A virus disease of *Phaseolus vulgaris* L. in the Transvaal

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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.

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ABSTRACT

Viral pathogens are known to infect Phaseolus spp. worldwide. A local survey was conducted to determine if viruses were associated with any disease symptoms in local varieties of Phaseolus spp. From the regions sampled, only flexuous rods and a spherical particle were isolated.

On the basis of particle morphology, differential host reactions and serological tests, one flexuous form was identified as BCMV. The identity was confirmed after a detailed investigation of viral properties and after using standard antiserum supplied from the World Reference Collection. Assay on Phaseolus vulgaris var. "Monroe" showed that the T.I.P. was 55°C to 60°C, the D.E.P. 10^{-3} to 10^{-4} and the L.I.V. one day. Purified capsid protein and nucleic acid components were analysed using PAGE to determine their molecular weights. The strain was determined using the scheme proposed by Dijfhout (1978). Results indicate that this virus may be related to the NL5 strain of BCMV.

The ELISA technique was used to determine if this isolated virus and cross-reacting viruses could be detected in bean seed. Results indicate that BCMV may probably be detected in seed-coats and embryos and occasionally in cotyledons. The ELISA results obtained correlated well with infectivity assays on Phaseolus vulgaris var "Bataaf" and suggests that a satisfactory routine assay for seed-screening could be established with further work.

List of Abbreviations

AMV	alfalfa mosaic virus
BBMV	broad bean mottle virus
BBSV	broad bean stain virus
BCMV	bean common mosaic virus
BPMV	bean pod mottle virus
BVN	black vein necrosis
BYMV	bean yellow mosaic virus
CCI	cylindrical cytoplasmic inclusion
CPG	controlled pore glass bead system
CMV	cucumber mosaic virus
CoMV	cowpea mosaic virus
C	crystal
EAKBMV	Echtes Acherbohnenmosaik Virus (broad bean true mosaic virus)
EM	electron microscope
ELISA	enzyme linked immunosorbent assay
f	filament
flex virus	flexuous virus particles
Fr I	fraction I host protein
HP	healthy plant
IP	infected plant
IgG	immunoglobin G (gamma immunoglobulin)
IEM	immunoelectronmicroscopy
LL	local lesion
LVN	local vein necrosis
mm	millimeter
MLO	mycoplasma like organism
M	mosaic
NLL	necrotic local lesion
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
PSV	pea seed borne virus
PTA	phosphotungstic acid

PVY	potato virus Y group
RBC	red blood cell
RPM	revolutions per minute
SG	starch grain
SV	spherical virus
SDS	sodium dodecyl sulphate
TEM	transmission electron microscopy
TMV	tobacco mosaic virus
TRIS-TGA	Tris-thioglycollic acid buffer
TNV	tobacco necrosis virus
UV	ultraviolet
VB	virus band
WP	wall proliferations
#	number

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CHAPTER 1

Introduction and Review of the Literature

Beans can comprise an important protein component in the human diet. Despite the plants' potential to produce several thousands of kilograms of beans per hectare (Coertze, 1978(a)) yields can be well below expected levels. This great difference between potential and actual yield is primarily a result of plant disease/insect complexes and soil nutritional problems encountered in the field (Schwartz and Gálves, 1980).

Beans are grown primarily in tropical and temperate zones throughout the world. Historically, the common bean has been one of the most important crops of tropical and sub-tropical areas since colonialization (Bird and Maramorosch, 1975). Along with corn, rice and other starchy crops it has been one of the staple components of the diet providing a good proportion of protein, enough to sustain human growth without supplementation.

Beans originated in Central Mexico, yet have only become of importance since the development of organized agriculture several hundred years ago. Through centuries of cultivation several varieties have evolved, adapting to the selection pressures imposed by disease and pests, consumers and growers. More recently however, manmade selections and crosses have helped improve the protein content and palatability (Echandi, 1975).

There are over 200 species of *Phaseolus* of which the most important cultivated species are: *P. lunatus* Mackie, *P. acutifolius* Freeman, *P. vulgaris* L. and *P. coccineus* Bukasar. The most successful varieties have been obtained from crosses using *Phaseolus vulgaris* as the female parent.

Beans are primarily produced by farmers of low economic resources and therefore on a small scale. In regions of Central and South America however, farms have become larger as these more temperate regions are also less affected by pathogens compared with farms in the tropical zones.

