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IDENTIFICATION AND CHARACTERISATION OF VIRUS DISEASE(S) OF GUAR IN SOUTH AFRICA

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A DISSERTATION SUBMITTED TO THE FACULTY OF SCIENCE, UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG, FOR THE DEGREE OF MASTER OF SCIENCE.

JOHANNESBURG 1989

ACKNOWLEDGEMENTS

I would like to thank my supervisors: Dr C Rey and Dr M M Sibara for their advice and encouragement during the execution of this work. Thanks also to Prof H Garnett for her advice. To Dr B Beck from White River for the supply of guar plants and seeds and to Mr G Pietersen of the Virology unit, Rietondale Research Station, Pretoria for the kind donation of seeds and antisera.

Thanks also due to Dr D Ariovich and Mrs V Harrera of the Microbiology department, Wits University, for their assistance.

I would like to acknowledge the financial assistance received in the form of the University Senior Bursary and the CSIR Bursary.

I would also like to thank Mrs J Visser for typing this manuscript.

Finally, I would like to thank my dear husband, Ran, for his continuous encouragement through this investigation.

DECLARATION

I declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

B. Ben Moshe

HEDVA BEN MOSHE

____ day of February 1989

ABSTRACT

Cyamopsis tetragonoloba (L) Taub., or guar, which is considered to be an important gum source for many industries, in Southern Africa, was found to exhibit symptoms of reduced leaf size, elongated green stems and sterility. These symptoms are unlike any other viral diseases reported in guar (Chester et al., 1944; Streets, 1948; Cooper, 1949 and Hansen and Lesemann, 1978) and have been named the "Green sterile" and "Little leaf" (B. Beck - pers. comm.) of guar.

The guar virus was characterised with regard to host range, symptomatology, transmission, physical properties, particle morphology, serology and molecular weight of the viral proteins. Based on these results the virus was identified as a member of potyvirus group. The causal agent has been identified as a flexuous viral particle (750 X 15nm), which is transmissible to several Phaseolus vulgaris plants by mechanical inoculation. Symptoms on indicator plants were mainly systemic red veins and chlorosis or hypersensitive I-gene reaction. Non-persistent aphid (Myzus persicae) (Sulzer) transmission was successful in some cases. Serological tests indicate a positive reaction with several potyvirus antisera, namely BCMV, BYMV, PVY and SMV. Polypeptide bands showed strong binding to these antisera. The molecular weight of the coat protein of guar virus was found to be 33,000 to 34,000 daltons, using PAGE.

LIST OF ABBREVIATIONS

Ab	-	Antibody
Ag	-	Antigen
BCMV	-	Bean common mosaic virus
BSA	-	Bovine serum albumin
BYMV	-	Bean yellow mosaic virus
CV.	-	Cultivar
CVP	-	Crystal violet pectate agar
DAS-ELISA	-	Double antibody sandwich ELISA
EDTA	-	Ethylenediamintetra - acetic acid
ELISA	-	Enzyme-linked immunosorbent assay
EM	-	Electron microscope
$F(ab')_2$	-	Antigen binding $F(ab')_2$ immunoglobulin fragments
Fig.	-	Figure
GAR	-	Goat-anti-rabbit
ICRISAT	-	International Crops Research Institute for the Semi-Arid Tropics
IEB	-	Immuno electron microscopy
IgG	-	Immuno globulin G
KB	-	King's medium B agar
mg	-	milligram
ml	-	millilitre
mm	-	millimetre
MS	-	Miller Schroth agar
nm	-	nano metre
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PBS-TPO	-	PBS-Tween-polyvinylpyrrolidone-Ovalbumin
PEG	-	Polyethylene glycol
Pers. comm.	-	personal communication
PTA	-	Phosphotungstic acid
PTA-ELISA	-	Plate Trapped Antigen ELISA

PVY	-	Potato virus Y
rpm	-	revolutions per minutes
SBMV	-	Southern bean mosaic virus
SDS	-	Sodium dodecyl sulphate
SMV	-	Soybean mosaic virus
TEM	-	Transmission electron microscopy
TEMED	-	N,N,N^1,N^1-tetramethyl-ethelene-diamine
TMV	-	Tobacco mosaic virus
TRSV	-	*Tobacco* ringspot virus
UA	-	Uranyl acetate
µg	-	micro-gram
µl	-	micro-litre
var.	-	variety
W/V	-	weight to volume
YDC	-	Yeast-extract-dextrose CaC_3 agar

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CHAPTER 1

INTRODUCTION AND REVIEW OF THE LITERATURE

Guar is the Hindi name for Cyamopsis tetragonoloba (L) Taub. The English name of the crop is Cluster bean.

Cyamopsis is thought to have originated in North Africa where several wild species exist (Hymowitz, 1972). The crop was taken to India and Pakistan, probably about 2 000 years ago, where guar has traditionally been used as a livestock feedstuff (Celanese report, 1984). It is grown in Africa for similar purposes but on a smaller scale. During the 1800's it was introduced into the southern United States of America where today approximately 300 000 hectares are under cultivation (Hansen and Lesemann, 1978). Commercial plantings of guar are currently made in India, Pakistan and the United States, principally for the production of the seed (Hymowitz, 1972).

1. GUAR PLANT

Guar is a summer annual leguminous shrub which may be cultivated in semi-arid regions. The guar plant varies in height from 0,5 to 3m, depending on the cultivar grown, the fertility of the soil and the climatic conditions.

Guar has a long tap root and nodules are found on both tap root and lateral roots. The stem is angular ribbed and hollow. The leaves are alternate, trifoliate with long petioles. The flowers are borne in dense, erect, axillary racemes. They are small, typically papilionaceous, varying in colour from purple to

white. Guar is largely self-pollinated. Elongated pods (50 to 75mm) appear along the main stem and are scattered throughout all the branches. The pods contain from 4 to 9 greyish-buff seeds. The gum, galactomannan, for which guar is prized, is contained in the large endosperm (Fig. 1), where it serves as a reserve food supply for the seedling when the seed germinates (Duke, 1981; Celanese report, 1984). The galactomannan polysaccharide of guar gum is a long chain, made up of mannose and galactose sugar molecules.

The endosperm weighs 35 to 40 per cent of the seed, whilst the embryo and cotyledons account for a further 45 to 50 per cent and the testa accounts for the remainder (Fig. 1).

1.1 Climatic requirements

The guar plant is drought-resistant. The crop grows best in areas with a minimum temperature of 27°C. High night temperatures, over 17°C are necessary to ripen the crop.

Guar requires 15 to 20 inches of rainfall during the growing season of the crop. Care must be taken not to provide excess water, as this markedly increases the susceptibility of guar to Alternaria leaf spot and bacterial blight (Duke, 1981; Celanese report, 1984; S.A. Institute for Crops and Pastures, 1975).

1.2 Soil requirements

Guar is grown best on fertile, medium to light sandy loam soils, with a pH greater than 6.0 and also on free draining subsoils.

It is recommended that guar seed is inoculated with Rhizobium inoculant of the cowpea type, group E (Celanese report, 1984).

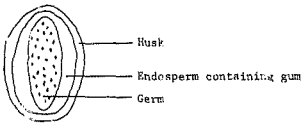


Figure 1: The wattle seed (S.A. Institute for Crops and Pasture, 1975)

The crop does require high levels of available phosphorous in the soil (Duke, 1981; Celanese report, 1984; S.A. Institute for Crop and Pastures, 1975).

1.3 Industrial potential

The valuable physical properties of guar gum only became known after the First World War. Previously, Carb-bean gum, animal glue and starches were the principal products relied upon for the purposes for which guar gum is now used (S.A. Institute for Crops and Pastures, 1975).

The gum, in the form of a mucilage, is increasingly being employed for recovery of uranium from sludges, in drilling for oil, in the paper, textile, pharmaceutical, food and other industries (Duke, 1981; Hymowitz, 1972). A by-product resulting after removal of the gum layer, is a very valuable, high protein feed supplement, used for all types of cattle feeding operations.

The United States has a limited production and most processors of guar are still dependent on Indian and Pakistan supplies for their requirements. Because of the steady progress in the local production of uranium, South Africa may become one of the world's principal users of guar gum. The government of South Africa has declared guar gum a strategic material (Chemserve Steinhall, 1986a; Celanese report, 1984).

1.4 Guar in South Africa

Guar was first used in South Africa in the late forties. At the time of the original uranium boom

guar replaced animal glue in filtration and extraction from slurries. South Africa leads the world in guar technology related to metallurgical applications (Chemserve Steinhall, 1986b).

Chemserve Steinhall is responsible for the local production of guar in South Africa. The agronomy programme includes the national states of KaNgwane, Lebowa, Gazankulu and Venda; as well as the white farming areas of the Lowveld. Seven hundred guar cultivars were imported and tested to select for drought and disease resistance through extensive field trials.

Apart from drought resistance and the advantageous nitrogen-fixing and soil-cleansing characteristics, the guar plant has good resistance to pests and diseases and production costs are generally low (Chemserve Steinhall, 1986a).

With the assistance of the KaNgwane Ministry of Agriculture, a demonstration farm of improved practices on black farmers' own land was established near Malekuti village (Eastern Transvaal). The company agronomist works with the farming families throughout the season and the farmer receives the entire crop after it has been harvested and weighed. In the first trials in 1983, there were only five farmers involved in the program, whilst in 1986 over 300 farmers were involved in the scheme (Chemserve Steinhall, 1986a).

1.5 Diseases of guar

The available literature describes bacterial and fungal pathogens of guar, but very limited information is presented on viral diseases (Mihail, 1982; Duke, 1981; Streets, 1968).

1.5.1 Viral diseases of guar:

1.5.1.1 Lethal virus of guar (Cyamopsis psoraloides).

A necrotic, lethal virus disease was found causing an estimated 75 per cent loss in an experimental field of guar in Oklahoma in 1944 (Chester et al., 1944). Symptoms comprised vein clearing, rolling, wilting, early stipple necrosis and abscission of young leaves; terminal necrosis, necrotic stem lesions, yellowing and abscission of older leaves; marked stunting of growth and ultimate death. The virus was transmissible mechanically. Symptoms were masked in hot weather, e.g. at 45 C (Streets, 1948).

1.5.1.2 Mosaic virus - unidentified

In 1944 this disease appeared in Maricopa County fields in the U.S.A. and caused losses in seed yield varying from 10 to 50 per cent (Streets, 1948).

The leaves showed a mosaic mottling as well as a reduction in the size of leaves and growth in general. The most striking symptom was the almost total suppression of seed pods. The virus was possibly spread by cotton aphid or melon aphid (Streets, 1948; Mihail, 1982).

1.5.1.3 Tobacco ringspot virus

First observed in 1944 in Oklahoma. The disease was reported as a new virus disease of guar and was later given the name "top necrosis" (Weiss, 1945; Cooper, 1949). The symptoms are top stem necrosis and light brown leaf lesions with dark margins.

1.5.1.4 Guar symptomless virus

Seeds from India, Pakistan, Belgian Congo and the U.S.A. contained a virus which induced characteristic red lesions on Chenopodium amaranticolor (Hansen and Lesemann, 1978). Since naturally infected and inoculated guar plants remained symptomless, the causal agent was named "guar symptomless virus". The virus belongs to the potyvirus group and was found to be transmitted through seeds (Mihail, 1982).

1.5.2 Bacterial and fungal diseases of guar

The principal bacterial and fungal diseases of guar are presented in Table 1.

1.6 Disease of guar in the Eastern Transvaal

Recently, disease symptoms appeared on guar plants in fields near White River in the Eastern Transvaal. These plants usually formed a few pods at the base of the stem. Thereafter flower formation took place normally, but the flowers rarely produced pods. From 300 seeds sampled, almost half (140) were distorted (50 per cent sterility) (Figs. 5 and 6). These sterile plants remained green long after normal plants had ripened. Leaf-size was also very much reduced compared to healthy one (Fig. 4). These symptoms were described by Dr B. Beck (pers. comm.) from White River and named "Green sterile" (Fig. 2) and "Little Leaf" (Fig. 3), respectively.

Table 1: Fungal and bacterial diseases of guar (Mihail, 1982)

Disease	Description	Causal agent
Bacterial Blight	Occurs mid-August when plants are flowering. Spread with monsoon in India and by rain. Leaf lesions: V-shaped with hydathode infection, dark circular spots moves from leaf to stem, acropetal defoliation, leaves with yellow ooze. Stem: linear brown/black streaks, internal discoloration, wilt. No fruit/flower symptoms.	Xanthomonas sp. X. cyamopsidis X. cyamophagus X. campestris
Leaf-spot	Gram-white bacteria with a single polar flagellum. Cause lesions 1-5mm diameter, round, dark border, light brown centre.	Pseudomonas syringae
Powdery Mildew	Endophytic; host specific Conidia 54 x 14.2. Occurs between August and October.	Oidiopis taurica Leveillula taurica
Anthracnose	Causes black spots on leaf and petioles. Produces acervuli with sete (dark purple); hyaline single celled conidia.	Colleototrichum capsici
	Produces yellow/green spots with dark center on leaves and pods. Seed borne.	Colleototrichum dematium f. truncata

Disease	Description	Causal agent
Wilt	Mycelial mat around stem in summer, wilt and root rot.	Sclerotium rolfsii
Alternaria leaf spot	Circular brown spots 3 to 6 diameter on leaves. Black flecks and oval spots on stem. Shriveled darkened seeds, 100 per cent infection. Seed borne.	Alternaria cucumerina Alternaria cyamopsidis Alternaria tenuisima
Stem and Root Rot	Water soaked lesions near hypocotyl.	Pythium myriotylum Pythium debaryanum
Root Rot	Causes root rot, leaf and stem blight.	Fusarium caeruleum
Streak	Black streak on stem, no flowering or pod set.	Fusarium moniliformi
Wilt	Yellowing, wilting. Roots with black lesions at soil line with internal brown discoloration.	Fusarium solani
Root Rot	Dark coalescing lesions on the roots. Death of plants.	Rhizoctonia sp.
Root Rot	Root-rot, wilting and death of plant in late summer.	Phymatotrichum omnivorum

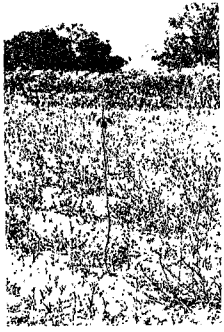


Figure 2: Infected guar var. ZDPS exhibiting green sterility symptoms in the field. Elongated green stem (EG) and few influorescences (arrow)



Figure 3: Infected guar var. ZDPS exhibiting "Little leaf" (LL) symptoms



Figure 4: Healthy guar var. ZDPS exhibiting normal-sized leaves (arrows)



Figure 5: Infected pod of guar showing two healthy (H) and two infected (I) seeds

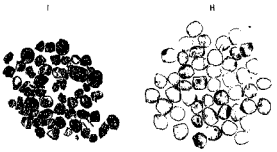


Figure 6: Infected (I) seeds of guar in comparison to healthy (H) seeds

2. PIGEONPEA IN SOUTH AFRICA

During a collection tour by ICRISAT (India) staff in South Africa, pigeonpea samples were gathered. Pigeonpea was found to be of rare occurrence. The southern most distribution of pigeonpea on the African continent is in locations mainly in the lower altitude areas of East and North-east Transvaal and Natal. Collected samples were found growing as an escape along the road and other samples were obtained from hedges or single plants in farm plots near houses (Van der Maesen, 1980).

Pigeonpea (Cajanus cajani (L.) Millsp.) plants showing symptoms of mosaic and sterility (Figs. 7 and 8), were brought from the Eastern Transvaal by Dr B Beck. The pigonpea plants were found growing in the edges of guar field near White River and showed similar sterility symptoms to the guar plants, but more severe. From 78 seeds sampled, 72 seeds were distorted (92 per cent sterility) (Fig. 9).

2.1 Sterility mosaic in pigeonpea

Sterility mosaic is one of the major disease problems of pigeonpea in the Indian subcontinent. The incidence of the disease seems to be on the increase and needs immediate remedial measures (Kannaiyan et al., 1981). The disease has so far been reported only from Burma, India and Thailand. Typical symptoms are mild mosaic, accompanied by little or no flowering at all.

Sterility mosaic is suspected to be caused by a virus and spread by the eriophid mite Aceria cajani Channalasavanna (Nene, 1972). The disease

spreads through infective mites disseminated by wind. After purification, long, thin, flexuous, rod-shaped, virus-like particles were observed, always in very low numbers. Sap transmission of the causal agent was unsuccessful (Reddy and Nene, 1981).

Development of resistant cultivars appears to be the most effective method of control. To meet this objective, a large-scale field screening program was undertaken in 1975 at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT Centre) and several resistant lines have been identified (Nene and Reddy, 1976; Nene et al., 1981).

3. VIRUS DISEASES OF BEANS

Bean common mosaic virus (BCMV), Bean yellow mosaic virus (BYMV), Southern bean mosaic virus (SBMV) and Soybean mosaic virus (SMV) represent some of the most important pathogens that infect beans worldwide (Table 2).

Schwartz and Galvez (1980) have shown that large yield losses can occur in beans affected by viruses such as BCMV and BYMV.

Viruses of beans have the potential for rapid spread and have established themselves worldwide due to their variable modes of transmission, e.g. mechanical transmission, transmission by vectors such as aphids, whitefly, beetles and leafhoppers or through seeds.

Chemical control strategies aimed at vectors, seed certification schemes and breeding for resistance can effectively control bean viruses (Neergaard, 1979).



