachis* sp; (2) the effect of cultural practices (including date of sowing, spacing and intercropping) on the incidence and spread of disease; and (3) avoiding sources of infection.

Diagnosis of Groundnut Virus Diseases

Various steps involved in the diagnosis of plant virus diseases (Bos 1976) are given in Table 1.

Table 1. Steps in the diagnosis of plant virus diseases*

1. Assessment of economic importance (incidence, distribution, and yield losses).
2. Transmission by grafting, sap inoculation, insects, nematodes, etc.
3. Inoculation to a series of test plants (preferably by mechanical sap inoculation) and back inoculation to a parallel range of test plants to check possible multiple infection and host range.
4. Identification of a host which consistently produces characteristic symptoms, especially local lesions (diagnostic host).
5. Identification of a systemically infected host which supports high virus concentration (for purification of viruses).
6. Determination of biological properties using local lesion, assay (TIP, LIV and DEP).
7. Examination under electron microscope (leaf dip, thin sections).
8. Testing by serological methods.
9. Development of methods to purify the virus.
10. Determination of physico-chemical properties and electron microscopy of purified virus.
11. Production of antiserum.
12. Testing of serological relationships with similar viruses occurring elsewhere.
13. Fulfillment of Koch's postulates, especially using purified virus.

.. Modified from Bos (1976).

Although it will eventually be necessary to diagnose virus diseases of minor importance, characterization of economically important groundnut virus diseases (bud necrosis, clump and peanut mottle) has to receive top priority.

Sap Inoculation

In initial stages, sap transmission of viruses present in crude groundnut leaf extracts could be achieved by adding reducing agents such as 2-mercaptoethanol to extracting buffers. In addition, maintenance of low temperature throughout the inoculation process, determination of optimum ionic strength and pH of phosphate buffer, and the selection of only young infected leaflets showing certain characteristic symptoms, have facilitated mechanical sap inoculation of all groundnut viruses isolated so far in India.

Diagnostic Hosts

A large number of hosts commonly used in the diagnosis of virus diseases have been secured and are being maintained. From these, diagnostic hosts have been selected for each of the virus diseases characterized at ICRISAT.

Serology

If virus antisera are available, serological techniques (Ball 1974; van Regenmortel 1978) offer effective means of diagnosis. They are rapid and can easily be standardized for the detection of specific viruses. Conventional serological techniques such as tube precipitin, micro-precipitin and precipitin ring tests have been used but have serious limitations for work with groundnut viruses. For instance, they were not successful when used for detection of TSWV in groundnuts because of limitations such as low virus concentration in plant extracts and lack of high titred antisera.

Three other serological techniques that have been used at ICRISAT with considerable success are Ouchterlony's agar gel double-diffusion (AGD); passive haemagglutination (PHA); and enzyme-linked immunosorbent assay (ELISA).

In the AGD test, antigen and antibody are allowed to diffuse into agar. A positive reaction results in the appearance of a thin white band

where antigen and antibody meet. The test is easy to perform and requires no specialized equipment (Ball 1974). It can be used to test several samples at the same time. By using the slight modification of incorporating 3,5-diiodosalycilic acid into the agar for dissociating long rod-shaped viruses, the test has been successfully employed to detect PMV and Cowpea mild mottle virus (CMMV) (Table 2).

The PHA test (Ball 1974), one of the most sensitive serological techniques, has been simplified and modified to prevent non-specific agglutination (Rajeswari et al„ in press). Glutaraldehyde-fixed red blood cells, after *treatment* with tannic acid, are coated with antiserum. Antibody sensitized red blood cells are then added to various dilutions of test solutions. The test is performed in lucite plates containing 'U'-shaped wells and in a positive reaction red cells agglutinate, forming a smooth mat with a serrated margin on the bottom of the well. In a negative reaction, red cells form a discrete red ring at the periphery of the well.

The PHA test is extremely sensitive, easy to operate, does not need specialized equipment or reagents, and requires much less antisera than the AGD test. The PHA technique can be used to detect viruses in crude plant extracts. The test has been successful in the detection of TSWV antigens in infected groundnut plants and in the thrips vector. The test has also been successfully used for the detection of other economically important virus diseases in India (Table 2).

Both AGD and PHA techniques were tried for detection of viruses in seeds but without success. The ELISA technique was acquired and successfully adopted for detection of PMV in seed (Reddy et al., in preparation). The ELISA test is by far the most sensitive and specific

Table 2. Characterization of important viral dlseases of groundnut in India.

characterization	Name of the virus			
	TSWV	PCV	PMV	CMMV
1. Serology				
Gel diffusion	?	+	+	+
Haemagglutination	+	+	+	+
ELISA		#	+	*
2. Electron microscopy				
Plant material	+	*	+	#
Purified virus	*	+	+	+
3. Transmission				
Mechanical	+	+	+	+
Vector	+	+	+	?
Seed		?	+	
4. Physicochemical properties				
Sedimentation coefficient	#	#	+	*
M.W. of protein	«	+	+	+
M.W. of nucleic acid	#	*	+	+
5. Host range	+	+	+	+
6. Biological properties				
TIP	+	+	+	+
LIV	+	+	+	+
7. Symptoms				
Groundnut	+	+	+	+
Diagnostic host	+	+	+	+