Bean production was introduced into South Africa during the first years of settlement at the Cape, that is, the last half of the 17th Century. The dry and green bean cultivars grown were developed from species originating in Central America (Coertze, 1977(a)). Today, these two distinct types of beans are still grown and the variation in quality of pods and seeds distinguishes their use. Green beans are therefore grown because the unripe pod is edible whereas those of dry beans are not and they are cultivated for seed.

The tropical origin of beans is reflected in their growing requirements as they can only be cultivated at temperatures ranging between 10°C and 35°C and they are sensitive to frost. Beans are therefore primarily produced in South Africa wherever these temperature requirements are met and where water is present in sufficient quantities (Coertze, 1977(b) and (c)). No statistics are available regarding the surface area of beans planted in the four provinces. In general, the following areas produce most of the beans grown in South Africa.

1.1. <u>Transvaal</u>

Beans are produced in two regions: throughout the year in the Lowveld yet only in the summer to late autumn in the remaining Transvaal regions owing to frost. In the Lowveld, the environs of Louw's Creek, Kaapmuiden and Malelane form a most important area where a large percentage of South African beans are produced. The Lowveld bridges the gap for

winter production for the entire country. In the Highveld, areas in Springs, Potchefstroom, Delmas, Klerksdorp and Ventersdorp play a major role in the production of fresh market green beans. The municiple areas of Pretoria, Vereeniging and the Witwatersrand are additional regions of fresh bean production for local markets. Smaller regions such as Rustenburg and Lydenburg also produce beans for local consumption.

1.2. Orange Free State

Bean production is of minor importance and only carried out on a small scale near the irrigation schemes of areas such as Ficksburg.

1.3. Natal

The coastal areas of Durban as well as northeast Natal form the major bean producing areas for the domestic market.

1.4. Cape

This is the second most important bean producing area in South Africa, the south-western Cape being involved in large scale production schemes.

Detailed surveys of the main markets in the Republic have estimated that thousands of tons of fresh green beans are consumed, processed and exported annually. The total green bean harvest as early as 1969 was valued at R2,25 million and evidence indicates that the value of the industry has increased considerably (Dry Bean Report, 1979).

Dry beans are produced on an even larger scale than fresh beans. However, when compared to world production (Table 1), the area is small.

Table 1: Dry bean production in the world (1975-1979)
(Schwartz and Gálvez, 1980)

Production areas	000 ha	Total production (tons)	Av. yields (kg/ha)
Brazil	3788	1973	521
Latin America	6486	3677	567
Africa	1961	1106	564
South Africa	69	64	727
Total for the World	23722	12392	522

Yields of beans are influenced by the season, area, growing conditions, pathogens and cultivar differences. Of all the different types of cultivars available, only a few can be used for production purposes in South Africa (Coertze, 1980). Changing weather conditions, poor soil fertility, insect pests and diseases are the most important factors limiting production (Schwartz et al., 1978). Tropical environmental conditions for example, encourage greater infection by pathogens such as fungi, bacteria, nematodes, mycoplasmas and viruses compared with temperate zones. More specifically, there are several viruses of economic importance that affect the genus Phaseolus (Table 2). Virus diseases such as bean golden mosaic may be restricted to specific geographical regions while others such as bean common mosaic, are worldwide in their distribution. Economically, these diseases affect bean production as they are difficult to control once established in a crop. Schwartz and Gálvez (1980) have shown that large yield losses can occur in those plants affected by viruses such as BCMV and BYMV (Table 3).