Figure 7: Pigeonpea exhibiting sterile stem (S) with almost no pods



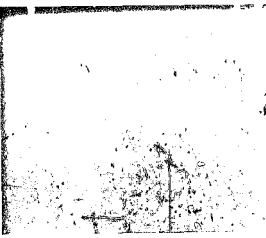
Figure 8: Pigeonpea leaves exhibiting mosaic (M) symptoms (arrows)



Figure 9: Infected seeds of Pigeonpea

Table 2: Virus diseases affecting beans (adapted from CMI/AAB descriptions from 1972 to 1984)

VIRUS	PARTICLE STRUCTURE AND SIZE	MODE OF TRANSMISSION	HOST RANGE	SYMPTOMOLOGY	GEOGRAPHICAL DISTRIBUTION
Bean diseases caused by filamentous particles					
Bean common mosaic virus	Flexuous filament 750nm long & 15nm wide	Sap, seed-borne, pollen-borne, transmitted non-persistently by several aphid species.	Restricted to Phaseolus spp. but can be transmitted to some members of the Chenopodiaceae and Solanaceae. Diagnostic species: Phaseolus vulgaris.	Causes 'common mosaic' which is associated with leaf malformation and rolling and 'black root' which is characterised by vascular necrosis and plant death. Severity of the symptoms depends on cultivar time of infection and environmental conditions.	World-wide
Bean yellow mosaic virus	Flexuous particle 750nm long	Sap and by many aphid species in a non-persistent manner	Infects leguminous plants and several non-legumes. Diagnostic spp.:- Phaseolus vulgaris, Pisum sativum, Vicia faba, Chenopodium amaranticolor, C. quinoa, Gomphrena globosa, Nicotiana tabacum, Petunia hybrida, Spinacia oleracea, Tetragonia expansa.	Causes mosaic disease in many legumes, apical necrosis in French bean, 'streak' in pea, rosette in Ornithopus sativus and white streak or latent infection in Gladiolus.	World-wide
Beet mosaic virus	Flexuous filament 730nm by 13nm.	Sap and by aphids in a non-persistent manner.	Host range fairly wide, mainly in the Chenopodiaceae, Solanaceae and Leguminosae. Diagnostic spp. Beta vulgaris, Spinacea oleracea.	Causes a mosaic disease.	World-wide
Clover yellow vein virus	Filamentous 760nm long	Sap and by aphids from various genera in a non-persistent manner.	Infects several species in the leguminosae and in 5 other families. Diagnostic spp.:- Chenopodium amaranticolor, C. quinoa, Nicotiana tabacum, N. clevelandii, Phaseolus vulgaris, Tetragonia expansa.	Causes veinal yellowing and mottling.	Britain, Canada



	PARTICLE STRUCTURE AND SIZE	MODE OF TRANSMISSION	HOST RANGE	SYMPTOMOLOGY	GEOGRAPHICAL DISTRIBUTION
[illegible]	Flexuous filament 750nm long.	Transmitted by several common aphid species. Seed-borne in cowpea.	Infects many species in Leguminosae and also members of the Amaranthaceae, Chenopodiaceae, Cucurbitaceae, Labiatae and Solanaceae. Diagnostic spp.:- Phaseolus vulgaris, Chenopodium amaranticolor, Glycine max, Ocimum basilicum, Petunia hybrida, Pisum sativum.	Causes severe mosaic of cowpea with vein banding, interveinal chlorosis leaf distortion, blistering and stunting.	Africa, Europe, Asia and U.S.A.
[illegible]	Flexuous particle [illegible] long.	Seed, sap, no known vectors.	Infects many different hosts, including members of the genus Phaseolus.	Causes mild leaf mottle, but occasionally severe systemic necrosis and chlorosis in cowpeas.	Eastern region of Ghana.
[illegible]	[illegible] particle [illegible]	Sap and by at least two species of aphids in a non-persistant manner.	Wide range of hosts, particularly in the Leguminosae. Diagnostic spp.:- Passiflora edulis, Phaseolus lathyroides and Phaseolus vulgaris.	Causes mosaics, ringspots, rugosity and distortion of leaves. Occurs naturally in P. vulgaris, Glycine max and other tropical legumes, but is of minor importance in these species.	Queensland, New South Wales, Western Australia and Surinam.
[illegible]	Flexuous filament [illegible] 750nm long	Sap and by several common aphid species. Is seed-borne in groundnuts.	Host range appears to be restricted to members of the Leguminosae. Diagnostic spp.:- Phaseolus vulgaris, Arachis hypogaea, Glycine max, Cassia occidentalis, Nicotiana clevelandii, Chenopodium amaranticolor.	Causes systematic mottling and necrosis.	S.E. USA, East Africa, N.E. Australia, Japan, West Malaysia, South America and Europe.

VIRUS	PARTICLE STRUCTURE AND SIZE	MODE OF TRANSMISSION	HOST RANGE	SYMPTOMOLOGY	GEOGRAPHICAL DISTRIBUTION
Potato virus T	Flexuous filament 640nm x 12nm	Sap	Limited host range, most susceptible species in Leguminosae, Solanaceae and Chenopodiaceae. Diagnostic spp.:- Phaseolus vulgaris, Chenopodium amaranticolor, C. quinoa, Datura stramonium, Nicotiana debneyi.	Causes chlorotic or necrotic local lesions.	Peru and Bolivia.
Potato virus Y	Flexuous particle 730nm x 11nm	Sap, aphids.	Host range limited to Solanaceae, Amaranthaceae, Chenopodiaceae, Compositae, Leguminosae.	Mild to severe mottle, streak, necrosis of veins, necrotic spots, infection variable depending on strains and plant host.	World-wide
Red clover vein mosaic virus	Tubular filamincus particle: 645nm long and 12nm wide (645 x 12nm)	Sap and transmitted by aphids in a non-persistent manner. Not common in seed.	Host range restricted to Amaranthaceae, Chenopodiaceae, Leguminosae and Solanaceae. Diagnostic spp.:- Chenopodium amaranticolor, C. quinoa, Gomphrena globosa, Pisum sativum, Trifolium dubium, T. pratense, Vicia faba and V. sativa.	Causes vein mosaic, streaking mosaic and stunting in various legumes.	Europe, North America and South Africa.

VIRUS	PARTICLE STRUCTURE AND SIZE	MODE OF TRANSMISSION	HOST RANGE	SYMPTOMOLOGY	GEOGRAPHICAL DISTRIBUTION
Soybean mosaic virus	Flexuous filament 750nm long	Sap, borne by seeds of Glycine max. transmitted non-persistently by many aphid spp.	Transmitted to Glycine max and members of the genera Phaseolus and Chenopodium. Diagnostic spp. Glycine max, Phaseolus vulgaris, P. lathyroides, Chenopodium album and C. quinoa.	Most varieties of Glycine max develop transient systemic vein-clearing followed by a rolling or distorting mosaic in younger leaves with dark green. Later puffed areas along the main veins and chlorosis between the dark coloured areas. Plants usually slightly stunted, pods are fewer, sometimes malformed. Some varieties develop progressive necrosis.	World-wide
White clover mosaic virus	Flexuous filament 740nm long and 13nm wide	Sap	Restricted to the Leguminosae. Diagnostic spp.:- Trifolium spp., Phaseolus vulgaris, Vicia faba, Vigna sinensis, Pisum sativum, Cucumis sativus.	Causes mosaic and mottle of different degrees and vein clearing.	North America, Europe and New Zealand.

4. TECHNIQUES USED FOR VIRUS IDENTIFICATION AND CHARACTERISATION

There are several basic procedures to follow when an unknown virus disease is being investigated (Hamilton et al., 1981). In the initial diagnostic phase one determines to which group the virus belongs. If the virus under investigation is not easily assigned to any known taxon, the diagnostic phase passes into the full descriptive phase in which every property possible is investigated.

The information sought during both the diagnostic and descriptive phases has been divided into two sections: firstly, what can be learned from crude virus preparations and, secondly, from purified virus preparations.

Using crude virus preparations the following properties of the virus may be elucidated:

(a) reproduction of the disease, using the isolated virus;
(b) host range studies and symptom expression;
(c) mode of transmission;
(d) electron microscopy, structure and size;
(e) biological properties;
(f) reactions with likely antisera.

Using purified preparations the following properties of the virus may be elucidated:

(a) fine structure of the particles in the electron microscope;
(b) serology;
(c) sedimentation properties;
(d) analysis of the nucleic acid and the coat protein.

(Hamilton et al., 1981; Bock, 1982)

By using these procedures, it can be determined if the virus concerned is a "new" virus or is identical with any known virus.

4.1 Host-range and symptoms

Most viruses that have been studied carefully are found to infect at least 30 different plant species. It is not known what determines the host range of a virus.

The host range of a virus and details of the symptoms it causes are specific characteristics of the virus and these characteristics are often used, together with others, to identify the virus (Gibbs and Harrison, 1976). However, according to Hamilton, et al. (1981), this is generally not a very precise or reliable guide to virus identification.

Preliminary host range studies are useful in discovering convenient laboratory hosts for subsequent virus multiplication, purification and infectivity tests (Hamilton, et al., 1981).

Most of the bean viruses (Table 2) are transmitted by inoculation of sap to members of the leguminosae primarily Phaseolus vulgaris species, as well as members of the genera Chenopodium and Nicotiana. The potyvirus group, which is the largest group among plant viruses, is transmissible by inoculation of sap to many leguminous species.

Host specificity is extremely important in infection and if its molecular basis was understood, host specificity could be of great importance in the control of plant disease (de Zoeten, 1981). Atabekov (1975)

has described the present status of research in the area of host specificity of plant viruses.

Many strains of a virus may have a very similar host range, while others may differ considerably. For example, many strains of TMV infect a wide range of hosts in the Solanaceae and other families (Matthews, 1970).

Changes ensuing from viral infection, however minor, may have a bearing on the physiology and/or morphology of the host, leading to a variety of abnormalities, the most readily perceptible of which are "symptoms", i.e. the outward visible expression of the disease (Giovanni and Marcello, 1985).

As a rule it is in the foliage of a plant that the most noticeable symptoms develop and the effects of different viruses on the leaves take many forms: mosaic diseases, ringspots, clearing of veins, leaf rolling, distortion and malformation (Smith, 1977). In addition, other symptoms such as necrosis, growth reduction, decreased or halting of flowering and seed production and tumours on the roots may occur (Bos, 1978).

Virus infections are influenced by several interacting variables, the majority of which are the virus genome, the host genome and the environment (Giovanni and Marcello, 1985). Sometimes many different strains of a virus occur and these may cause quite different kinds of disease in the same host plant under the same conditions. Plant factors, such as age and genotype, or environmental factors (e.g. light, temperature, water supply, humidity, nutrition, time of day or time of year) may also affect symptom development (Matthews, 1970).

4.2 Transmission studies

4.2.1 Mechanical transmission

Mechanical inoculation produces small temporary wounds in the plant through which virus particles can enter and infect the cells. Gentle abrasive powders, such as carborundum or celite, are often used to increase the number of infections produced (Stephen, 1984). The viruses that are most readily transmitted by mechanical inoculation are those that reach the largest concentrations in plants and can infect the epidermis as well as other tissues (Matthews, 1970; Gibbs and Harrison, 1976). The success of mechanical inoculation depends on numerous factors, including virus purity and stability, pH and ionic strength of inoculum, type, age and physiological condition of the host and also environmental conditions before or after inoculation. Such environmental conditions may include keeping plants in the dark or in warm air before inoculation (Stevens, 1983; Kado, 1972).

4.2.2 Transmission by aphids

Aphids constitute the largest group of known plant virus vectors. Viruses transmitted by aphids can usefully be separated into groups, differing in the length of time the viruses persist in the aphids (Table 3).

Table 3: Comparison between non-persistent and persistent viruses (Gibbs and Harrison, 1976)

Terms	Non-Persistent viruses (*Stylet-borne*)	Persistent viruses (circulative)
Persistence	Persisting for a few hours (usually less than four)	Persisting for more than 100 hours, in some instances for the life of the vector.
Acquisition and inoculation feeding periods	Seconds	Hours
Latent period	None	12 hours or more
Location of virus in the vector	Viruses carried on the *stylets of* the insect.	Viruses which pass through the gut wall into the haemolymph of the vector and back to salivary glands *before* the insect becomes infective.
Location of virus in the plant	The virus is acquired from and inoculated to cells of epidermis.	The virus is concentrated in the *phloem of* the plant.

Terms	Non-Persistent viruses (Stylet-borne)	Persistent viruses (circulative)
Transmission	Mechanically transmissible.	Not mechanically transmissible.
Symptoms	Mosaic symptoms.	Leaf-rolling and yellowing symptoms.
Specificity	Specificity between the virus and species of vector is not developed. Usually one virus is transmitted by several or many aphid species.	Transmitted by only one or a few aphid species.

4.2.3 Seed transmission

Most viruses seem not to be transmitted through true seed or pollen, but at least thirty are transmitted in this way. With most virus/host combinations the proportion of seedlings infected through the seed is usually small, but in some instances, e.g. BCMV, up to 83 per cent of the seeds of diseased plants may be infected (Reddick and Stewart, 1919).

Many factors affect seed transmission (Shepherd, 1972):

(a) Environmental factors - Temperature is the most important environmental factor influencing transmission.

(b) Host and virus genotype.

(c) Infection timing - for seed transmission to occur, plants must in general be infected before the ovules are fertilised.

Viruses can invade the seed embryo (e.g. Prunus necrotic ringspot virus) or are found in and on the testa (e.g. TMV). Not all viruses cause symptoms in the plants they infect through the seed (Bos, 1977). The reason for the failure of most plant viruses to be transmitted through seed is their inability to replicate and their degradation in meristematic tissue. They also appear not to be able to enter the seed if they are restricted to the phloem (Gibbs and Harrison, 1976).

4.3 Purification procedures

Purification is often the major stumbling block in virus characterisation. Purified preparations are used for characterisation by electron microscopy, serology, determination of the physicochemical properties of the virus, its protein and nucleic acid components and for the production of high quality antisera (Hamilton, 1981). There are as many purification procedures as there are viruses which have been purified (Franki, 1972). Various methods used for purifying plant viruses have been described (Steere, 1959; Brakke, 1967 a,b; Ackers and Steer, 1967; Venekamp, 1972; Schumaker and Rees, 1972; Noordam, 1973). The object of purification is to produce a preparation containing only infective virus particles, but this is rarely achieved (Gibbs and Harrison, 1976). It is important to choose a host plant in which the virus reaches a high concentration (Smith, 1977).

The main steps in the purification of a plant virus are:

(a) Extraction of the sap: infected tissue is usually homogenised in a blender in the presence of a suitable buffer. Buffers that are commonly used are phosphate, citrate, borate and EDTA.

In order to preserve particle integrity and infectivity, agents such as sodium sulfite, mercaptoethanol, ascorbic acid and polyphenol-oxidase inhibitors are added (Van Regenmortal, 1982).

(b) Clarification of crude sap to remove host plant material such as treatment with chloroform, diethyether, carbon tetrachloride, n-butanol or ethanol.

(c) Concentration of the virus usually performed by ultracentrifugation or precipitation with polyethylene glycol or salts.

4.4 Preservation of virus material

Once a virus has been propagated and purified, it is important to store it under conditions in which its infectivity and antigenic properties remain unaltered (Van Regenmortel, 1982). The methods that were found most successful are based on desiccation, freezing, or lyophilisation of virus material (McKinney and Silber, 1968; Hollings and Stone, 1970; Grivell et al., 1971; Purcifull et al., 1975).

The addition of 1 per cent sodium azide to leaf tissue has been shown to protect viruses against microbial

degradation and to preserve their antigenicity (Gooding and Tsakiridis, 1971). Many plant viruses can be preserved for many years in small pieces of infected leaf material dried and stored over calcium chloride in stoppered bottles at 4°C (Bos, 1977; Bos and Benetti, 1979).

Freezing infected tissue or purified virus preparations at -20°C, or in dry ice or liquid nitrogen, has also been used successfully with many viruses (Rochow et al., 1971; Best, 1961; Silber and Burk, 1965). Addition of 10 per cent glycerol to purified preparations before freezing preserves the antigenic activity of some viruses (Paul and Querfurth, 1979). Lyophilisation of infected plant sap in the presence of glucose and peptone, followed by storage under vacuum at room temperature, has also been used (Hollings and Stone, 1970).

4.5 Electron microscopy

Electron microscope studies of crude extracts or purified preparations are the most important first steps in the identification of a plant virus and a great deal of subsequent investigations will depend on these results (Bock, 1982). According to Brandes and Berck (1965) viruses with elongated particles are easily assigned to a taxonomic group, because the size range of most groups of such viruses is sufficiently distinct from that of other groups. Spherical virus particles in crude extracts are very difficult to see under the electron microscope. Determination of particle size can be made from crude plant extracts, using the leaf dip method or after purification. The advantage of measurements employing crude plant

extracts is to avoid breakage of particles that often occurs after purification (Hitchborn and Hills, 1965).

4.5.1 Negative staining

The negative staining technique was first introduced by Hall (1955) and developed by Brenner and Horne (1959) and Almeida and Howatson (1963). Examination of preparation by electron microscopy, using the negative staining procedure, is one of the fastest ways to assign a particular virus to a taxonomic group (Horne, 1967). The most widely used negative stains are solutions of sodium phosphotungstate, uranyl acetate, uranyl formate, ammonium molybdate or sodium silicotungstate (Gibbs and Harrison, 1976). The particles of some viruses are destroyed or damaged in Phosphotungatic acid (PTA) (Hamilton et al., 1981). The essence of the process is that the stain does not react with the particle but merely surrounds it, outlining it as electron-dense material (Milne, 1972).

4.6 Inclusion bodies

A major cytological effect of virus infection is the development of inclusion bodies. They are located in either the cytoplasm or the nucleus of infected cells (Martelli and Russo, 1977). The type of inclusions formed is characteristic of the particular virus, but one virus may induce the formation of several different types of inclusion, even in the same cell (Gibbs and Harrison, 1976). Many viruses do not form inclusion bodies (Rubio-Huertos, 1972). Inclusion bodies are visible by light microscopy but electron microscopy examination has given a new insight into ultrastructure and composition of the viral inclusion bodies (Matthews, 1970).

Four general types of inclusion bodies are recognised (Smith, 1977; Bos, 1978):

(a) amorphous bodies, or "X" bodies;
(b) crystalline inclusion;
(c) abnormal cell organelles;
(d) inclusions usually seen only by electron microscopy that consist of viral coat protein or other unidentified protein.

Various normal structures may resemble virus-induced inclusion (Newcomb, 1967; Price and Thompson, 1967; Marinos, 1967). Pinewheel inclusions are characteristic for many Potyviruses (Edwardson, 1966; Edwardson et al., 1968; Edwardson, 1974).

4.7 Enzyme-linked immunosorbent assay (ELISA)

ELISA technique is used as a routine detection of plant viruses. The ELISA test has the following advantages (Stephen, 1984):

(a) Extreme sensitivity - allows the detection of as little as 1-10ng/ml virus (Clark et al., 1967a; Gugerli, 1978; Reeves et al., 1978; Devergne et al., 1978; Voller et al., 1976).

(b) Applicability to large numbers of samples, as investigations of the incidence of virus infection in crops (Thresh et al., 1977).

(c) Economy in use of high cost antisera.

(d) Semi-automatable.

(e) Quantitative (Clark, 1981; Jaegle and Van Regenmortel, 1985; Marco and Cohen, 1979).

(f) Independent of virus morphology.

(g) Independent of virus concentration.

Typical applications include the routine indexing of *fruit trees* in the field for the presence of Apple mosaic virus (Clark et al., 1976b), Plum pox virus (Adams, 1978), Prunus *necrotic* ringspot virus (Barbara et al., 1978) and Citrus tristeza virus (Bar-Joseph et al., 1979); testing of potato *leaves, tubers* and sprouts for the presence of Potato virus Y and Potato virus A (Maat and De Bokx, 1978b), Potato leaf roll virus (Maat and De Bokx, 1978a; Gugerli, 1980; Tamada and Harrison, 1980) and Potato virus X (De Bokx et al., 1980); and detection of Tobacco ringspot virus in individual soybean seed (Lister, 1978).