+ = Positive result, - - Negative result. *= Not performed, ? >> Data Inconclusive

serological technique now available for detection of plant viruses (Clark and Adams 1977; Voller et al. 1976). The procedure is simple and rapid. The y-globulins extracted from antisera, are absorbed to welts of a special microtiter plate. Test samples, including crude plant extracts, purified viruses and extracts from seed, are added to the wells. If the test sample contains specific viral antigens, theseare bound tothe y-globulins coated on the inner surface of the well. Thetest samples are washed away and enzyme-conjugated y-globulins are added to the wells. The labelled antibodies bind to the viral antigen already bound to the y-globulins coated on the plastic surface. Finally, a substrate for the enzyme, which was used earlier for conjugating '/-globulins is added to the well. The color change in the substrate is proportional to the amount of enzyme present, which in turn is proportional to the viral antigen concentration.

The two major limitations of ELISA are the need for high titered antisera and specialized reagents and plates for performing the test. Using the ELISA technique, it has been possible to screen nearly 1000 kernels for presence of PMV in two days. It would take nearly one month to field plant seed and score visually for PMV symptoms. A small portion of the cotyledon is adequate for detecting the virus. In addition, PMV could be detected in crude plant extracts d iluted to 1:10000.

Experiments are under way to employ ELISA for the detection of other groundnut viruses and especially for monitoring field collected viruliferous vector populations.

Electron Microscopy

Electron microscopy is an essential technique for the detection and identification of plant viruses. An electron microscope has recently been installed at ICRISAT and facilities are available for fixation, embedding and thin sectioning of plant material. Purified preparations of PCV, PMV and CMMV have been examined. Tomato spotted wilt virus and PMV could be localized in thin sections of infected plant material.

Purification

Purification of plant viruses is essential to produce antisera, for determining physicochemical properties and for electron microscopy. Purification of viruses requires expensive laboratory equipment such as a refrigerated superspeed centrifuge, an ultracentrifuge, a spectrophotometer and a gradient scanner. In addition, expertise is required for virus purification. However, with the aid of a refrigerated superspeed centrifuge it would be possible to partially purify viruses and prepare electron microscope grids for examination at ICRISAT.

Several physicochemical techniques are now available for separating virus particles from the normal constituents of their host cell, and the art of purification is to exploit these techniques so as to produce highly infective virus preparations as free as possible from host material. Groundnut tissue contains an excess of tannins which normally interfere in virus purification. At least one more suitable host has been discovered for each one of the groundnut viruses characterized at ICRISAT for use in virus purification. Various buffers, with specific ionic strength and pH values, have been used successfully to stabilize viruses in the initial purification steps which involve extraction from the leaves, clarification with organic solvent, precipitation with polyethylene glycol (PEG) and subsequent resuspension of PEG precipitates. Further purification has been achieved in rate and quasi-equiiibrium zonal density gradient centrifugation in sucrose solutions.

Purification techniques specific for PMV, PCV and CMMV have been developed to obtain high virus yields and high specific infectivity with no detectable impurities (Table 3). Tomato spotted wilt virus is known to be one of the most difficult viruses to purify, but a purification method developed at ICRISAT should soon be available.

Physicochemical Properties

Specialized skills and experience, and special equipment, are required to characterize viruses by physico-chemical methods. These techniques usually complement the results of electron microscopy and serology but are indispensable in determining relationships among similar viruses and in distinguishing strains. Molecular weight determination of viral proteins and nucleic acids employing polyacrylamide gel electrophoresis (Adesnik 1971; Maizel 1971; Reddy and Black 1973; Reddy and

Table 3. Virus purification methods davalopad at ICRISAT Center.

To obtain:	For:
High virus yields	Peanut mottle virus
No detectable impurities	Cowpea mild mottle virus
High specific infectivity	Peanut green mosaic virus
	Peanut clump virus
	Tomato spotted wilt virus

MacLeod 1976) has now become an indispensable tool for rapid characterization of viruses.

Chemical characterization has been successfully employed at ICRISAT to distinguish the morphologically identical PMV and peanut green mosaic virus (both belong to the potato virus Y group) and the morphologically similar CMMV which belongs to the Carla Virus group) (Table 2).

General

The important criteria employed in the characterization of groundnut viruses are given in Table 4. A series of occasional papers, describing details of all the steps involved in each of the techniques employed for the diagnosis of groundnut viruses at ICRISAT, is under preparation.

Table 4. Diagnosis of virus diseases.