Table 2: Principle virus diseases affecting beans

VIRUS	TRANSMISSION AND VECTORS	DIFFERENTIAL HOSTS	SYMPTOMS	PARTICLE	DISTRIBUTION
Bean pod mottle virus	Leaf beetle, sap	Glycine max. Only host range members are limited to members of the Leguminosae primarily Phaseolus vulgaris species.	causes severe leaf mosaic, mottled pods. Plants become severely stunted	isometric 30 nm in diameter	southern and eastern U.S.A.
Bean yellow mosaic virus	aphid species, sap, seed occasionally	affects leguminous and often non leguminous hosts. Diagnostic species are members of the genera Phaseolus, Pisum, Chenopodium and Vicia.	causes mosaic diseases in many legumes, necrosis symptoms for some plant species	flexuous particles 750 nm long	worldwide
Broad bean mottle virus	sap, vectors not known	host range little studied Infects mostly legumes. The diagnostic species are members of genera Phaseolus Vicia, Pisum, Nicotiana and Chenopodium.	causes chlorotic mosaic and vein clearing in most hosts	isometric 26 nm in diameter	England
Broad bean stain virus	seed, sap, vectors not yet known	hosts restricted to the Leguminosae; diagnostic species are members of the genera Phaseolus, Pisum, and Chenopodium	causes chlorotic mottle and mosaic of infected leaves. Also necrotic staining of seed testa	isometric 25 nm in diameter	Europe and North west Africa
Broad bean true mosaic (BABBMV)	seed (common), sap, vectors not yet known	only hosts known are legumes. Diagnostic species are members of the genera Vicia, Pisum, Phaseolus and Chenopodium	commonly causes mosaic symptoms in bean crops	isometric	Europe and North west Africa

VIRUS	TRANSMISSION AND VECTORS	DIFFERENTIAL HOSTS	SYMPTOMS	PARTICLE	DISTRIBUTION
Cowpea aphid borne mosaic virus	aphids, seed, sap	infects many species in the Leguminosae. Members of the genera Chenopodium, Nicotiana and Phaseolus are also affected	causes a severe mosaic of cowpea with vein banding, inter-veinal chlorosis leaf distortion blistering and stunting	flexuous particles 750 nm long	occurs in Africa, Europe, Asia, U.S.A. and Pacific area
Pea enation mosaic virus	sap, aphids	restricted host range, infecting members of the families Leguminosae, Solanaceae, Chenopodiaceae	infected plants develop mosaic, translucent spots and enations	isometric 30 nm in diameter	northern temperate regions
Bean mild mosaic virus	sap, beetles	infects many cultivars of Phaseolus vulgaris. Affects other leguminous hosts; host range is restricted	mild mosaic symptoms accentuated when virus occurs with mixed infection	isometric 28 nm in diameter	El Salvador, Columbia
Potato virus Y	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	flexuous particle 730 nm x 11 nm	worldwide
Cowpea severe mosaic virus	beetle, weeds, sap	host range limited to members of the Leguminosae and members of the Chenopodiaceae	chlorotic, necrotic lesions on inoculated leaves, mosaic, mottle, blistering and distortion of leaflets, some plants collapse	isometric 25 nm in diameter	U.S.A., Brazil, Peru; mainly South America

VIRUS	TRANSMISSION AND VECTORS	DIFFERENTIAL HOSTS	SYMPTOMS	PARTICLE	DISTRIBUTION
Cowpea mottle virus	beetle, seed, sap	host range restricted to members of the Solanaceae and Leguminosae	mottling or bright yellowing in some plants, leaves distorted, stunted, witches broom syndrome. Systemic mosaic	isometric 30 nm in diameter	only reported in Nigeria
Bean golden mosaic virus	whitefly, weeds, sap	host range restricted to members of the Leguminosae only	characteristic leaf colouring stunted plants, malformed pods	geminate, 25 nm in diameter	worldwide in tropical zones
Southern bean mosaic virus	sap, seed, beetles	restricted to the Leguminosae. Infects several varieties of Phaseolus vulgaris	necrotic lesions, vein banding, mosaic	isometric 29 nm in diameter	America, Africa
Cowpea mild mottle virus	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	flexuous particle 650 nm long	eastern region of Ghana
Tobacco necrosis virus	sap, soil borne by fungus Olpidium brassicae, not in seed	infects many different hosts. Diagnostic species and assay species are members of the genus Phaseol...	causes various symptoms including tulip necrosis, bean stipple streak, bark split symptoms in trees	isometric particle 26 nm in diameter	worldwide
Tobacco mosaic virus	sap, and externally on seed, but not by vectors.	transmissible to many hosts. Diagnostic species are members of the genus Nicotiana	mosaic, veinclearing, distortion, blistering of infected leaves	helically symmetrical particles	worldwide