There are basically three enzyme-linked immunosorbent assays that have become *established* in plant virology:

4.7.1 The double antibody sandwich (DAS) ELISA

Clark and Adams (1977) described the standard double antibody sandwich (DAS) ELISA technique. Virus-specific antibodies are adsorbed to a solid surface, such as the wells of a polystyrene Microtiter plate. A test sample, suspected to contain the viral antigen, is incubated with the antibody-sensitised solid phase. Viral antigens recognised by the antibodies are *serologically bound*. Enzyme-labeled specific antibodies are added after the test sample is removed. The labeled antibodies *bind* to the viral antigen already

bound to the coating antibodies and form the double antibody sandwich. Enzyme substrate is added. The colour change in the substrate is proportional to the amount of enzyme present, which in turn is proportional to antigen concentration (Lax-Joseph and Garnsey, 1981).

DAS ELISA is extremely sensitive, but is also very strain specific (Koenig, 1978; Barbara et al., 1978; Lister and Rochow, 1979; Rochow and Carmichael, 1979; *Bar-Joseph and* Salmon, 1980; Uyemoto, 1980). The DAS ELISA method is preferred when separate detection of serologically related viruses in mixed infections is required (Koenig and Paul, 1982a).

4.7.2 Indirect *ELISA*

The indirect ELISA was introduced by Voller and Bidwell (1977) and has been used by many virologists, including Rybicki and Von Wechmar (1981), Koenig (1981) and Bar-Joseph et al. (1979). Indirect *ELISA systems* follow a double antibody sandwich process, except that the second layer of specific antibody raised in rabbit is not enzyme labelled. The label is introduced as a conjugated anti-rabbit gamma globulin in a further step. The indirect ELISA overcomes the extreme specificity of DAS ELISA and is useful for the detection of distant serological relationships which may not be demonstrable by any precipitin test (Rybicki and Von Wechmar, 1985; Lommel et al., 1982; Koenig and Paul, 1982b).

4.7.3 Compound or sandwich indirect ELISA ($F(ab')_2$ELISA)

This ELISA modification introduced by Barbara and Clark (1982) and is used by Bar-Joseph and Malkinson (1980) and Van Regenmortel and Burchard (1980). This technique combines the advantages of an indirect assay with those of DAS-ELISA. Antigen is trapped on a solid phase as in DAS Eliza but the $F(ab')_2$ part of the gamma globulin molecule is used. The trapped virus is detected, using unlabelled gamma globulin and this in turn is detected, using an immunoglobulin-based enzyme conjugate specific for the Fc portion of the IgG.

4.7.4 Detection of virus in aphids by ELISA

ELISA can be used to detect viral antigen in aphids, e.g. Cucumber mosaic virus (Cera et al., 1978), Pea enation mosaic virus (Fargette et al., 1981), Potato leafroll virus (Clarke et al., 1980; Tamada and Harrison, 1981), Potato virus Y (Raccah et al., 1981) and Maize rayado fino virus has been detected by ELISA in the leafhopper Dalbulus maidis (Rivera et al., 1981).

4.8 Polyacrylamide gel Electrophoresis (PAGE)

SDS-PAGE is the principal technique used to determine the number of viral polypeptides and their molecular weights (Laemmli, 1970; Maizel, 1971). The problem of protein degradation during virus purification and storage is especially significant in its effect on estimates of polypeptide number by SDS-PAGE (Koenig et al., 1970; Hiebert and Mcdonald, 1973; Al Ani et al., 1979). The molecular weight of an unknown protein is estimated by comparing its mobility with those of other

proteins of known molecular weight (Shapiro et al., 1967; Weber et al., 1972; Alper et al., 1984). 10 to 50µg of proteins of purified virus produce distinct bands using SDS-PAGE (Paul, 1974). SDS increases the resolving power of bands and prevents zone dispersion (Chen and Chramback, 1980).

5. AIMS

The aim of this investigation was to ascertain the causal agent of the disease of guar bean whose symptoms of growth reduction, "little leaf" and sterility were suspected to be of a viral nature, since preliminary tests showed no signs of a bacterial infection.

The objectives of the present study were to

(1) establish whether bacteria were responsible for the disease;

(2) confirm the presence of the virus(es) by indicator studies, i.e. transmission of the virus by aphids and mechanical inoculation to host plants;

(3) detect the virus, using ELISA procedures with different antisera to common virus diseases of beans and to identify the group to which guar virus is serologically related;

(4) purify the virus, following determination of particle size and shape in the electron microscope as well as molecular weights of viral proteins, using polyacrylamide gel electrophoresis;

(5) Ascertain if the guar virus had any resemblance to the causal agent of sterility mosaic in pigeonpea.

CHAPTER 2

MATERIALS AND METHODS

1. CONDITIONS FOR GROWTH OF GUAR

The guar plants were grown in a mixture of sterilised soil, compost and vermiculate in a ratio of 1:1:1. The seeds were planted in soil not deeper than 0.3cm. The pots were 30cm deep to allow the long root of guar to develop. After the seeds germinated, about seven days later, the seedlings were watered with a suspension of Rhizobium cowpea E group (Appendix 1). The plants were watered only twice a week because excess water increases susceptibility to diseases and regularly supplied with essential nutrients by the application of a balanced mineral solution ("Chemicult"). The guar plants were kept in a growth cabinet with a temperature of 28°C and 60 per cent humidity.

2. PLANT MATERIAL

Leaf material showing symptoms (Figs. 2 and 3) was collected from fields of guar near White River in the Eastern Transvaal (Table 4). Leaf material which showed no virus symptoms in the field ("Healthy") was also collected (Fig. 4) (Table 5). In addition, seeds suspected of being infected with virus and healthy seeds were provided by Dr Brian Beck.

Table 4: Infected guar varieties

Varieties	Symptoms
Tx-79-2741	"Little leaf"
Tx-70-2741	"Little leaf" + sterility
HSB-130	Green sterile
G-120	Green sterile
SL-100	Green sterile
Lewis	Green sterile
ZDPS	Green sterile
ZDPS	"Little leaf"

Table 5: Healthy guar varieties

Varieties		Percentage germination
201	Lewis	88
202	TX-78-3726	72
203	TX-78-3695	76
204	TX-71030-4-1-2-2B	84
205	TX-78-3337	60
206	TX-77-3347	80
207	HSB-130	52
208	HFG-75	36
209	HFG-363	68
210	Esser	64
211	ZDJ	64
212	G120	60
213	TX-79-2741	36
214	SL-100	68
215	ZDPS	84.6
216	Z-207-1-1	76
217	TX-76-3285	55.5
218	Katch-8	96

3. IDENTIFICATION OF BACTERIA

The procedure followed for identification of bacteria is depicted in Fig. 10 (Schaad, 1980):

(a) Growth on common bacteriological media
Small sections of infected guar leaves TX 79-2741 were sterilised by placing them in 20 per cent Formalin for 10 minutes and then in 70 per cent Alcohol for 15 minutes. After rinsing in sterile water, the sections were placed onto nutrient agar media (Appendix II). Plates were observed for bacteriological growth.

(b) Gram stain
The colonies grown on nutrient agar were stained with Gram-stain (Appendix II).

(c) Selective media
1. YDC agar
Colonies were grown on YDC agar (Appendix II) and observed for colour of colonies.

2. KB agar
Colonies were grown in KB agar (Appendix II) and observed for fluorescent pigment.

4. TRANSMISSION STUDIES

4.1 Indicator plants

Indicator plants are used to confirm the presence of a virus in another plant which shows symptoms. They are also useful in studying the host range of a virus. The virus must be laboratory transmissible, either by sap or insects.

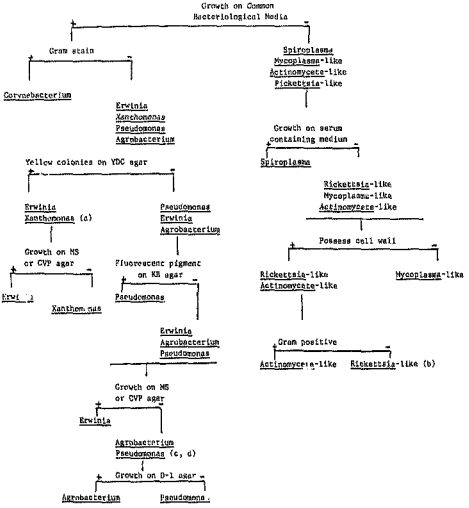


Figure 10: Differentiation of commonly isolated genera of plant pathogenic bacteria (adapted from Schaad, 1980)

a Colonies usually mucoid
b *Usually observed under phase optics only*
c Some Pseudomonas will grow on N.S. colonies differ in colour
d Pectolytic forms will grow on CVP but differ in growth characteristics

Four types of indicator plants are desirable (Gibbs and Harrison, 1976):-

One in which a stock culture of the virus can be maintained for long periods; a second in which characteristic symptoms are produced and which can be used to aid detection and diagnosis; a third which is a source of large amounts of virus; and a fourth for assaying infectivity, preferably by counting visible primary infections.

In this host study plants were chosen on the basis of their susceptibility to viral infection. The families Leguminosae, Solanaceae, Labiatae, Chenopodiaceae and Curcubitaceae are "susceptible families" (Hollings, 1966). The list of indicator plants used in this investigation are presented in Table 6.

Table 6: List of indicator plants used in the determination of host range

Indicator plant	Family
Nicotiana clevelandii Gray	Solanaceae
Nicotiana glutinosa L.	Solanaceae
Nicotiana tabacum var. Samsun L.	Solanaceae
Datura stromonium L.	Solanaceae
Petunia hybrida Vilm.	Solanaceae
Pisum sativum L. var. Greenfeast	Leguminosae
Chenopodium amaranticolor	Chenopodiacea
Chenopodium quinoa	Chenopodiacea
Lycopersicum esculentum L.	Solanaceae
Vicia faba L.	Leguminosae
Glycine max L.	Leguminosae
Lupinus albus	Leguminosae

Indicator plant	Family
Vigna unguiculata var. Black eye	Leguminosae
Phaseolus vulgaris L.	Leguminosae
var. The Prince	Leguminosae
var. Bountiful	Leguminosae
var. Double white setrler	Leguminosae
var. Top crop	Leguminosae
var. *Seminole*	Leguminosae
var. Contender	Leguminosae
var. Canadian wonder	Leguminosae
var. Wintergreen	Leguminosae
var. Golden podded wax	Leguminosae
var. Monroe	Leguminosae
var. Natal round yellow sugar	Leguminosae
var. Genuine cornfield	Leguminosae
var. Lazy housewife	Leguminosae
var. Aman...	Leguminosae
var. Nep 2	Leguminosae
var. Peru 0251	Leguminosae
var. Sanilac	Leguminosae
var. Diacol calima	Leguminosae
var. Pinto	Leguminosae
Phaseolus sp. (broad beans)	Leguminosae
var. Aquadulce	
Phaseolus sp. (runner beans)	
var. Blue peter	Leguminosae
var. Abundance	Leguminosae
var. Everybearing	Leguminosae

4.2 Mecahnical inoculation

Infected guar leaves vars. TX-79-2741, HSB-130, G-120, SL-100, Lewis and ZDPS showing symptoms of sterility and "little leaf" (Table 4) were macerated in 2-5 parts (W/V) 0.01M potassium phosphate buffer pH 7.0 (Appendix III) and a small amount of celite (50 to 100mg/ml) in a mortar and pestle. This inoculum was then gently rubbed over the surface of susceptible (10 to 14 day-old first trifoliate leaves) indicator plants (Table 6) as well as healthy guar plants. After inoculation, leaves were rinsed under a slow stream of distilled water to increase the points of entry of the virus (Smith, 1977). Inoculated plants were kept in temperatures of 25°C and 30°C. Infected leaves were harvested 3 weeks after the inoculation of primary leaves. As control, healthy indicator plants were rubbed with distilled water and celite.

4.3 Aphid transmission

According to Böck (1985) the guar virus could not be transmitted by white fly (Bemisia tabaci). In this investigation transmission studies were performed with Myzus persicae (Sulzer), which is the vector of 50 different viruses (Smith, 1977).

Aphids reared on healthy chinese cabbage plants in an insect cage. The plants were kept at temperatures between 15 to 20°C and continuous illumination. These conditions suppress the development of winged aphids which are not easy to handle and not such efficient vectors (Stephen, 1984).

4.3.1 Non-persistent aphid transmission

Virus-free aphids were brushed into a petri-dish, starved for 1 hour in a cool shaded place and given an acquisition period of 2 minutes on infected guar var. TX-79-2741. Aphids were then transferred to healthy guar and indicator plants: Chenopodium quinoa, Chenopodium amaranticolor, Pisum sativum, Nicotiana glutinosa, Nicotiana tabacum, Nicotiana clevelandii, Glycine max, Phaseolus vulgaris vars. Bountiful, Double white settler, Prince and Top crop. At least five aphids were transferred to each separate indicator plant. The aphids were allowed a transmission feed of 1 hour, in a small cage which clipped onto a small area of leaf.

In order to avoid damage, feeding aphids were disturbed by gently touching their antennae or breathing on them to withdraw stylers. Once the stylets were withdrawn the aphid can be carefully picked up with a slightly moistened paint brush.

The vector aphids were then killed by spraying with an aphicide (Pirimor/efekto) and test plants were allowed to grow for symptom observation.

4.3.2 Persistent aphid transmission

Aphids were handled in the same way as for non-persistent virus transfer, except that no starvation period was required before acquisition. In addition, the acquisition period and inoculation feed were longer (24 hours).

In a parallel control test, the aphids were fed on non-infected guar leaves var. TX-79-2741 and transferred to indicator plants using the relevant acquisition and transmission feeding times. This control test was made in order to ensure that the symptoms which developed were not simply due to feeding of vectors or extraneous factors (Stephen, 1984).

4.4 Seed transmission

Pods from infected guar varieties (Table 4) showing symptoms of "little leaf" stunting and sterility were collected, as well as pods from indicator plants (Table 6) infected by mechanical inoculation and by aphid transmission. Seeds were removed and sown in pots. Seedlings were observed for virus symptoms for 8 weeks at 25°C and 30°C. As control, healthy seeds were sown.

5. BIOLOGICAL PROPERTIES OF VIRUS

5.1 Longevity in vitro

Different viruses behave differently when allowed to age in vitro at room temperature. Longevity in vitro is defined as the time for which crude juice kept at room temperature remains infective (Stephen, 1984).

Infected guar sap var. TX-79-2741 was maintained at room temperature and daily inoculations were made to the indicator plant Phaseolus vulgaris var. Top crop at 30°C.

5.2 Thermal inactivation point

The thermal inactivation point of a virus in crude extract is the temperature required for complete inactivation following a 10 minutes exposure to this temperature (Stephen, 1984). 2ml volumes of infected guar sap var. TX-79-2741 were placed into thin-walled test tubes. Each tube was subjected to the following different temperatures for 10 minutes:

20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 70°C, 80°C

by placing it in a water bath. Each sample of heat-treated sap was inoculated onto Phaseolus vulgaris var. Top crop at 30°C.

5.3 Dilution end-point

Determination of the dilution of crude sap extracts at which infectivity is lost is a useful pointer in virus characterisation (Stephen, 1984). Infected guar sap var. TX-79-2741 was diluted with 0.01M Potassium phosphate buffer pH 7.0 as follows:

1/1, 1/10, 1/100, 1/1000, 1/10000

and separate mechanical inoculations from each tube dilution were made to test plant Phaseolus vulgaris var. Top crop.

6. VIRUS PURIFICATION

Infected guar plants vars. TX-79-2741 and ZDPS were used for virus purification procedures. In addition, purification was done with infected Pigeonpea and Phaseolus vulgaris var. Contender mechanically inoculated with the infected guar sap.

6.1 Procedure 1

The method of Jafarpour et al. (1979) was used. This method has been reported to give the highest yield of virus and the best purity (Cowper, 1983; Fox, 1984).

One hundred and fifty grams of infected leaf tissue was homogenised with a Waring blender in 450ml cold 0.5M potassium phosphate buffer, pH 7.2 (Appendix III). Crude extracts were filtered through two layers of cheesecloth and clarified by shaking for 5 minutes with an equal volume of chloroform. The emulsion was centrifuged at 8000 rpm for 25 minutes in a J14 rotor of a Beckman JS-21 centrifuge. Virus was precipitated from the collected supernatant by adding solid polyethylene glycol 6000 (PEG) to an 8 per cent (W/V) level, plus 0.5 per cent (W/V) sodium chloride (Nacl). Stirring was continued for 30 minutes at 4°C, after which the extract was incubated overnight at the same temperature. The precipitate was collected by centrifugation for 20 to 30 minutes at 8000 rpm, and the pellet was resuspended for 4 hours in 1/10 of the original volume with 0.025M phosphate buffer, pH 7.2 (Appendix III). The virus was then subjected to two cycles of differential centrifugation, a low speed centrifugation of 8000 rpm for 30 minutes, followed by a high speed centrifugation of 27,000 rpm for 2 hours in a Ti 40 rotor in a Beckman ultracentrifuge. Pellets were resuspended in 0.2 to 0.5ml 0.025M potassium phosphate buffer pH 7.2.

6.2 Procedure 2

This method is for Potyviruses (Pietersen, pers. comm.). Infected leaves were homogenised in a Waring blender in cold 0.5M K_2H/KH_2PO_4 pH 8.4 and 0.5 per cent (W/V) Na_2SO_3. 350g of leaves in 1000 ml of buffer.

Crude extracts were filtered through two layers of cheesecloth. The filtrate was centrifuged at 4500 rpm for 10 minutes. Triton X-100 2 per cent (W/V) was added to the supernatant and stirred for 15 minutes at 4°C. Then 4 per cent (W/V) PEG 8000 and 0.1M NaCl were added and stirring was continued for 1 hour at 4°C. After a centrifugation at 7000 rpm for 10 minutes, the pellet was resuspended in 50ml 0.1M Borate/Kcl pH 8 (Appendix III) and stirred for at least 1 hour at 4°C. The virus was subjected to a low speed centrifugation of 7000 rpm for 10 minutes. The supernatant was added to 25ml tubes underlayed with 5ml/tube 30 per cent (W/V) sucrose in 0.1M borate/Kcl pH 8.0, and given a high speed centrifugation at 27 000 rpm for 2.5 hours at 4°C. The pellets were resuspended in 0.1M borate/Kcl buffer pH 8 overnight at 4°C, with tubes tilted to cover pellets. (The rotors mentioned in procedure 1 were used.)

6.3 Procedure 3

The method of Alper et al. (1984) was used.