Identification depends on
Serology
Electron microscopy
Transmission
Physicochemical properties
Host range
Symptomatology

Management of Virus Diseases

With the exception of PCV and CMMV the vectors of all groundnut viruses, characterized at ICRISAT, have been identified (Table 5). Studies on various factors contributing to the multiplication and spread of vectors have provided us with ways and means of managing the diseases. For instance, cultural practices (date of sowing, and plant spacing) have been successfully employed to reduce losses from bud necrosis (TSWV). In addition, identification of vectors and virus-vector relationship have been helpful, in the diagnosis of TSWV and PMV. Large scale methods for screening germplasm have been developed and sources of resistance have been identified for some viruses.

Table 5. Vectors of virus diseases identified at ICRISAT Center.

1. Bud necrosis (Tomato spotted wilt virus)	: *Scirtothrips dorsalis* *Frankliniella schultzei*
2. Peanut mottle	: *Aphis craccivora* *Myzus persicae*
3. Peanut clump	: Nematodes (?)
4. Yellow spot (Tomato spotted wilt virus?)	: *Scirtothrips dorsalis*
5. Peanut green mosaic	: *Aphis gossypii* *Myzus persicae*

Groundnut Virus Research in the SAT

The techniques described for detection, identification and purification of viruses require elaborate and expensive equipment (Table 6) and availability of highly trained scientific and technical staff. The virus laboratory at ICRISAT and a relatively small number of other laboratories in the SAT are so equipped. It would not be practical to set up such laboratories in all areas of the SAT where research on groundnut viruses is considered desirable. However, the absence of a fully equipped and staffed virus laboratory does not meanthat useful research ongroundnutviruses cannot be undertaken.

Groundnut virologists from ICRISAT, or from other institutions where specialized virus research is being undertaken, could visit different areas of the SAT and in collaboration with

Table 6. Requirements for virology research.

I.	Maintenance and transmission
	*Glass or screenhouse
	*Autoclave
II.	Serology
	*Clinical centrifuge
	*Hot water bath
	Special chemicals, plates
III.	Production of antisera
	*Animal house
	*Rabbits
IV.	Diagnosis
	*Diagnostic hosts
	Chemical characterization
	Electrophoresis apparatus
	•Spectrophotometer
V.	Purification
	•Ref. superspeed centrifuge
	Ultracentrifuge
	Gradient scanner
VI.	Electron microscopy
	Fixing and embedding
	Electron microscope
	Vacuum coating device
	Ultra microtome

* Essential

national scientists carry out surveys to determine the occurrence and distribution of important groundnut virus diseases. The basic technology for such work could readily be prepared at ICRISAT and taken to the survey areas. This would include a supply of seed of diagnostic hosts, antisera for use with PHA and ELISA techniques and fixatives to prepare tissues for eventual electron microscopy.

Antisera can be stored for long periods at low temperature without considerable loss of their titers. Gluteraldehyde-fixed red blood cells can be held at room temperatures for at least a week, without impairing their suitability for sensitization; and if kept at low temperatures they are suitable for use in the PHA test after 3 months of storage.

If it were desired to test seeds or plant tissues for the presence of PMV, the ELISA technique could be employed. At ICRISAT, y-globulins and enzyme labelled y-globulins could be prepared and taken to the laboratory where tests were to be done. These preparations can be kept at room temperature for 10 days without damage and stored at low temperature for over a year.

Where no electron microscope facility is available locally, it would be possible to fix and embed plant tissues for later sectioning and examination at ICRISAT. Where no facilities for fixation and embedding exist, it would be sufficient to infiltrate portions of plant tissues with gluteraldehyde; this process being carried out at reduced atmosphere pressure. Such materials could be shipped to ICRISAT, or another laboratory with electron microscopy facilities.

Problems could arise where an important virus disease was of relatively restricted distribution and where no fully equipped virus laboratory was available to carry out virus purification and production of antisera.

Irrespective of the presence of a similar disease in India, it would not be possible for such work to be carried out at the ICRISAT Center because of plant quarantine laws prohibiting the importation of live viruses. This problem could be solved if virus laboratories in technically advanced countries where groundnuts are not grown could cooperate in purification and antisera production. A number of such laboratories have already shown interest in such cooperation.

Cooperation is also envisaged between virus laboratories in the exchange of antisera, seed of diagnostic hosts, and other materials useful in virus identification. Every effort should be made to expedite publication of research findings and in particular to make available data on new techniques.

An important part of the work of ICRISAT is the collection, recording and dissemination of research data and the provision of specialized training and opportunities for cooperative research. As already mentioned, papers are being prepared on the various techniques used in the groundnut virus research laboratory. Training can be given on these techniques and on other relevant techniques in the associated fields of entomology (identification and control of virus vectors), plant breeding (screening of germplasm and production of resistant cultivars) and cytogenetics (utilization of wild *Arachis* species as sou rces of resistance to virus diseases). It can also be arranged for virologists to make visits of varying duration to ICRISAT to discuss collaborative projects, acquire exper-

tise in specific techniques, or to process their own research materials.

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