5 grams of infected leaves were homogenised in 40ml of a solution containing 20ml of 0.1M phosphate buffer, pH 8.2, 0.1 per cent 2-mercaptoethanol, and 20ml of chloroform (Appendix III). The mixture was centrifuged at 2 000g for 10 minutes. Polyethylene glycol (4 per cent W/V: M.W.6000) (PEG) and 0.2M NaCl were added

to the aqueous phase and centrifuged after 1 hour at 12 000g for 15 minutes.

The pellet was resuspended in the phosphate buffer and brought to $^{1}/_{10}$ of the initial volume. After additional precipitation with PEG and NaCl and low speed centrifugation, the resulting pellet was resuspended in 0.2 to 0.5ml of phosphate buffer and centrifuged at 2 000g for 10 minutes, and the supernatant kept. All procedures were conducted at 4°C.

The supernatant, containing virus particles was subjected to further clarification by extraction of polysaccharides.

6.4 Polysaccharide extraction

To 1ml of purified virus were added:

1 volume 2.5M K_2HPO_4 and

1 volume ethylenglycolmonomethylether (EGME).

The mixture was shaken for 3 minutes and centrifuged at 6500 rpm for 10 minutes. The upper aqueous phase was dialized overnight in 0.05M Borate/Kcl pH 8,0 and freeze-dried. The resulting pellet was resuspended in 500µl Borate/Kcl buffer pH 8,0 (or any other buffer according to the purification procedure used).

7. PRESERVATION OF VIRUS MATERIAL

Wherever possible, purified virus preparations were used immediately to avoid contamination and inactivation of the virus particles. However, when storage was necessary, a drop (0.05ml) of 1 per cent

sodium azide, which is an antimicrobial compound, was added to virus preparation and kept at 4°C. Infected guar leaves, pigeonpea and mechanically infected indicator plants were also used, fresh where possible, but when storage was necessary they were freeze-dried for 24 hours and stored over calcium chloride.

8. ELECTRON MICROSCOPY

All electron microscopy methods were performed with all infected guar varieties (Table 4), infected pigeonpea and indicator plants challenged with the guar virus by mechanical inoculation (Table 6), or by aphid transmission (4.3). Healthy guar varieties, pigeonpea and indicator plants were used as controls. The negative stains which were used:

2 per cent aqueous phosphotungstic acid (PTA) pH 6.5 and 2 per cent aqueous uranyl acetate (Appendix IV). Formvar-carbon or collodion-coated grids were employed (8.1, 8.2).

8.1 Preparation of copper grids

Formvar for copper grids (300 mesh) was prepared for TEM by dipping slides vertically into a beaker containing 0.3 per cent formvar in chloroform. The slides were removed with a smooth, rapid movement. A sharp blade was used to outline the edge of the formvar film and this was floated off onto distilled water. Copper grids were dropped onto the formvar film. The formvar-coated grids were scooped out of the water, using a piece of wire mesh taped to a slide and were carbon-coated after drying and finally stored in a dessicator.

8.2 Preparation of collodion grids

The film was cast on a clean water surface by letting fall a single drop of 2 per cent collodion in amyl acetate. The solution spreads and the solvent evaporates, leaving a film which was skimmed off to clean the water surface. A second similar film was then cast and grids dropped onto the collodion film, which was then collected by the same method as the formvar-coated grids.

8.3 Leaf-dip method

The leaf-dip method in this study was based on Ball (1971: 1974). The grid was clamped in forceps and loaded with one drop of negative stain and a freshly cut edge of leaf run through the drop on the grid over a period of about 30 seconds. The grid was drained and allowed to dry before examination under the JEM 100S TEM.

8.4 Leaf squash method

This method provides excellent results for many high-concentration viruses, but leaf squash preparations contain, as well as virus, much cell debris (Stephen, 1984). Squares 3mm in size were cut from a leaf and macerated in drops of cold 0.5M PPB pH 7.2 (Appendix III). Grids were placed face down on the drops and left for 30 minutes and then rinsed in distilled water. The grids were then placed face down on drops of negative stain for 3 minutes and then rinsed with distilled water. Grids were blotted dry and viewed under the electron microscope.

8.5 Purified virus preparation

Drops of purified virus were placed on dental wax. Formvar-carbon or collodion coated grids were inverted onto these, left for 30 minutes and then rinsed in a stream of distilled water. Grids were placed onto drops of negative stains (Appendix IV) for 3 minutes, rinsed in distilled water, blotted dry on filter paper and viewed under the JEM 100S TEM.

8.6 Transmission electron microscopy

The examination of biological material in the electron microscope necessitates the use of special preparative techniques. These techniques have enabled the study of virus in situ, the position of viruses in cells and the changes they may cause in cells.

The following were the steps used in the preparation of material for transmission electron microscopy (Provvidenti and Cobb, 1975):

(1) Fixation - 2mm leaf sections were cut and immersed in 2.5 per cent gluteraldehyde solution (Appendix IV) in small bottles. The material was left to fix for 1 hour on a rotor and then left in 0.05M sodium cocodylate buffer overnight. Leaf material was post-fixed with 1 per cent osmium tetroxide for 1 hour, then rinsed 3 times with buffer for 10 minutes each.

(2) Dehydration - The fixed material was dehydrated, using the following ethanol series for 15 minutes each: 30 per cent, 50 per cent, 75 per cent, 90 per cent and twice 100 per cent.

(3) Infiltration - Dehydrated material was placed in a mixture of Propylene oxide and Epoxy resin (Appendix IV) as follows:

Propylene oxide - 20 minutes
1:1 Propylene oxide/Epoxy resin - 30 minutes
1:3 Propylene oxide/Epoxy resin - 30 minutes
Pure Epoxy resin - 4 hours.

(4) Embedding - One piece of material was placed in each plastic boat which was filled with fresh pure Epoxy resin (Appendix IV). Specimens were polymerised for 48 hours at 60°C.

(5) Sectioning - Blocks were sectioned on a Reichert ultramicrotome and sections of 70 to 150nm were collected in 300 mesh copper grids. Sections were stained with 1 per cent uranyl acetate and lead citrate (Appendix IV) (Venable and Coggleshall, 1965). Sections were viewed under the JEM 100S TEM.

3.7 Inclusion bodies

The following plant virus inclusion techniques were used (Christie and Edwardson, 1977; Christie and Edwardson, 1986):

(a) Epidermal strips of infected leaves were placed in 5 per cent Triton X-100 for 5 to 10 minutes and then in calcomine orange/Luxol brilliant green stain (1 drop calcomine orange, 8 drops Luxol brilliant green, 1 drop H_2O) for 10 minutes. The strips were rinsed in 95 per cent ethanol and fixed in acetate for 1 minute.

(b) Epidermal strips were placed into Azure A stain (9 drops azure A, 1 drop 0.2M Na_2HPO_4) for 10 minutes then rinsed twice with ethanol. Strips were then mounted in euparal and viewed under the light microscope.

8.8 Immuno electron microscopy (IEM)

Immune electron microscopy (IEM) is the term generally used for techniques that detect, by electron microscopy, the specific binding of antibody to antigen (Milne and Luisoni, 1977).

Purified guar virus vars. TX-79-2741 and ZDPS as antigen and the following antisera:

SCMV, BYMV, PVY, SMV and SBMV

were used in four different IEM techniques. The various procedures were compared with control grids where healthy purified guar sap was employed.

8.8.1 The Derrick technique

(Derrick, 1972, 1973a, b; Brlansky and Derrick, 1974, 1979; Derrick and Brlansky, 1976; modified by Milne and Luisoni, 1977)

The above antisera (8.8) were diluted 1:10, 1:100 and 1:1000 in 0.1M phosphate buffer pH 7.0. 2µl of the diluted antisera were placed on grids held in forceps and these were incubated in a humid box for 5 minutes at room temperature. The grids were then washed with 20 consecutive drops of buffer and drained, but not dried, and drops of virus sample (2 to 4µl) were added. The drops were incubated on the grids for 15 minutes as above and the grids were then washed with 20 drops of buffer, 30 drops of water and 5 drops of 2 per cent uranyl acetate. The preparations were then drained and dried.

8.8.2 Leaf dip serology
(Ball, 1971, 1974)

A drop of antiserum diluted 1:100 in 0.001M ammonium acetate was placed on a grid and a freshly cut edge of guar infected leaf was held in the drop for a few seconds. Then the drop was air-dried and stained with 2 per cent PTA pH 6.8 for 10 minutes.

8.8.3 Decoration

In this method introduced by Yanagida and Ahmad-Zadeh (1970) and used by Milne and Luisoni (1975, 1977) and Milne and Lesemann (1978), virus is adsorbed onto grids, washed and then the antiserum is added. Decoration, or the covering of antigenic sites by the antibody molecules, occurs.
Drops of virus extract were put on a carbon-formvar grid for 10 seconds, rinsed with phosphate buffer and incubated for 15 minutes at room temperature with drops of antisera (8.8) used directly or diluted 1:10 in phosphate buffer. The grids were then rinsed with water and negatively stained in 2 per cent aqueous uranyl acetate.

8.8.4 Clumping
(Anderson et al., 1961; Lafferty and Oertelis, 1961)

The antisera (8.8) were diluted 1:10 and 1:100 in 0.1M phosphate buffer pH 7, and 10µl drops placed on a glass slide. The infected guar leaf samples were crushed in the drops of antisera and the slides incubated in a humid box for 15 minutes at room temperature. A grid held in forceps was allowed to touch the drop and then

washed with 20 drops of phosphate buffer, 30 drops of distilled water and finally 5 drops of 2 per cent uranyl acetate, before draining and drying.

If a purified virus suspension was used, it was mixed with the diluted antisera, incubated for 15 minutes and than processed as before.

9. SEROLOGY

Since Preliminary double diffusion test (Ouchterlony method) and Radial diffusion technique (Slack and Shepherd, 1975) using infected guar and the different antisera (9.1) were unsuccessful, the present investigation concentrated on different ELISA procedures which gave better results.

9.1 Antisera

Antisera to known viruses were kindly donated by the following:

BCMV, BYMV, PVY, SMV. SBMV (adjusted to 1mg/ml) by G Pieterson, Rietondale Research Centre were used in indirect ELISA tests.

BCMV, (1:4096) BYMV, (1:16,384) TRSV (1:4096) by A Brunt Glasshouse Crops Research Institute, England, were used in PTA ELISA tests.

9.2 Grid titration

Before use in routine ELISA testing, the optimal dilution for each reagent (sample, gamma globulin and

conjugate) was determined experimentally, using a chequer-board titration (Voller et al., 1976):

Sample concentration: 1/10, 1/20, 1/40, 1/80, 1/160
Antiserum concentration: 1/500, 1/1000, 1/1500, 1/2000
Conjugate concentration: 1/1000, 1/2000, 1/5000, 1/10 000
Optimal concentration was taken as the lowest dilution, giving a high absorbance A405 value for infected samples and a low value for healthy controls.

9.3 Indirect ELISA method

The indirect ELISA procedure for virus detection was as follows:

1. 200µl of purified virus suspension or crude extract from infected guar leaves (Table 4) infected pigeonpea and mechanically infected indicator plants (Table 6) was diluted 1:20 in 0.05M sodium carbonate buffer pH 9.6 (coating buffer) (Appendix V) and added to each well of a polystyrene microtitration plate. Sap extracted from healthy leaves was used as a control. Coating buffer was added to 6 wells to act as a buffer control. Plates were thoroughly washed 3 times with PBS-Tween (Appendix V) for 3 to 5 minutes, after three hours incubation at 37°C.

2. 100µl of 1 per cent Bovine serum albumine (BSA) was subsequently added and incubated for 1 hour at room temperature before being rinsed with washing buffer.

3. 200µl of Purified IgG (9.7) (1mg/ml) diluted 1:1500 in virus buffer (Appendix V) were added to the wells. The plates were incubated overnight at 4°C and were washed as before.

4. 200µl of Alkaline phosphatase labelled goat anti-rabbit IgG (1:7 000, Copper Biomedical) diluted 1:1000 in virus buffer or Alkaline phosphatase labelled Protein A, was added to the wells. Plates were incubated for 3 hours at 37°C and were washed as before.

5. 200µl of p-nitrophenylphosphate substrate in Diethanolamine buffer was added to each well and plates were incubated at room temperature for 30 minutes.

The reaction was stopped by addition of 50µl of 3N NaOH solution. The plates were read at 490nm wavelength on an ELISA plate reader.

9.4 $F(ab')_2$ ELISA method

The following sample preparation was followed: 8g of leaf material was macerated in 10ml of 0.0[illegible] Na[illegible]-buffered saline (PBS) pH 7.4, 0.05 per cent Tw[illegible], 2 per cent polyvinylpyrrolidone and 0.2 per cent ovalbumin (extraction buffer) using an ultraturrox homogeniser. Samples were centrifuged at 8 000 rpm for 5 minutes, using an MSE Hi Spin 21 centrifuge and the supernatant used in $F(ab')_2$ ELISA.

1. 200µl of specific $F(ab')_2$ fragments of BCMV, BYMV and SBMV diluted in 0.05M coating buffer pH 9.6 was placed in the wells of a polystyrene microtitration plate and incubated at 4°C overnight.

2. Unabsorbed $F(ab')_2$ fragments were removed by flooding wells once with PBS-Tween and immediately removing this, using a Titertek Microplate washer

(Flow Inc., Conn., U.S.A.), followed by 3 soaking cycles of 3 minutes each.

3. 200µl samples were placed in each of two wells of a plate, along with similarly prepared healthy control plant samples and 0.1 per cent formalin inactivated virus-infected sap as positive controls and incubated 4 hours at 30°C.

4. After washing, as above, 200µl of the specific immunoglobulins (1mg/ml) diluted in extraction buffer was placed in all the wells and incubated at 4°C overnight.

5. After washing, 200µl at a 1:500 dilution of goat-antirabbit Fc immunoglobulin-alkaline phosphatate conjugate (GAR-Fc conjugate) was placed in each well and incubated 4 hours at 30°C.

6. Plates were then washed and incubated with 200µl of a freshly prepared 1µg/1ml p-nitrophenol phosphate substrate in 10 per cent diethanolamine pH 9.8. The hydrolysed enzyme substrate concentration was determined colorimetrically after 30 minutes by measuring absorbtion at 405nm, using a Titertek multiscan photometer.

9.5 Plate-trapped antigen (PTA) ELISA method
(Mowat and Dawson, 1987)

A simple and rapid procedure of enzyme-immuno assay (PTA-ELISA) was used in an attempt to detect the virus in guar. Virus antigen in crude leaf extracts as adsorbed directly to a solid phase, allowed to react with unfractionated antiserum and the antigen-antibody

complex detected with a conjugate of Protein A and enzyme.

1. Test samples of healthy and infected guar (Table 4) and pigeonpea leaves in carbonate buffer (5ml/g leaf) were added undiluted and diluted 1:10 to wells of microtitre plates and incubated for 15 minutes at 37°C.

2. Antisera (9.1) diluted 1:1000 with virus buffer (Appendix V) (PBS-TPO) were incubated in the wells for 30 minutes at 37°C.

3. Protein A-alkaline phosphatase (ALP) conjugate in virus buffer was added and incubated for 30 minutes at 37°C.

4. Enzyme substrate, p-nitrophenyl phosphate in 10 per cent diethanolamine at pH 9.8 was added. Plates were washed between each stage with PBS-Tween.

9.6 Detection of virus in aphids by indirect ELISA

The method of Tamada and Harrison (1981) was used. Groups of 20 aphids were fed on infected guar plants for 20 minutes. Aphid extracts were prepared by grinding one or more individuals in a final volume of 0.25ml extraction fluid (0.05M phosphate buffer pH 7.0) in the presence of carborundum. All aphid samples were kept in -70°C before grinding and extracts were incubated at 37°C for 1 hour, then centrifuged for 10 minutes at 8000 rev/min before transferring the supernatant fluids to wells in microtitre plates. The indirect ELISA method was then performed.

9.7 Purification of immunoglobulin G

A highly purified preparation of IgG was used in the indirect ELISAS.

A simple and convenient method for serum fractionation is Protein A-Sepherose affinity chromatography (Miller and Stone, 1978).

Protein A isolated from the Cowan strain of Staphylococcus aureus binds specifically to the Fc region of IgG. A very pure preparation of IgG can be obtained from whole sera using affinity chromatography on a protein A-Sepharose CL-4B column.

1-2ml of crude antisera BCMV, BYMV, PVY, SBMV and SMV and then equal volume of buffer A were passed through 0.5 inch glass wool in Pasteur pipette (Appendix V). The antiserum-buffer mixture was loaded into the column. The column was washed with 5 to 10 volumes of buffer A and the flow rate adjusted to one drop per 15 seconds. 3ml samples were collected and absorbance was read until it drops off near zero. The IgG fraction was eluted off the column with buffer B (Appendix V) and flow rate was adjusted to one drop per 7 seconds. 3ml samples were collected and absorbance was checked until it reached near zero.

The high readings were collected, pooled and dialised against double distilled H_2O at 4°C for 1 hour, then in PEG 6000 to concentrate the IgG. When concentrated to desired volume (1 to 2ml) the antibody solution was dialysed against PBS overnight. Protein concentration was adjusted to 1 mg/ml, where at OD_{280} = 1.4, IgG = 1 mg/ml.

9.8 Immuno-electroblot (IEB)

Enzyme-assisted immunoelectroblotting (IEB) entails SDS-PAGE of virus or sap preparation, electrophoretic transfer of the resolved polypeptides to a nitrocellulose membrane and probing the membrane with antivirus serum (Towbin et al., 1979; Rybicki and Von Wechmar, 1982; Pappas et al., 1983; Bode et al., 1984). The technique allows characterisation of antigen, both by their molecular weights and by their antigenic reactivity. Because antigen detection is by an indirect enzyme immune reaction, distant serological relationships can be detected.

In this study the Western blot method of Hibi and Saito (1985) was used:
Nitrocellulose blots were soaked in 1 per cent Bovine serum albumin (BSA) in PBS buffer pH 7.4 (Appendix V) for 60 minutes at 37°C with shaking. After washing 4 x 5 minutes in PBS-Tween, blots were incubated on a shaker with the antisera BCMV, BYMV, PVY, SMV and SBMV (diluted 1:200 in PBS) for 3 hours at 37°C. After washing 4 x 5 minutes in PBS-Tween and 1 x 5 minutes in AB buffer (Appendix V), GAR-alkaline phosphatase diluted 1:2000 in AB buffer was added and blots were incubated for 2 hours, on a shaker at room temperature, again washed 3 x 15 minutes in AB buffer and 2 x 20 minutes in AP Buffer (Appendix V). Then the substrate NBT + BCIP (Appendix V) was added, and blots were incubated in the dark until bands appear. Blots were washed in 10mM Tris-5mM EDTA pH 7.5 and dried at 80°C in vacuum oven for 2 minutes.

10. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

10.1 10 per cent gel

10 per cent gels were prepared according to the method in Appendix VI. Samples were prepared by mixing semi-purified viral preparations from infected leaves with splitting solution in a ratio 1:1 and heating this in a boiling water bath for 5 minutes. 10µl samples were loaded onto the gel and 10µl of molecular weight markers (Pharmacia), in order to determine the molecular weight of the migrated proteins. Healthy leaf extracts were run as controls. The gel was electrophoresed at 30mA for 3 hours or when the bromo-phenol blue band reached the bottom of the gel. The gels were stained in comassie blue or silver stain (Appendix VI) (Wray et al., 1981). After destaining, gels were scanned on an LKB 2202 ultrascan laser densitometer and the molecular weight of the proteins determined.

10.2 Gradient gel

Same procedure as the 10 per cent gel was used but a gradient gel from 5 per cent to 15 per cent was employed (Appendix VI).

CHAPTER 3

RESULTS

1. IDENTIFICATION OF BACTERIA

The methods for identification of bacteria are depicted in the diagram (Fig. 10).

1.1 Growth on common bacteriological media

Growth on nutrient agar was observed. Colonies were white. Only one type and colour of colonies was found.

1.2 Gram stain

The bacteria isolated (1.1) were found to be gram negative. Since Corynebacterium (Fig. 10) is gram positive, the bacteria were suspected to be either Erwinia, Xanthomonas, Pseudomonas or Agrobacterium.

1.3 Colonies on YDC agar

White colonies were observed on YDC agar. Since Erwinia and Xanthomonas produce yellow colonies (Fig. 10) the bacteria were suspected to be either Pseudomonas, Erwinia or Agrobacterium.

1.4 Fluorescent pigment on KB agar

A fluorescent pigment was found in colonies grown on KB agar. According to Figure 10 positive result for fluorescence indicates that the bacterium was Pseudomonas.

Pseudomonas causes numerous plant diseases with diverse symptoms, including vascular wilts, stem cankers, soft rots, blossom and twig blights, leaf spots, tumors or galls (Schaad, 1980). However, these symptoms were not observed on infected guar plants, so it was concluded that this bacterium was resident, but not the causal agent of the disease.

2. INDICATOR PLANT STUDIES

2.1 Mechanical inoculation

Results are presented in Table 7. Mechanical inoculation, using infected leaf extracts (Chapter 2, 4.2) of TX-79-2741 or ZDPS varieties on indicator plants, gave different symptoms from the virus natural guar host. No symptoms were observed on guar plants inoculated mechanically with infected guar sap.

The most common symptoms at 25°C and 30°C were characterised by small necrotic red lesions, appearing 5 days after inoculation followed by red veins and chlorosis (Fig. 17). After 14 days virus symptoms were most severe. The symptoms were sometimes systemic (observed on the first two leaves inoculated and then spread to new leaves) in the following:

Phaseolus vulgaris vars. Wintergreen (Fig. 18), Seminole (Fig. 21), Abundance (Fig. 26), Top crop (Fig. 13) and Golden podded wax (Fig. 22). Only on P. vulgaris vars. Nep 2 (Fig. 11) and Contender (Figs. 16 and 17) did the reaction appear at 30°C and was restricted to inoculated leaves. This may indicate in these plants the presence of a hypersensitive reaction. Leaf curling and malformation symptoms were observed in Phaseolus vulgaris vars. Prince (Fig. 14), Double

white settler (Fig. 15), Natal round yellow sugar (Fig. 24), Blue peter (Fig. 25) as well as Amanda, Bountiful and Lazy housewife.

Most of the symptoms appeared at 30°C in a growth cabinet with constant humidity (70 per cent) and light (1500 lux) conditions.

Many plants (Table 7), which developed symptoms at 30°C, did not show any symptoms at 25°C. Symptoms were not produced in other indicator plants, except Vigna unguiculata and Lycopersicum esculentum, i.e. symptoms were mainly restricted to varieties of the genera Phaseolus. Therefore, host range studies indicated that the guar virus may possibly be restricted to viruses that infect the bean family.

Table 7: Symptoms on indicator plants produced by mechanical inoculation

Indicator host	Symptoms produced by mechanical inoculation	
	at 30°C	at 25°C
Phaseolus vulgaris		
var. Monroe	No symptoms	Red vein & chlorosis
var. Amanda	Leaf curling	No symptoms
var. Nep 2	Hypersensitive reaction of red vein & chlorosis, leaf curling and death	No symptoms
var. Peru 0251	No symptoms	No symptoms

Indicator host	Symptoms produced by mechanical inoculation	
	at 30°C	at 25°C
Phaseolus vulgaris (cont)		
var. Sanilac	No symptoms	No symptoms
var. Diacol calima	No symptoms	Red vein & chlorosis
var. Top crop	Systemic red vein & chlorosis	No symptoms
var. Prince	Leaf curling	No symptoms
var. Bountiful	Leaf curling	Red vein & chlorosis
var. Double white settler	Leaf curling & malformated leaves	No symptoms
var. Pinto	No symptoms	No symptoms
var. Contender	Hypersensitive reaction of red vein & chlorosis	No symptoms
var. Wintergreen	Systemic red vein & chlorosis. Pods with red spots.	No symptoms
var. Seminole	Systemic red vein & chlorosis	Red vein & chlorosis
var. Golden podded wax	Systemic red vein & chlorosis	Red vein & chlorosis

Indicator host	Symptoms produced by mechanical inoculation	
	at 30°C	at 25°C
Phaseolus vulgaris (cont)		
var. Canadian wonder	No symptoms	No symptoms
var. Lazy housewife	Leaf curling	No symptoms
var. Genuine cornfield	Red vein & chlorosis	No symptoms
var. Natal round yellow sugar	Leaf curling & yellowing	No symptoms
Phaseolus sp. (Runner beans)		
var. Blue peter	Leaf curling & mosaic	No symptoms
var. Everybearing	No symptoms	No symptoms
var. Abundance	Systemic red vein & chlorosis, leaf curling, sterility, no pods	No symptoms
Phaseolus sp. (Broad beans)		
Aquadulce	No symptoms	No symptoms
Chenopodium quinoa	No symptoms	No symptoms
Chenopodium amaranticolor	No symptoms	No symptoms

Indicator host	Symptoms produced by mechanical inoculation	
	at 30°C	at 25°C
Pisum sativum	No symptoms	No symptoms
Glycine max	No symptoms	No symptoms
Vigna unguiculata	No symptoms	Red local lesions
Nicotiana clevelandii	No symptoms	No symptoms
Nicotiana glutinosa	No symptoms	No symptoms
Nicotiana tabacum var. Samsun	No symptoms	No symptoms
Lycopersicum esculentum	Malformation & blisters	No symptoms
Datura stromonium	No symptoms	No symptoms
Petunia hybrida	No symptoms	No symptoms
Vicia faba	No symptoms	No symptoms
Lupinus albus	No symptoms	No symptoms



Figure 11: Phaseolus vulgaris var. Nep 2 exhibits a hypersensitive raction of red veins (RV) and chlorosis (CHL) leaf curling (LC) and death following mechanical inoculation at 30°C

(a)



(b)

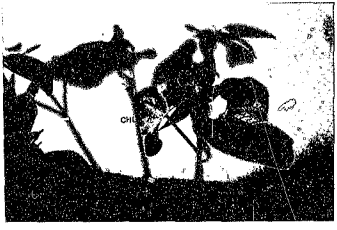


Figure 12 (a & b) P. vulgaris var. Top crop exhibits symptoms of necrotic lesions (NL), red veins (RV) and chlorosis (CHL) following mechanical inoculation at 30°C.



Figure 13: Systemic infection on P. vulgaris var. *Top crop*. New leaves with red veins (RV) and chlorosis (CHL) following mechanical inoculation at 30°C.



Figure 14: P. vulgaris var. Prince exhibits symptoms of leaf curling (LC) and epinasty following mechanical inoculation at 30°C



Figure 15: P. vulgaris var. Double white settler exhibits symptoms of leaf curling (LC) epinasty and malformated leaves following mechanical inoculation at 30°C



Figure 16: P. vulgaris var. Contender exhibits a hypersensitive reaction of red veins (RV) one week after mechanical inoculation at 30°C



Figure 17: P. vulgaris var. Contender exhibits a hypersensitive reaction of red veins (RV) and chlorosis (CHL) two weeks after mechanical inoculation at 30°C

(a)



Figure 18 (a & b) [illegible] *vulgaris* var. Wintergreen exhibits symptoms of red veins (RV) and chlorosis [illegible] on new leaves (systemic infection) following mechanical inoculation at 30°C

(b)





Figure 19: P. vulgaris var. Wintergreen exhibits symptoms of red veins (RV) and chlorosis (CHL) following mechanical inoculation at 30°C

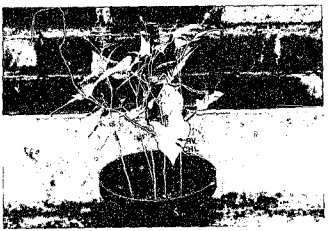


Figure 20: P. vulgaris var. Wintergreen exhibits malformed pod with red lesions (RL) following mechanical inoculation at 30°C



Figure 21: P. vulgaris var. Seminole exhibits systemic red veins (RV) and chlorosis (CHL) symptoms on new leaves following mechanical inoculation at 30°C

(a)



(b)



Figure 22: P. vulgaris var. Golden podded wax exhibits
(a) Systemic red veins (RV) and chlorosis (CHL) following mechanical inoculation at 30°C
(b) Symptoms of malformation and blisters (BL) on new leaves following mechanical inoculation at 30°C

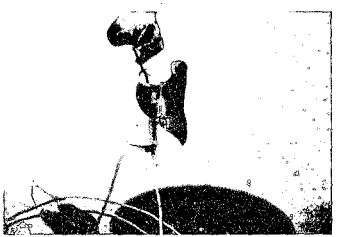


Figure 23: P. vulgaris var. Genuine cornfield exhibits symptoms of red veins (RV) and chlorosis (CHL) following mechanical inoculation at 30°C

(a)



(b)



Figure 24 (a & b): P. vulgaris var. Natal round yellow sugar exhibits symptoms of leaf curling (LC) epinasty and yellowing following mechanical inoculation at 30°C

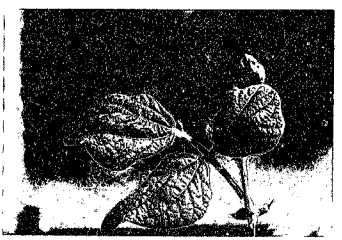


Figure 25: Phaseolus sp. (Runner beans) var. Blue peter exhibits symptoms of leaf curling (LC), blisters (BL) and chlorosis (CHL) following mechanical inoculation at 30°C

(a)



(b)



Figure 26 (a & b): Phaseolus sp. (Runner beans) var. Abundance exhibits systemic symptoms of red veins (RV) and chlorosis (CHL) on new leaves following death after mechanical inoculation at 30°C



Figure 27: Lycopersicum esculentum exhibits symptoms of malformation and blisters (BL) following mechanical inoculation at 30°C



Figure 28: P. vulgaris var. Monroe exhibits symptoms of red veins (RV) and chlorosis (CHL) following mechanical inoculation at 25°C. Some senescent leaves were also observed



Figure 29: P. vulgaris var. Diacol calima exhibits symptoms of red veins (RV) and chlorosis (CHL) following mechanical inoculation at 25°C



Figure 30: P. vulgaris var. Bountiful exhibits symptoms of red veins (RV) and chlorosis (CHL) following mechanical inoculation at 25°C

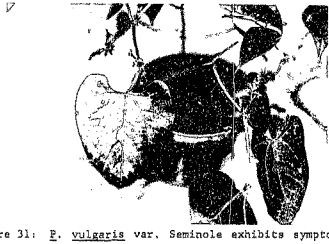


Figure 31: P. vulgaris var. Seminole exhibits symptoms of red veins (RV) and chlorosis (CHL) following mechanical inoculation at 25°C



Figure 32: P. vulgaris var. Golden podded wax exhibits symptoms of red veins (RV) and chlorosis (CHL) following mechanical inoculation at 25°C

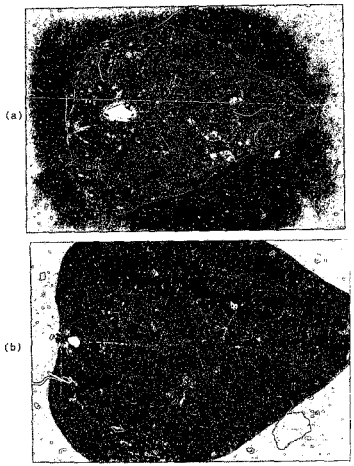


Figure 33 (a & b): Vigna unguiculata exhibits symptoms of red local lesions (LL) and chlorosis (CHL) following mechanical inoculation at 25°C

2.2 Transmission by aphids

Results are presented in *Table 8. Aphid studies* were performed in a persistent and a non-persistent manner (Chapter 2, 4.3). Indicator plants (Table 8) infected with the persistent way of transmission show no symptoms, except rolling and small blisters which appear also on control plants tested with virus-free aphids. These symptoms were due to feeding of the vector.
Symptoms of malformation of leaves and mosaic were produced on the following indicator plants, using the non-persistent way of transmission: Phaseolus vulgaris vars. Bountiful (Fig. 35), Double white settler (Fig. 36), Prince (Fig. 37), *Top crop* (Fig. 38) and Glycine max (Fig. 34).

No symptoms were observed on guar plants using aphid transmission from infected guar plants.

Because it was too difficult to maintain aphid cultures, mechanical inoculation was preferred for virus transmission during the research.

Table 8: Symptoms on indicator plants produced by aphid transmission

Indicator host	Symptoms produced by aphid-transmission	
	Persistent	Non-persistent
Glycine max	*No symptoms*	Leaf malformation blisters and mosaic
Phaseolus vulgaris var. Bountiful	No symptoms	Leaf malformation and mosaic
Phaseolus vulgaris var. Double white settler	No symptoms	Leaf malformation and mosaic
Phaseolus vulgaris var. Prince	No symptoms	Leaf malformation and mosaic, white necrotic lesions.
Phaseolus vulgaris var. Top crop	*No symptoms*	Leaf malformation and mosaic
Chenopodium quinoa	No symptoms	No symptoms
Chenopodium amaranticolor	No symptoms	No symptoms
Pisum sativum var. Greenfeast	No symptoms	No symptoms
Nicotiana glutinosa	No symptoms	No symptoms
Nicotiana tabacum var. Samsun	No symptoms	No symptoms
Nicotiana clevelandii	No symptoms	No symptoms



Figure 34: Glycine max exhibits symptoms of malformation, blisters (BL) and mosaic (M) following non-persistent aphid transmission

(a)



(b)



Figure 35 (a & b): P. vulgaris var. Bountiful exhibits symptoms of malformation following non-persistent aphid transmission (arrows)

(a)

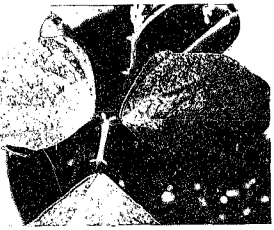


(b)



Figure 36 (a & b): P. vulgaris var. Double white settler exhibits symptoms of mosaic (M) and malformation following non-persistent aphid transmission

(a)



(b)



Figure 37: P. vulgaris var. Prince exhibits symptoms of
(a) Malformation and mosaic (M) following non-persistent aphid transmission
(b) white necrotic lesions (NL)

(a)



(b)

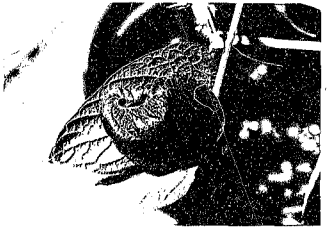


Figure 38 (a & b): P. vulgaris var. Top crop exhibiting symptoms of malformation following a non-persistent aphid transmission (arrows)

2.3 Seed transmission

Guar varieties (Table 4) grown from infected seeds did not show the symptoms of the virus disease ("Little leaf", stunting and sterility). No visible difference appeared between plants grown from infected and healthy guar seeds.

Seeds were collected from pods of indicator plants infected mechanically or aphid transmitted (Tables 7 and 8) with the virus. Plants grown from these seeds appeared healthy, without any virus symptoms.

Mechanically inoculated Phaseolus vulgaris var. Wintergreen exhibited red necrotic lesions and produced pods with red necrotic lesions (Fig. 20). Seeds from these pods exhibiting symptoms, when germinated, gave rise to plants showing leaf malformation and chlorosis (Fig. 39).

(a)



(b)

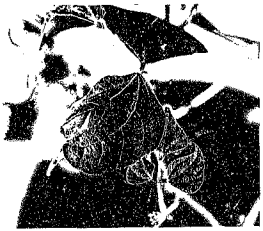


Figure 39 (a & b): Malformated leaves and mosiac (M) of P. vulgaris var. Wintergreen grown *from infected seeds*

BIOLOGICAL PROPERTIES OF THE VIRUS

Inoculation of infected guar sap ZDPS onto Phaseolus vulgaris var. Top crop indicated that the longevity in vitro was 2 to 3 days, the thermal inactivation point was 55°C to 60°C and the dilution end point was 10^{-3} to 10^{-4}. Red local lesions followed by red vein and chlorosis (Fig. 12) appeared about 5 days after the inoculation.

4. VIRUS PURIFICATION

Fresh leaves of infected guar varieties TX79-2741 and ZDPS, brought from the Eastern Transvaal and showing severe symptoms of "little leaf" stunting and sterility, were found to be the most suitable source plants for virus purification. The virus particles could not have been observed in the electron microscope after purification of frozen infected guar leaves (-20°C), nor fresh guar leaves grown from infected seeds and indicator plants infected with the guar virus.

As symptoms on indicator plants suggested that the guar virus may be Bean common mosaic virus, Soybean mosaic virus or other related potyviruses, purification procedures were selected accordingly. Virus purification from infected leaf material were made, using the procedures documented in Chapter 2, 6. Procedure 1 (Chapter 2, 6.1) is known to be a most successful method for isolating BCMV (Jafarpour et al., 1979). Procedure 2 (Chapter 2, 6.2) is a general method for potyviruses (Piesersen G. - pers. comm.) and procedure 3 (Chapter 2, 6.3) is especially for BYMV (Alper et al., 1984).

Comparison of virus yields using procedure 1 and procedure 3 showed that about 0.5mg per 100g of leaves

was obtained. Using procedure 2, a virus yield of 0.06mg per 100g of leaves was obtained.

The absorption spectrum of purified virus from infected guar TX-79-2741 had a maximum at 260nm (0.12) and a minimum at 246nm (0.11) (Fig. 40). The ratio of absorbance A260:A280 was 1.25 and the ratio of A260:A230 was 0.7.

Pigeonpea leaves and pods were purified as well and used in ELISA tests and polyacrylamide gel electrophoresis.

5. DETECTION OF VIRUS PARTICLES BY ELECTRON MICROSCOPY

Virus particles were scarcely observed in electron microscope leaf dip preparations (Chapter 2, 8.3). No virus particles were observed in leaf dips from infected guar varieties (Table 4). Virus particles could not be observed in leaf dip preparations from indicator plants infected mechanically (Table 7) or by aphids (Table 8) as well as infected pigeonpea.

Using leaf squash preparation (Chapter 2, 8.2) from mechanically infected Phaseolus vulgaris var. Wintergreen pod (Fig. 42), long flexuous rods were observed in the electron microscope.

Better observation of the virus particles was achieved with purification of fresh guar leaves. The results indicated that procedure 1 (Chapter 2, 6.1), purification of infected guar TX-79-2741 and procedure 3 (Chapter 2, 6.3), purification of infected guar ZDPS,

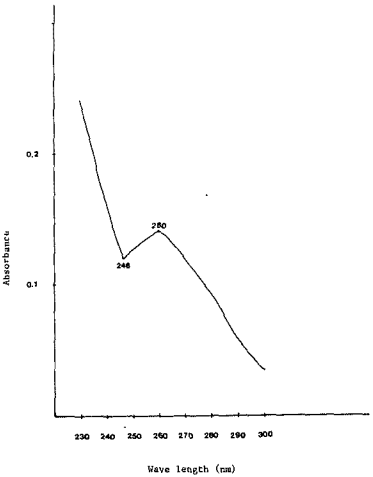


Figure 40: Absorption spectrum of a purified preparation of virus (*Jafarpour et al.*, 1979) maximum at 260nm and minimum at 246nm

gave the best isolation of particles (Figs. 41(a&b),43). Virus-like particles from purified infected pigeonpea, using purification procedure 3, were observed in the electron microscope (Fig. 44).

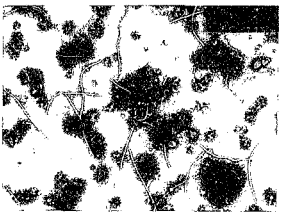
The micrographs showed the virus to be a long flexuous rou-shaped virus. The mean width and length of 30 particles was measured and calculated according to the following formula:

$$\frac{\text{Size of particle (mm)} \times 10^6 \text{nm}}{\text{Size of print}} \times \text{magnification}$$
$$\text{Size of negative}$$

No standards were used in particle length determination. Particles were on average 750nm in length and 15nm in width. The particle dimensions obtained from infected guar (Figs. 41(a&b), 43(a&b)) and mechanically infected indicator plant, P. vulgaris var. Wintergreen (Fig. 42(a&b)) was similar, showing that the guar virus was transmitted successfully. Virus-like particles were observed in a purified pigeonpea preparation (Fig. 44). These particles were also flexuous and were 650nm long and 10nm wide.

The particles were isolated in low numbers, indicating that the virus probably occurs in relatively low concentrations within the plants.

No virus particles were observed using thin sections (Chapter 2, 8.6) and immunoelectronmicroscopy (Chapter 2, 8.8). Inclusion bodies were not observed in light microscopy using the method described in Chapter 2, 8.7.



(a) Magnification x29,000



(b) Magnification x 58,000

Figure 41 (a & b) An electron micrograph of guar TX-79-2741 virus particles following purification procedure 1 (2 per cent PTA)

(a)

(b)

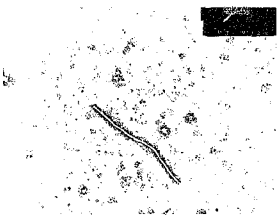


Figura 42 (a & b): An electron micrograph of virus particles from infected pod of Phaseolus vulgaris var. Wintergreen with red spots (Fig. 20) (x47,000; 2 per cent PTA)

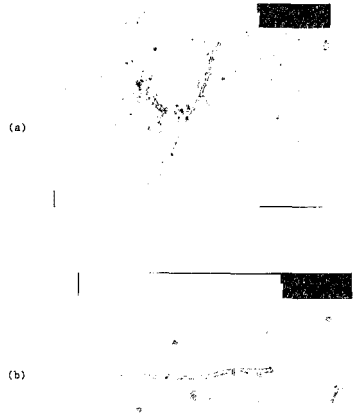


Figure 43 (a & b): An electron micrograph of virus particles following purification procedure 3 of infected guar ZDPS
(a) (x58,000; 2 per cent PTA)
(b) (x117,000; 2 per cent PTA)

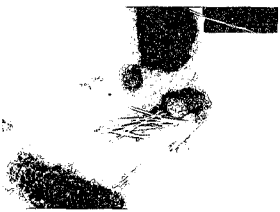


Figure 44: An electron micrograph of virus-like particles following purification procedure 3 from infected pigeonpea (x47,000; 2 per cent PTA)

6. SEROLOGY

6.1 Grid titration

Table 9: Grid titration

	Antiserum	1:1500	1:1000	1:500	1:1500	1:1000	1:500	1:1500	1:1000	1:500	buffer
	Conjugate		1:1000			1:2000			1:5000		
Infected sap	1:10	1.376	1.252	1.531	0.651	0.753	0.996	0.619	0.654	0.707	1.046
	1:20	1.111	1.397	1.735	0.702	0.801	1.026	0.618	0.864	0.711	1.119
	1:40	1.225	1.455	1.985	0.740	0.990	1.137	0.751	0.685	0.742	0.779
	1:80	1.240	1.478	1.855	0.732	0.860	1.070	0.754	0.764	0.756	0.877
Healthy sap	1:80	0.402	0.578	0.673	0.249	0.316	0.444	0.490	0.492	0.450	0.328
	1:40	0.193	0.330	0.663	0.262	0.304	0.476	0.488	0.570	0.458	0.514
	1:20	0.157	0.196	0.505	0.222	0.259	0.427	0.383	0.516	0.428	0.167
	1:10	0.229	0.301	0.670	0.298	0.345	0.498	0.564	0.577	0.508	0.305

The following concentrations of reagents were used:
Infected and healthy sap of guar ZDPS in the following dilutions with coating buffer:
1:10, 1:20, 1:40, 1:80.

Antiserum BCMV in the following dilutions with PBS-TPO
1:500, 1:1000 1:1500.

Protein A-Alkaline phosphatase conjugate in the following dilutions in PBS-TPO:
1:1000, 1:2000, 1:5000.

Coating buffer with the following dilutions of Ab/conjugate:

500/1000, 500/2000, 500/5000, 1000/1000, 1000/2000, 1000/5000, 1500/1000, 1500/2000, 1500/5000.

According to Table 9, the best dilution to work with were:

Ag	1:20
Ab	1:1500
Conjugate	1:1000

6.2 $F(ab')_2$ ELISA

Six h lthy indicator plants and six mechanically infected indicator plants (Table 10) were tested in $F(ab')_2$ ELISA according to the method described in Chapter 2, 9.4. The following antisera were used:

BCMV, BYMV, SBMV.

The results are shown in Table 10. The following samples: mechanically inoculated Pisum sativum and infected Double white settler were BCMV positive. Mechanically inoculated Pisum sativum also appeared to be positive for BYMV even though it was symptomless. Infected guar sample was positive to BCMV and BYMV.

6.3 INDIRECT ELISA

6.3.1 Crude extracts of guar varieties

Different guar varieties leaves were tested in the indirect ELISA method (Chapter 2, 9.3) and the results are presented in Table 11. The concentrations of the antigen, antisera and Protein A-Alkaline phosphatase used in this test was according to the results in grid titration test (Chapter 3, 6.1).

Table 10: $F(ab')_2$ ELISA readings

Sample	BCMV	BYMV	SBMV
Mechanically inoculated			
Pisum sativum	0.710	0.788	0.125
Infected Prince 3	0.114	0.331	0.036
Infected Prince 2	0.168	0.268	0.035
Infected Top crop	0.112	0.165	0.058
Infected Bountiful	0.113	0.332	0.024
Infected Double white settler	0.800	0.307	0.075
Infected Guar TX-79-2741	1.204	1.077	0.323
Healthy Pisum sativum	0.199	0.181	0.012
Healthy Prince 3	0.052	0.134	0.001
Healthy Prince 2	0.131	0.189	0.025
Healthy Top crop	0.103	0.085	0.018
Healthy Bountiful	0.085	0.293	0.026
Healthy Double white settler	0.091	0.236	0.012
Healthy guar TX-79-2741	0.164	0.222	0.290
Positive Control	1.999*	1.172	0.411

A_{405} values of samples in $F(ab')_2$ ELISA after 30 minutes enzyme hydrolysis.

* Values had gone off absorption scale

the results showed that all guar varieties (Lewis, TX-79-2741, ZDPS and HSB-130) were positive to BCMV and SMV.

6.3.2 Purified leaves and pods of guar

Purified healthy and infected leaves of guar ZDPS using the three purification procedures (Chapter 2, 6.1, 6.2

and 6.3), as well as healthy and infected guar pods were tested with the indirect ELISA method. *Virus* samples were diluted 1:40 in coating buffer. The following antisera: BCMV, BYMV, SMV, PVY and TRSV were *diluted 1:1500 in PBS-TPO.* Protein A-Alkaline phosphatase was diluted 1:5000 in PBS-TPO. The results are presented in Table 12.

Purified guar virus preparations were positive to BCMV, BYMV, SMV and PVY. Positive results were higher for purified virus, using purification procedure 1 (Chapter 2,6.1).

6.3.3 Crude extracts of pigeonpea

Infected pigeonpea sap, serially diluted in coating buffer (Table 13) as well as healthy pigeonpea sap were tested in indirect ELISA method. The antisera BCMV and BYMV were diluted 1:1500 in PBS-TPO and protein A-Alkaline phosphatase 1:1000 in PBS-TPO. The results *are presented in Table 13. Infected pigeonpea* was positive to BCMV and BYMV.

6.3.4 Comparison between protein A-Alkaline phosphatase and GAR Alkaline phosphatase in indirect ELISA

Infected and healthy sap of guar ZDPS were tested by indirect ELISA. Samples were diluted 1:20 in coating buffer and the following antisera BYMV, SMV and PVY diluted 1:1500 in PBS-TPO. Protein A-Alkaline phosphatase and GAR Alkaline phosphatase diluted 1:1000 in PBS-TPO were compared. Results are presented in Table 14. Infected guar readings were higher with Protein A and the healthy control was lower than with GAR.

Table 11: Indirect ELISA readings of guar varieties

	SAMPLE	BCMV	SNV	SBMV
Infected sap	Lewis green sterile	0.662	0.680	0.081
	TX-79-2741 Chlorotic	0.553	1.369	0.095
	TX-79-2741 stunting	0.535	1.284	0.080
	TX-79-2741 green sterile	0.419	0.394	0.056
	ZDPS "little leaf"	0.554	0.859	0.096
	HSB - 130 green sterile	0.649	0.672	0.087
	ZDPS - green sterile	0.318	0.323	0.085
Healthy sap	Lewis	0.125	0.137	0.066
	HSB-130	0.100	0.113	0.081
	TX-79-2741	0.126	0.143	0.064
	ZDPS	0.150	0.150	0.075

Buffer control 0.060

Absorbance readings are means from six replicates

Table 12: Indirect ELISA readings of purified guar virus

SAMPLE	BCMV	BYMV	SMV	PVY	TRSV
Purified virus from infected guar leaves ZDPS (procedure 1)	0.792	0.633	0.608	0.590	0.232
Purified healthy leaves of guar ZDPS (procedure 1)	0.179	0.151	0.183	0.157	0.173
Purified virus from infected guar leaves ZDPS (procedure 2)	0.202	0.234	0.239	0.212	0.235
Purified healthy leaves of guar ZDPS (procedure 2)	0.134	0.153	0.144	0.148	0.117
Purified virus from infected guar leaves ZDPS (procedure 3)	0.384	0.320	0.375	0.368	0.212
Purified healthy leaves of guar ZDPS (procedure 3)	0.187	0.142	0.122	0.127	0.096
Purified virus from infected guar pods ZDPS (procedure 3)	0.324	0.331	0.358	0.352	0.282
Purified healthy pods of guar ZDPS (procedure 3)	0.188	0.176	0.163	0.171	0.139
Buffer controls	0.046	0.044	0.043	0.046	0.044

Table 13: Indirect ELISA readings of pigeonpea

Sap dilutions \ Ab	Infected Pigeonpea		Healthy Pigeonpea	
	BCMV	BYMV	BCMV	BYMV
1:5	0.402	0.745	0.093	0.328
1:10	0.610	0.848	0.107	0.247
1:20	0.715	1.190	0.113	0.286
1:40	0.765	1.254	0.118	0.299
1:80	0.780	1.246	0.119	0.298

Buffer control 0.072

Table 14: Comparison between Protein A-Alkaline phosphatase conjugate and goat anti-rabbit conjugate in indirect ELISA

	Protein A		GAR	
	Healthy	Infected	Healthy	Infected
BYMV	0.187	0.408	0.259	0.260
SMV	0.123	1.491	0.206	0.307
PVY	0.095	0.226	0.125	0.127

6.4 Plate-trapped antigen (PTA) ELISA

6.4.1 Crude extracts of guar varieties and pigeonpea

Different infected varieties of guar leaves were tested in PTA-ELISA, (As well as infected pigeonpea) (Table 15). As controls, healthy guar varieties and healthy pigeonpeas were tested. Crude sap (5ml/1g leaf) was used undiluted. The following unfractionated antisera diluted 1:1000 in PBS-TPO were used: BCMV, BYMV, TRSV. Protein A-alkaline phosphatase 0.025u/ml in PBS-TPO was added. Results are presented in Table 15. Infected guar varieties and infected pigeonpea were positive to BCMV, BYMV and TRSV.

6.4.2 Purified guar leaves and pods and purified pigeonpea

Purified virus samples from infected guar leaves and pods, as well as leaves from infected pigeonpea, were tested in PTA-ELISA. Healthy controls were also tested. Virus samples were diluted 1:40 in coating buffer. BCMV and BYMV were diluted 1:1000 in PBS-TPO. Each antiserum from two different sources. Protein A-Alkaline phosphatase conjugate was diluted 0.025u/ml in PBS-TPO. Results are presented in Table 16.

Purified virus samples from guar and pigeonpea were positive to BCMV and BYMV.

6.5 Detection of virus in aphids by ELISA

There was no difference in absorbance readings between viruliferous and non-viruliferous aphids, using the indirect ELISA method.

Table 15: PTA ELISA readings of guar varieties and pigeonpea

SAMPLE	BCMV	BYMV	TRSV
Infected guar Lewis	0.270	0.385	0.269
Infected guar ZDPS	0.432	0.604	0.413
Infected guar ZDPS ("Little leaf")	1.426	1.587	0.577
Infected guar HSB-130	1.252	1.426	0.589
Infected pigeonpea	1.621	1.461	1.212
Healthy guar Lewis	0.241	0.256	0.186
Healthy guar ZDPS	0.185	0.216	0.158
Healthy guar ZDPS	0.157	0.165	0.159
Healthy guar HSB-130	0.120	0.175	0.132
Healthy pigeonpea	0.153	0.131	0.166
Buffer controls	0.036	0.042	0.045

Table 16: PTA ELISA readings of purified virus from guar and pigeonpea

SAMPLE	BCMV		BYMV	
	ENGLAND	G PIETERSEN	ENGLAND	G PIETERSEN
Purified virus from guar pods (Alper et al., 1984)	1.700	0.972	1.731	1.999
Purified virus from guar leaves (Alper et al., 1984)	0.786	0.773	1.242	1.999
Purified virus from guar leaves (Jafarpour et al., 1979)	1.999	1.999	1.999	1.999
Purified virus from pigeonpea leaves (Alper et al., 1984)	1.030	0.951	1.178	1.725
Purified BCMV	1.999	1.99[illegible]	0.116	0.117
Purified healthy guar pods (Alper et al., 1984)	0.138	0.139	0.137	0.148
Purified healthy guar leaves (Alper et al., 1984)	0.137	0.145	0.132	0.136
Purified healthy guar leaves (Jafarpour et al., 1979)	0.132	0.148	0.135	0.131
Purified healthy pigeonpea leaves (Alper et al., 1984)	0.131	0.138	0.136	0.133
Buffer controls	0.085	0.071	0.051	0.062

7. POLYACRYLAMIDE GEL ELECTROPHORESIS

7.1 Estimation of the molecular weights of viral proteins

The log 10 of markers of known molecular weight was plotted against distance migrated by the proteins (in mm). In this way a standard curve was constructed from which the unknown molecular weights of the viral protein bands could be determined (Fig. 48).

SDS-PAGE of semi-purified infected guar ZDPS leaf extracts (lanes 6 and 7) revealed several polypeptides not presented in healthy leaf samples (lanes 4 and 5) (Fig. 45). Lane 10 is semi-purified BCMV. An upper band of 95,000 daltons was observed in infected guar as well as bands appearing at 47-48,000 daltons, 43,000 daltons, 36-37,000 daltons and 33-34,000 daltons molecular weight. Five polypeptides bands were also observed from infected pod extracts (lane 5) but not from healthy pods (lane 4) (Fig. 46). An upper band of 95-80,000 daltons followed by two bands between 36 and 40,000 daltons and two lower bands (23,000 and 26,000 daltons) were observed.

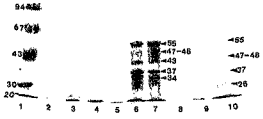


Figure 45: Ten per cent Polyacrylamide gel of infected and healthy guar leaves

lane 1 Molecular weight markers (X 10^3)
lane 4 Healthy guar leaves ZDPS (purification procedure 3)
lane 5 Healthy guar leaves ZDPS (purification procedure 1)
lane 6 Infected guar leaves ZDPS (purification procedure 3)
lane 7 Infected guar leaves ZDPS (purification procedure 1)
land 10 BCMV - England

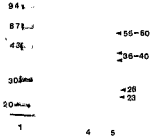


Figure 46: Five to fifteen per cent Polyacrylamide gradient gel of infected and healthy guar pods

lane 1 Molecular weight markers (X 10^3)
lane 4 Healthy guar pods (purification procedure 3)
lane 5 Infected guar pods (purification procedure 3)

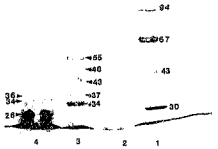


Figure 47: Ten per cent Polyacrylamide gel of infected and healthy guar leaves

lane 1 Molecular weight markers (X 10^3)
lane 2 Healthy guar leaves ZDPS (purification procedure 1)
lane 3 Infected guar leaves ZDPS (purification procedure 1)
lane 4 BCMV (England)

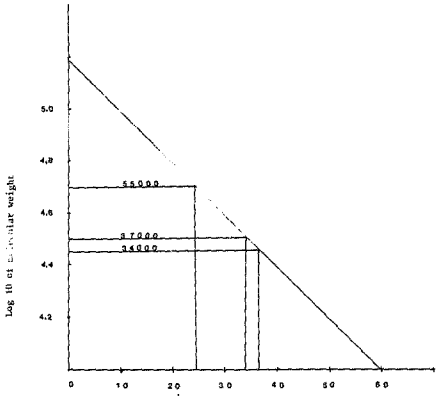


Figure 48: Estimation of the molecular weight of guar viral proteins

7.2 Immuno-electro blot

Purified virus (purification procedure 1) preparations from infected guar plants ZDPS and purified BCMV were run on PAGE gels (Fig. 47). Gels were treated with the following antisera: BCMV, BYMV, SMV and PVY. Immunoblots with BCMV antiserum showed strong binding to several polypeptide bands of BCMV (Fig. 47) mainly one at 26,000 daltons and two bands at 34-35,000 and 36-37,000 daltons molecular weight. Infected guar samples (lane 2, fig. 49) exhibited strong binding of BCMV antiserum to polypeptides with a molecular weight of 33-34,000 and 36-37,000 daltons and the upper 55,000 dalton band. Little or no binding was observed in the healthy guar leaf (lane 1, Fig. 49). Strong specific binding was again observed, to polypeptide bands at 33-34,000, 36-37,000 and 55,000 daltons from infected guar leaves (lane 2), employing PVY (Fig. 50), BYMV (Fig. 51) and SMV (Fig. 52).

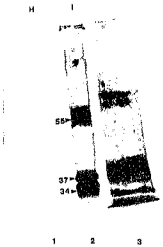


Figure 49: Immunoblot of purified healthy (H) and infected (I) guar preparations using BCMV (1:200 in PBS) antiserum

lane 1 healthy guar
lane 2 infected guar
lane 3 BCMV



Figure 50: Immunoblot of purified healthy (H) and infected (I) guar preparations using PVY (1:200 in PBS) antiserum

lane 1 healthy guar
lane 2 infected guar
lane 3 BCMV

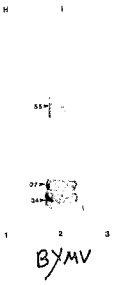


Figure 51: Immunoblot of purified healthy (H) and infected (I) guar preparations using BYMV (1:200 in PBS) antiserum

lane 1 healthy guar
lane 2 infected guar
lane 3 BCMV

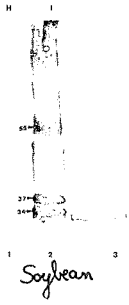


Figure 52: Immunoblot of purified healthy (H) and infected (I) guar preparations using SMV (1:200 in PBS) antiserum

lane 1 healthy guar
lane 2 infected guar
lane 3 BCMV

CHAPTER 4

DISCUSSION AND CONCLUSION

Although the symptoms of the disease of Cyamopsis tetragonoloba (guar) in South Africa appeared to be of a viral nature, preliminary tests for the identification of bacteria were carried out. A bacterium identified as Pseudomonas (Chapter 3, 1) was isolated from infected guar leaves. None of the symptoms associated with this bacterium, e.g. vascular wilts, stem cankers, leaf spots, soft rots, tumors or galls, blossom and twig blights were observed on infected guar plants. It was thus concluded that this bacterium was merely resident and not the causal agent of the disease. Consequently this investigation was aimed at elucidating the viral nature of the disease using techniques such as host range studies, virus purification, electron microscopy and serology. From these studies a potyvirus has been shown to be associated with the virus symptoms of "Green sterile" and "Little leaf".

Many trials were made to establish the optimal growth conditions of the guar plant (Chapter 2, 1). Deep pots were used in order to allow long guar roots to develop properly and high temperatures (28°C-30°C) were found to be essential for the guar growth. Although these optimal conditions were maintained, guar plants propagated in growth cabinets, were about 30cm in height, compared to 1m height for those growing under field conditions. Plants grown from seeds obtained from infected guar pods did not express the virus symptoms. In fact, there was no difference in appearance between healthy and infected guar grown from seeds.

Table 17: Varieties in South Africa tested and being tested for I-Gene (G. Pietersen pers. comm.)

Dominant I-Gene Resistance	Recessive I-Gene No Resistance	$Bc2^2$ Gene
Nep 2	Heuningberg	Teebus
Kamberg	Majuba	
Nuweveld	Bonus	
Carioca	Natal speckled	
A 40	Skurweberg	
Seafarer	Broadacres	
Top crop	Umvoti	
Contender	Lazy housewife	
Wintergreen		
Cape		
GV 2		
Harvester		
Seminole		
Strike		
Unknown		
Witsa		
Blue peter		
Flo		
Forté		
Labrador		
Provider		

Since only 50 per cent of guar seeds (Fig. 5) were able to germinate, its possible that these may be the healthy seeds which do not carry the virus. Therefore, it was necessary to use fresh material, i.e. guar plants showing symptoms of "Little leaf" and "Green-sterile" which were brought from the field in the Eastern Transvaal. When frozen guar leaves, or guar plants grown from infected seeds, were used, ELISA tests, host range inoculation and purification did not show satisfactory results.

No symptoms were observed on mechanically inoculated guar using infected guar sap, however, indicator plants inoculated mechanically, using sap (extracts) from infected guar, developed symptoms of red necrotic veins and chlorosis (Figs. 11, 12, 13, 16, 17, 18, 19, 21, 22a, 23 & 26) after one week, implying the presence of a sap transmissible agent. Symptoms were produced only at temperatures of 30°C and most of the indicator plants failed to produce symptoms at 25°C (Table 7). Symptoms were almost restricted to varieties of the genus Phaseolus, which indicates that the guar virus may possibly be restricted to viruses that infect the bean family. According to Table 19 the following bean varieties which had been used in our investigation have I-gene resistance: P. vulgaris vars. Nep 2, Top crop, Contender, Wintergreen and Seminole. The I-gene resistance implies that below a temperature of 29°C these varieties would not exhibit symptoms when challenged with BCMV pathotypes and a hypersensitive reaction would only occur at temperatures above 29°C (Kyle et al., 1988). In our investigation, Nep 2, Top crop, Contender and Wintergreen indeed showed symptoms only at a temperature of 30°C (Table 7), thereby suggesting that a hypersensitive reaction had occurred. However, on Top crop, Wintergreen and Seminole, symptoms also became systemic (Figs. 13, 18 and 21) and only on Nep 2 and Contender was the reaction restricted

to inoculated leaves (Figs. 11 and 17). These results indicate that the virus causes a hypersensitive reaction in some varieties at certain temperatures, but in others the symptoms spread to uninoculated leaves and it would appear that the virus isolated in this study does not behave exactly in the same manner as the BCMV pathotypes tested by Kyle et al. (1988). In the case of SMV or BCMV pathotypes NL-3, NL-5 and NL-8 (Drijfhout, 1978) the hypersensitive reaction is independent of temperatures which differs from the other potyviruses, e.g. Blackeye cowpea mosaic virus, Cowpea aphid-borne mosaic virus, Watermelon mosaic virus-2 and most pathotypes of BCMV (Kyle, 1988). It would, therefore, appear that even within the potyvirus group there is a range of symptoms with respect to the I-Gene reaction. Kelly et al., (1982) reported a new BCMV strain associated with the Sanilac cv. Electron microscope studies revealed the presence of the *flexuous rod-like virus particles characteristic of* BCMV in leaf tissue infected with the new strain. The new strain induces systemic top necrosis at relatively moderate temperatures (24 to 26°C), in commercial cultivars carrying 1-Gene resistance. The new strain which overcame the dominant I-Gene resistance found its way to Europe via snap bean seed multiplications by the Europeans in East Africa (Silbernagel et al., 1982). Genotypes with dominant I-gene will progress from necrotic local lesion to spreading veinal necrosis on the primary leaf ("road map"). In our investigation indicator plants which have the I-gene and were infected with the guar virus showed symptoms of veinal necrosis only at 30°C, meaning that the guar virus has not overcome the I-gene resistance.

Symptoms of red vein necrosis and chlorosis, curling and malformation were similar to those described by Bos (1971) for BCMV. He indicated that the nature and severity of symptoms vary greatly, depending on cultivar, time of infection and environmental conditions. French bean (Phaseolus vulgaris) is the common natural host. Sensitive cultivars may show a reddish rugose mosaic, rolling or curling (Bos, 1971). According to Bos (1971) BCMV produces no obvious local reaction in Chenopodium quinoa or C. amaranticolor, while Vicia faba and Pisum sativum have been reported to be immune to BCMV. Contradictory to Bos (1971), Noordam (1973) found C. quinoa a highly susceptible local lesion host of BCMV. Such differences in observation may be attributable to variable reactions of the BCMV strains. According to Drijfhout (1978), there are 15 strains of BCMV.

The reaction of certain P. vulgaris indicator plant, to the isolated virus from guar, was not similar to the local strain of BCMV (B. Eddington, personal communication). Noticeably P. vulgaris var. Sanilac is susceptible to BCMV, producing necrotic lesions or necrotic veins and chlorosis, and P. vulgaris cv. Peru 0251 is hypersensitive to BCMV above 26°C. In this study, Sanilac and Peru 025[illegible] exhibited no symptoms at either 25°C or 30°C. Comparing the virus to local BCMV strains may be useful. The appropriate comparison will be to strains prevalent in the area where the guar seed was produced.

Comparisons of symptoms induced by the guar virus in the Eastern Transvaal with other reported viruses were established. Top necrosis is a virus disease of guar

first observed by Chester in 1944. The leaf symptoms are yellowing, stunting, occasionally vein-clearing or a faint oak-leaf pattern and still more rarely, a light chlorotic mottling. Small chlorotic depressions on the leaflets became necrotic as the leaves became older (Cooper, 1949). An unidentified mosaic virus of guar, found in 1973 in Maricopa Country field in America exhibits mosaic mottling on leaves plus a reduction in the size of leaves and growth, as well as almost total suppression of seed pods (Streets, 1948). These symptoms were similar in some respects, i.e. reduction in leaf size and pods suppression to the symptoms in this study, which infected guar plants exhibit.

"Guar symptomless virus" has been so named since naturally infected and inoculated guar plants remained symptomless. According to Hansen and Lesemann (1978) this virus induced red lesions on the inoculated leaves of C. amaranticolor. In this investigation symptoms do not develop on C. amaranticolor and the naturally infected guar exhibits "Green sterile" and "Little leaf" symptoms. Since the causal agent was not re-isolated from the indicator bean plants, further transmission studies to other indicator hosts such as tomato and other cowpea varieties may prove useful.

Aphid transmission performed using Myzus persicae (Sulzer) to several P. vulgaris varieties (Table 8) showed that the virus was transmitted non-persistently. P. vulgaris varieties (Figs. 34-38) exhibited malformation and mosaic symptoms following the non-persistent aphid transmissions, BCMV, as the guar virus is also transmitted non-persistently by aphids (Zettler and Wilkinson, 1969). No symptoms were observed on healthy guar plants challenged with the virus, using aphid transmission. The mosaic virus isolated from the Maricopa Country fields in America is

also suspected to be transmitted by the cotton or melon aphid (Aphis gossypii) which is also able to transmit bean mosaic virus (Streets, 1948). No further work on this virus has been reported. Since aphid transmission studies were performed using only Myzus persicae, it would be useful to catch aphids in the guar fields and use these in transmission studies.

From the studies so far it would appear that the guar virus is neither mechanically nor aphid transmitted to guar but is transmitted chiefly to certain Phaseolus sp. vars. Monroe, Amanda, Diacol calima, Nep 2, Top crop, Prince, Bountiful, Double white settler, Contender, Wintergreen, Seminole, Golden podded wax, Lazy housewife, Genuine cornfield, Natal round yellow sugar, Blue peter, Abundance and Glycine max (Tables 7 and 8). Since aphid transmission and mechanical transmission yielded different symptoms, and since infected pods from var. wintergreen, when germinated, gave plants with leaf malformation and chlorosis but not red veins, there is a possibility of more than one virus strain. Transmission studies from infected guar to healthy guar were unsuccessful, but perhaps symptoms only appear later on mature guar plants in the field (1m height) and not on small guar plants grown in pots in growth cabinets.

Biological properties of virus were also used as a guide in identification. Estimation of thermal inactivation point (T.I.P.), dilution end point (D.E.P) and longevity in vitro (L.I.V) were made using Phaseolus vulgaris var. Top crop. The T.I.P was between 55°C and 60°C, the D.E.P between 10^{-3} - 10^{-4} and L.I.V was two to three days.

The results for the biological properties correspond to those documented for BCMV by Bos (1971): The T.I.P. is usually about 60°C but can vary depending on virus source, strain and environmental conditions. The D.E.P. is between 10^{-3} and 10^{-4} and sap retains infectivity at room temperature for one to four days.

Seed transmission was only achieved through mechanical inoculation of P. vulgaris var. Wintergreen (Fig. 39). It must be pointed out that only a proportion of seeds from infected pods were able to germinate. The heavily infected pods exhibits 50 per cent necrotic, wrinkly seeds (Fig. 5), which do not germinate and may well carry the virus. Indirect ELISAS (Tables 11 and 12) also indicate a positive reaction of infected guar pods with several potyvirus antisera, namely BCMV, BYMV, PVY and SMV. It appears, therefore, that about 50 per cent of the seeds are sterile and virus-infected, as is the case with other symptomless virus (Hansen and Lesemann, 1978).

Leaf dips from infected leaves failed to show the presence of viral particles, possibly due to low virus concentration. Purification methods and with fresh guar material were both successful. Using purification methods I and II developed by Jafarpour et al. (1979) and Alper et al. (1974) respectively, virus yields of 0.5mg per 100g fresh leaves were obtained.

The absorption spectrum of purified virus (Fig. 40) had a maximum at 260nm and a minimum at 246nm. The A260:A280 was 1.25 and the Amax:Amin was 1.11. According to Hollings and Brunt (1981) the u.v absorbance spectra of potyviruses are typical of nucleoproteins, with a maximum at 260 to 262nm and

minimum at 240 to 246mm. The A260:A280 ratios of 1.14-1.25, and Amax:Amin ratios of 1.11-1.27 are typical of nucleoproteins containing five to six per cent nucleic acid.

The yield of virus was low (0.5mg per 100g of leaves), probably because of aggregation with host protein and large amounts of phenolic compounds and polysaccharides in mature guar plants. Bos (1971) also reported the sedimenting of BCMV in association with chloroplasts. Most reports for purification of potyviruses indicate that only small amount of virus can be obtained and that methods for purifying one virus cannot be applied to other members of the same group. The advantage of Alper et al. (1984) purification method 3, compared to Jafarpour et al. (1979) purification method 1, was the use of very small amounts of leaves (20g). It must be emphasised that purified virus could be achieved only when unfrozen infected leaves from the field were used. Many attempts which were made to purify the virus from frozen leaves or leaves from plants from infected seeds growing in growth cabinet, were unsuccessful. Probably the guar virus is unstable during freezing and the infected seeds which germinate may not contain the virus.

The purification procedures of Jafarpour et al. (1979) and Alper et al. (1984) yielded flexuous virus particles of 750nm length and 15nm width (Figs. 41 and 43) which bear close resemblance to members of the potyvirus group. The best preparations were obtained by staining with two per cent phosphotungstic acid rather than with two per cent uranyl acetate.

Hansen and Lesemann (1978) found in negatively stained crude sap from infected guar, elongated flexuous

particles with a length of 761nm and a width of 12nm. Particle properties of other guar viruses (Chapter 1, 1.5) have not as yet been reported.

Ultra-thin sections from infected guar leaves and indicator plants infected with the virus did not reveal the presence of the virus, nor inclusion bodies, possibly due to low virus concentration. Inclusion bodies may accumulate at a certain stage of virus infection in some cells only, so further investigation at the appropriate stage may yet reveal these bodies.

Light microscopic examination of inclusion bodies was unsuccessful and further work in the area needs to be done. According to Hansen and Lesemann (1978) ultra-thin sections from guar infected with "guar symptomless virus", revealed inclusion bodies containing pinwheel and scroll elements.

The sensitive technique of immune electron microscopy also failed to detect the virus. IEM, with several viruses, for example bean yellow mosaic and bean common mosaic viruses, has failed to improve detection, compared to conventional electron microscopy (Milne, 1980). Bean yellow mosaic and bean common mosaic viruses are morphologically and biologically similar to virus particles isolated from infected guar in this study.

According to Cohen et al. (1982) the efficiency of trapping of four viruses tested depended markedly on the pH of the virus extract. They suggest that for each virus and host the extraction and testing procedures have to be worked out separately. According to Milne and Luisoni (1977), many variables can be

tested in the various methods described (Chapter 2, 8.8), involving incubation times of the serum or the virus, their optimal concentrations, the temperature of incubation and influence of agitation. In our investigation IEM was performed only on samples where no potyvirus was observed (frozen material). The potyvirus were found only in extraction from fresh field material with which IEM could have been successful. Unfortunately fresh leaves were not available throughout the year.

Since symptoms on indicator plants, mode of transmission, biological properties and particle morphology indicated that the guar virus may belong to the potyvirus group, serological relationships were studied, using antisera from various sources. According to Hollings and Brunt (1981), a virus is considered to be a definitive member of the potyvirus group if it

(1) has filamentous particles of characteristic size and properties;

(2) is transmitted non-persistently by aphids

(3) induces the formation of characteristic cytoplasmic inclusions ("pinwheels");

(4) is clearly serologically distinguishable from morphologically similar viruses.

Serological relationship to other definitive potyviruses is not an essential criterion for membership of the group.

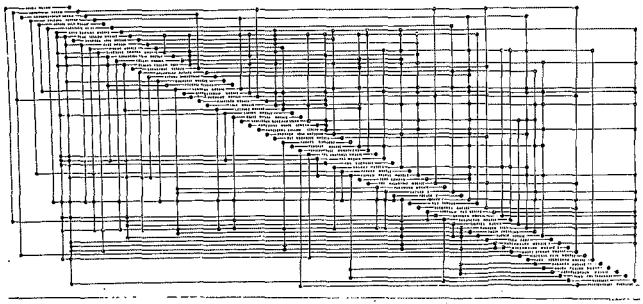


Figure 53: Serological relationships of some viruses in the potato Y group (adapted from Edwardson, 1974)
below *diagonal-related*
above diagonal-unrelated

As indicated by Van Regenmortel and Burckard (1980), the indirect ELISA procedure is better suited for diagnostic work because of its wide specificity. The antisera which were available by donation in this investigation are common viruses of the potyvirus group, which infect bean plants. *Indirect* ELISAS gave strongly positive absorbance reading for infected guar varieties ZDPS, HSB-130, Lewis and TX-79-2741 using BCMV, BYMV, PVY and SMV antisera. According to Figure 53, these viruses are serologically related. The results were positive, using crude extract (Table 11) or purified virus preparation from leaves and pods (Table 12).

The *rapid and simple procedure of* PTA-ELISA with the aid of unfractionated antisera gave some positive readings with BCMV and BYMV, using crude extract from guar varieties (Table 15) as well as purified virus (Table 16). Infected sap reacted with antisera to TRSV (Table 15). This is a nepovirus and the possibility of mixed infection should be investigated. According to Mowat and Dawson (*1987*) the *incubation regime adopted* is a compromise between the convenience of performing *the test as a* continuous operation and the need for it to be sensitive enough to detect or identify the test viruses. Yet, in this investigation there was no difference in results between indirect ELISA and this shorter procedure, the PTA-ELISA, which was more convenient and rapid.

$F(ab')_2$ ELISA *gave also positive* reading for infected guar var. TX-79-2741 using BCMV and BYMV antisera (Table 10).

According to Mowat and Dawson (1987) when virus-free extracts of leaf were tested by PTA-ELISA and $F(ab')_2$ ELISA, using the same antisera, similar absorbance readings were obtained by both forms of ELISA. Antisera was not available in sufficient quantity and hence direct ELISA could not be done and only broad antigenic specificity could be found by indirect ELISAS.

Protein A-Alkaline-phosphatase was found to be superior to Goat anti-rabbit conjugate. The positive readings were higher with Protein A-Alkaline phosphatase using the same antisera (Table 14).

There was no difference in absorbance readings between virus-carrying aphids and virus-free aphids. Gera et al. (1978) detected a viral antigen in aphids carrying a non-persistent virus, even on a single aphid, with ELISA test and Tamada and Harrison (1981) found potato leafroll virus in Myzus persicae. According to Tamada and Harrison (1981), the temperature affects the behaviour of the virus and may cause rapid degradation of potato leafroll virus particles in the gut at 25°C-30°C than at 15°C. Since the aphids in our investigation were not kept under a constant controlled temperature, the guar virus could have been degraded.

The strong serological relationship of the isolated virus from guar to the following potyviruses: BCMV, BYMV, PVY and SMV was confirmed with immunoblotting (Fig. 47), where strong binding took place of BCMV antiserum to polypeptide bands appearing at 33-34,000 daltons, 36-37,000 daltons and an upper 55,000 dalton molecular weight. Similar binding, but not as strong, was observed with PVY (Fig. 50) BYMV (Fig. 51) and SMV (Fig. 52). From SDS-PAGE gels, five polypeptide bands

present in infected guar leaves (Fig. 45) and pods (Fig. 46) but not in healthy extracts, were observed. From the molecular weights of BCMV proteins (34-35,000 and 36-37,000 daltons, Fig. 49) and BYMV (34,000 daltons, Alper et al., 1984; Stein et al., 1986) and SMV (30,700 and 29,244 daltons, Diaco et al., 1986), it appears as if the coat protein of the guar virus may be the 33-34,000 dalton band which is consistent with these potyviruses. The 36-37,000 dalton polypeptide band appears in both infected guar and BCMV (Fig. 45 and 49). The slower moving band (55,000 daltons) may possibly be inclusion body proteins, as in the case of BYMV, where two bands of 50-55,000 and 69,000 daltons were shown to be cytoplasmic cylindrical inclusion proteins (Alper et al., 1984). However, further electronmicroscopical investigations are necessary to establish the presence of inclusion bodies in infected guar.

Pigeonpea plants which were grown in the edges of guar field, showing symptoms of mosaic and sterility were suspected to be a source of infection in guar. Purification procedure 3 revealed virus-like particles (Fig. 44). Purified preparations as well as crude extract gave positive readings, using BCMV and BYMV antisera (Table 13 and Table 16). Sterility mosaic of pigeonpea has been reported in the Indian subcontinental (Kamaiyan et al., 1981) and further investigation has to be done to compare the virus from pigeonpea in South Africa to those found in India, and to establish whether pigeonpea acts as a primary source for guar infection under South Arican field conditions. The manner in which such transmission takes place needs also be be elucidated.

The morphology of the virus-like particles observed in extracts of guar with "Green sterile" and "Little leaf" symptoms is similar in structure to the potyvirus, "guar symptomless virus (GSV)", isolated from seeds from India, Pakistan and the U.S.A. (Hansen and Lesemann, 1978). However, red lesions developed on C. amaranticolor with GSV and not with the "Green sterile", "Little leaf" guar virus. Host range studies with Phaseolus vulgaris varieties were unsuccessful with GSV, whereas the guar virus in Southern Africa was transmissible to several P. vulgaris varieties. Unfortunately researchers working on GSV did not check for symptoms in guar under field conditions (Hansen, pers. comm.); so at present it is not possible to ascertain the relationship between GSV and the virus isolated in this study.

The disease of guar in Southern Africa is apparently associated by a transmissible virus of the potyvirus group, since antisera to several viruses of this group react positively with crude extracts of diseased plants. Identification of the causal agent requires fulfilling Koch's postulates, i.e. to isolate the causal agent and to show that when reintroduced into guar it causes the same disease. Since transmission in growth cabinets were unsuccessful, field trials could be carried out. Until such transmission to guar is achieved, the definitive causal agent of guar "Sterile" and "Little leaf" disease cannot be established. The only conclusion is that this disease is associated with a BCMV-like potyvirus which was isolated from guar plants (Fig. 41).

Since four antisera to various potyviruses reacted positively to extract of diseased plants as mentioned

previously, the possibility of a mixed infection by BCMV and another virus cannot be ruled out.

Establishing virus purity and identity needs to be carried out in the future.

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APPENDIX I

Medium 79 - *for* Rhizobium cowpea E group (Dr Beck - pers. comm.).

5g Mannitol
0.25g K_2HPO_4
0.1g $MgSO_4 \cdot 7H_2O$
0.05g Nacl
50ml yeast extract (make up a stock solution 10g yeast extract/1ℓ distilled water)
500ml distilled water.

The above were mixed together and autoclaved.

The Rhizobium cultures were kept on a gentle shaker in 25°C and transferred to a fresh medium every three weeks.

APPENDIX II

IDENTIFICATION OF BACTERIA (Schaad, 1980)

King's Medium B agar (KB)

	per l
Proteose peptone (Difco)	20.0g
$K_2HPO_4 \cdot 3H_2O$	2.5g
$MgSO_4 \cdot 7H_2O$	6.0g
Agar	15.0g
Glycerol	15.0ml

Gram stain

A thinly spread bacterial film was dried in air on a clear slide and then flamed to fix the bacteria. The smear was flooded with crystal violet solution for 1 minute, washed in tap water an dried. Then flooded with iodine solution for 1 minute and again washed and dried. Decolorisation was made with ethyl alcohol for 30 seconds, rinsed in tap water and counterstained with safranin solution for 10 seconds, then washed again and dried.

Hucker's ammonium oxalate crystal violet

Solution A.	Crystal violet (Difco)	2.0g
	Ethyl alcohol (95 per cent)	20.0ml
Solution B.	Ammonium oxalate (Fisher)	0.8g
	Distilled water	80.0ml

Solutions A and B were mixed and filtered.

Gram's modification of Lugol's solution

Iodine	1.0g
Potassium iodine	2.0g
Distilled water	300.0ml

Decolorizer

Ethyl alcohol 95 per cent

Counterstain

Stock solution

Safranin	2.5g
Ethyl alcohol 95 per cent	100.0ml

Working solution

Stock solution	10.0ml
Distilled water	90.0ml

Nutrient agar (NA)	g/l
Beef extract	3.0
Peptone (Difco)	5.0
Glucose	2.5
Agar	15.0

Yeast extract-dextrose-$CaCO_3$ (YDC)

	g/l
Yeast extract	10.0
Dextrose	20.0
Calcium carbonate	20.0
Agar	15.0

Gram's modification of Lugol's solution

Iodine	1.0g
Potassium iodine	2.0g
Distilled water	300.0ml

Decolorizer

Ethyl alcohol 95 per cent

Counterstain

Stock solution

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Beef extract	3.0
Peptone (Difco)	5.0
Glucose	2.5
Agar	15.0

Yeast extract-dextrose-$CaCO_2$ (YDC)

	g/ℓ
Yeast extract	10.0
Dextrose	20.0
Calcium carbonate	20.0
Agar	15.0

APPENDIX III

Buffers

1. 0.01M Potassium Phosphate Buffer, pH 7.0

200ml glass distilled water was added to 0.35g K_2HPO_4
200ml glass distilled water was added to 0.272g KH_2PO_4

When dissolved, the KH_2PO_4 solution was added to the K_2HPO_4 solution till a pH of 7.0 was reached.

2. 0.5M Potassium Phosphate Buffer pH 7.2

500ml glass distilled water was added to 43.75g K_2HPO_4
500ml glass distilled water was added to 34.00g KH_2PO_4.

The two solutions were mixed, the volume measured and 0.5M urea added as well as 1 per cent Na_2SO_3, 0.5 per cent 2-mercaptoethanol and 0.05M EDTA.
The pH was adjusted to 7.2.
Approximately 1.2ml of this buffer was added to 1g of fresh plant tissue.

3. 0.025M Potassium Phosphate Buffer pH 7.2

500ml glass distilled water was added to 1.7g KH_2PO_4
500ml glass distilled water was added to 2.19g K_2HPO_4

The two solutions were mixed, the volume measured and 1M urea, 0.1 per cent mercaptoethanol added and the final solution adjusted to pH 7.2.

4. 0.1M Borate/KCl Buffer pH 8.0

500ml glass distilled water was added to 3.09g Boric acid
500ml glass distilled water was added to 19.06g Borax ($Na_2B_4O_7 \cdot 10H_2O$).

The Borax solution was added to the Boric acid solution till a pH of 8.0 was reached. 0.1M KCl was also added.

5. 0.1M Phosphate buffer pH 8.2

0.1M solution of $NaH_2PO_4 \cdot 2H_2O$ (31.2g in 1000ml ddH_2O)
0.1M solution of Na_2HPO_4 (28.39g in 1000ml ddH_2O)

The two solutions were mixed and pH adjusted to 8.2.

APPENDIX IV

Transmission Electron Microscopy

1. Negative stains

(a) Phosphotungstic acid (PTA)

The stain was prepared by dissolving the solid dodecatungstophosphoric acid in glass distilled water to give 2 per cent (W/V) solution.
The pH was adjusted to 6.8 with 1N NAOH, centrifuged or filtered to clarify and stored in the dark.

(b) Uranyl acetate

The stain was prepared by dissolving the solid in distilled water to give 2 per cent (W/V) solution without pH amendment. The solution was kept in a dark bottle and refrigerated.

2. Preparation of thin sections for TEM (Provvidenti and Cobb, 1975; Venable and Cogglehall, 1965)

Materials

(a) 0.05M Sodium cacodylate buffer pH 7.2 (1.07g/100ml glass distilled water).

(b) 25 per cent stock gluteraldehyde solution diluted to 2.5 per cent with sodium cacodylate buffer.

(c) 4 per cent Osmium tetroxide stock solution diluted to 1 per cent with sodium cacodylate buffer.

(d) Epoxy resin:

The following components were mixed together:

DDSA	40g
Araldite	14.8g
Epon B12	20.6g
DMP 30 2.5 per cent	1.87g

3. Staining of thin sections

(a) Method

Drops of 1 per cent uranyl acetate were placed on dental wax layed on wet filter paper in a petri dish. Grids were inverted onto the drops for 20 minutes, then rinsed with distilled water and drained. The grids were allowed to dry on filter paper for 1 hour and then transferred onto drops of lead citrate for 15 minutes, rinsed with distilled water and dried.

(b) Materials

1. 1 per cent Uranyl acetate.

2. Lead citrate

Sodium citrate	1.76g
Lead nitrate	1.33g

These two components were added to 20 to 30ml distilled water and shaked vigorously for 1 hour, followed by 30 minutes occasional shaking. Lead nitrate was converted to a milky suspension of lead citrate. 8ml of fresh 1N NAOH were added to the milky suspension which changed to a clear one. Distilled water was added up to 50ml.

APPENDIX V

THE ELISA TECHNIQUE (Voller and Bidwell, 1977; Barbara and Clark, 1982; Mowat and Dawson, 1987)

(a) Materials

1. Coating buffer (0.05M sodium carbonate at pH 9.6)

Na_2CO_3	1.59g
$NaHCO_3$	2.93g
NaN_3	0.20g

Make up to 1 litre and adjust the pH to 9.6 with Hcl.

2. PBS (phosphate buffered saline)

Nacl	8.0g
KH_2PO_4	0.2g
$Na_2HPO_4 \cdot 12H_2O$	2.9g
KCl	0.2g
NaN_3	0.2g

Make up to 1 litre and adjust the pH to 7.4

3. PBS-Tween Washing solution

PBS, pH 7.4 with 0.05 per cent Tween 20

4. Virus buffer (also used as conjugate buffer) (PBS-TPO)

PBS-Tween	1ℓ
Polyvinylpyrrolidone (mw 10,000)	20g
Ovalbumin	2g

5. Substrate buffer

10 per cent diethanolamine adjusted to pH 9.8 with HCl.

diethanolamine	97ml
gd H_2O	800ml
NaN_3	0.2g

Make up to 1 litre with H_2O. Adjust to pH 9.8 with HCl.

6. 3M NaOH

120g NaOH/1 litre gd H_2O

7. 1 per cent BSA: (Bovine serum albumin)

II. Plystyrene microtitre plates.

III. Test samples: purified virus, sap extracts.

IV. Enzyme: Alkaline - phosphatase.

V. Substrate: 2-nitrophenyl phosphate in substrate buffer (1mg/ml).

Immunoelectroblot (Hibi and Saito, 1985)

Ab buffer

PBS-Tween
0.2 per cent BSA
2 per cent Polyvinylpyrrolidone

AP buffer pH 9.5

0.1M Tris
0.1M Nacl
5mM $MgCl_2$

Substrate

1mg nitro blue tetrosolium (NBT) was dissolved in 3ml AP buffer by mixing vigorously for 1 to 2 minutes, then centrifuged for 5 minutes at 10,000 rpm. 0.5mg 5-bromo-4 chloro- 3 indolylphosphate (BCIP) was dissolved in 10µl N,N-dimethylformamide by mixing with a vortex mixer for 1 minute and added dropwise with gentle mixing into the NBT solution.

Protein A-Sepharose affinity chromatography (Miller and Stone, 1978)

Buffer A pH 8.0

0.016M boric acid
0.012M NaCl
0.025M NaOH
0.1mM PMSF (= Phenylmethylsulfonyl-fluorid)
0.02 per cent NaN_3

Buffer B pH 3.0

0.1M glycine-Hcl
0.02 per cent NaN_3

Protein A column

10ml slurry of protein A-Sepharose CL-4B packed in K 9/15 (0.9 x 15cm) column, equilibrated with buffer A.

APPENDIX VI

SDS-PAGE (Shapiro et al., 1967; Weber et al., 1972)

I. Preparation of gels:

Reagents:-

(a) Acrylamide stock solution

22.2g acrylamide) dissolved in distilled
0.6g bis-acrylamide) water to 100ml.

May be filtered if cloudy.

(b) 10 per cent SDS made up in 100mls glass distilled water.

(c) Temed (N,N,N^1,N^1 - tetramethylethylene diamine)

(d) 10 per cent Ammonium Persulphate, freshly made.

(e) Lower gel buffer: 1.5M Tris-HCl buffer. (18.15g Tris base adjusted to pH 8.8 with 6N HCl, final volume 100ml).

(f) Upper gel buffer: 0.5M Tris-HCl buffer. (6g Tris base adjusted to pH 6.8 with 6N HCl, final volume 100ml)

10 per cent lower gel

Acrylamide stock solution	13.5ml
gd H_2O	8.4ml
Lower gel buffer	7.5ml
10 per cent SDS	0.3ml

Degas for 5 to 10 minutes, then rapidly add:-

Temed	0.03ml
Ammonium persulphate	0.3ml

The above components were mixed well, poured into gel holders and left for 1 hour to polymerise.

Upper gel (stacking gel)

Acrylamide stock solution	1.35ml
gd H_2O	6.0ml
Upper gel buffer	2.5ml
10 per cent SDS	0.1ml

Mix and add:-

Temed	0.01ml
Ammonium persulphate	0.1ml

Stacking gel was layered on top of the 10 per cent lower gel ($\pm$ 2cm) and left to polymerise. Once set, the gel cassettes were removed and placed in an electrophoresis vessel containing bath buffer (28.8g glycine, 6.0g tris, 2g SDS, water to 2000ml).

Gradient gel	5 per cent gel	15 per cent gel
Acrylamide	3.5ml	10.5ml
1.5M Tris buffer pH 8.8	5ml	5ml
+ 0.4 per cent SDS	0.2ml	0.2ml
gd H_2O	11.4ml	4.4ml
Ammonium persulphate	75µl	75µl
Temed	8µl	8µl

II. Sample preparation

(a) Splitting solution

Glycerol	10ml
Mercaptoethanol	5ml
10 per cent SDS	30ml
0.5M Tris buffer pH 6.8	12.5ml

Volume brought to 100ml with gd H_2O and a pinch of bromophenol blue added until colour is dark blue.

A ratio of 1:1 of the above solution and of the purified virus sample was used. All samples were heated in a boiling water bath for 5 minutes prior to layering on to the gel.

(b) Molecular weight markers

Component	Molecular weight (Daltons)
Phosphoralase b	94,000
bovine serum albumin	67,000
Avalbumin	43,000
Carbonic anhydrase	30,000
Soybean trypsin inhibitor	20,100
lactalbumin	14,400

III. Staining of gels

(a) Coomassie Brilliant blue 2g
Methanol 500ml
Acetic acid 100ml
gd water 400ml

Gels were placed in the stain and left overnight on a shaker.

(b) Silver staining (Wray et al., 1981)

1. Gels were soaked in 50 per cent methanol overnight.

2. Solution A: 0.8g Silver nitrate was dissolved in 4ml d H_2O.
Solution B: 21ml of 0.36 per cent Sodium hydroxide were mixed with 1.4ml of 14.8M ammonium hydroxide.
Solution C: Solution A was added dropwise into solution B with constant stirring and increased to 100ml with H_2O.

3. The gels were stained in solution C for 15 minutes with gentle agitation.

4. Gels were washed in deionized water for 5 minutes with gentle agitation.

5. Developer was prepared· 2.5ml of 1 per cent citric acid were mixed with 0.25ml of 38 per cent formaldehyde and increased to 500ml with water.

6. Gels were soaked in the developer until bands appear (less than 10 minutes).

7. Gels were washed with H_2O and placed in 50 per cent methanol to stop reaction.

IV. Destaining of gels

Methanol	500ml
Acetic acid	100ml
dH_2O	400ml

Gels were destained for 2 hours at 37°C.



Author Ben-moshe Hedva
Name of thesis Identification And Characterisation Of Virus Disease(s) Of Guar In South Africa. 1989

PUBLISHER:
University of the Witwatersrand, Johannesburg
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