VIRUS	TRANSMISSION AND VECTORS	DIFFERENTIAL HOSTS	SYMPTOMS	PARTICLE	DISTRIBUTION
Cucumber mosaic virus	sap, aphids, seed, dodder	host range is wide. Trannsmitted to several members of the genera Cucumis, Nicotiana, Phaseolus and Vigna	mosaic, blight, woodiness, fern leaf, variable symptoms depending on the plant infected	isometric, 30 nm in diameter	worldwide in temperate regions
Soybean mosaic virus	sap, aphids, seed	restricted host range to Glycine max and members of the genera Phaseolus, Chenopodium	causes mosaic of soybeans	flexuous particles 750 nm	worldwide
Alfalfa mosaic virus	sap, aphids, seed	affects primarily species of the genera Nicotiana, Chenopodium, Phaseolus, and Pisum	causes various mosaics, mottles and malformations in lucerne and other hosts	spherical to bacilliform particles 60 nm in size	worldwide
Red clover vein mosaic virus	sap, aphids, not common in seed	host range is restricted to the families Leguminosae Solanaceae, Chenopodiaceae, Amaranthaceae	causes vein mosaic, mosaic, streaking and stunting in various legumes	filamentous, tubular particles 645 x 12 nm	widely distributed in Europe, North America, and South Africa
Bean common mosaic virus	sap, aphids, seeds, pollen	transmitted to several members of the Leguminosae (especially Phaseolus vulgaris varieties) Chenopodiaceae, and some Solanaceae	causes common mosaic with leaf malformation and rolling, black root symptoms, vascular necrosis and death depending on strain and host	flexuous filamentous particle 750 nm long	worldwide

Adapted from Kranz et al (1977) and CMI/AAB descriptions of plant viruses

Table 3: Estimated bean yield losses attributed to BCMV and BYMV (Schwartz and Gálvez, 1980) for 1999.

Disease		Estimated Yield Loss
1. BCMV	USA	53 - 68%
	Latin America	16 - 95%
2. BYMV	Brazil	48 - 85%

1.5. Some viruses affecting beans worldwide

Bean common mosaic virus was first observed in 1899 by Iwanoski (in Drijfhout, 1978) and became one of the first virus diseases to be reported. Since then, this seedborne virus has been reported worldwide. BCMV may incite three types of symptoms, namely mosaic, systemic necrosis or local lesions. Symptom expression and the severity of the disease is determined by virus strain, age of the plant, climatic conditions, time of infection and the bean cultivar planted. Yield loss is attributable to a lower number of pods being borne by infected plants (Hampton, 1975).

Bean yellow mosaic virus causes symptoms that are similar to BCMV but far more severe. With infection, considerable reductions in pod number and seed yield occur (Schwartz and Gálvez, 1980).

BCMV and BYMV can be considered to be two of the most important pathogens transmitted in a non persistent manner by aphids (Watson, 1972).

Bean golden mosaic virus has become a serious problem in tropical areas of the world. Infected plants may readily be recognised by their leaf colour and the presence of stunted and malformed pods.

Bean pod mottle virus occurs frequently, usually affecting seedlings. The plants become severely stunted, often exhibiting a characteristic witches broom effect. Yield can be severely affected.

Bean golden mosaic virus and bean pod mottle virus are examples of pathogens transmitted by whitefly and beetles respectively. Also, weeds such as Euphorbia sp. and Sida sp. may serve as resevoir hosts from which these viruses are transmitted to new crops by mechanical inoculation.

Bean rugose mosaic virus and Bean southern mosaic virus are also important yield-reducing viruses, transmitted by yet another means. They are mechanically transmitted by chrysomelid beetles of the genera Cerotoma and Diabrotica. Yields are reduced as plants become stunted and blistered and pods become affected by water-soaked lesions.

These viruses therefore represent some of the most important pathogens that infect beans worldwide. As indicated, they may be spread by several means: mechanical inoculation, vectors such as aphids, beetles, whitefly and leafhoppers, or they are carried in seed. Transmission of viruses is an important consideration in the epidemiology of any disease and the success with which bean viruses have established themselves worldwide is probably related to their variable transmission mechanisms. In general, bean viruses have the potential for rapid spread (Costa, 1969; Jafarpour et al., 1979).

Virus diseases of beans occuring in South Africa were first documented by Klesser (1960; 1961), symptomatology being used for the identification of all the viruses. Only BCMV and BYMV appeared to be definitely present, although various viruses were listed by other names. Several "new viruses" were therefore described. It is quite possible however that

factors such as test plant differences, climatic and environmental conditions could have accounted for the variation in symptoms recorded and that no new viruses were indeed present.

To date viruses have remained of little apparent economic importance in Transvaal bean crops. The pathogens causing outbreaks of diseases of legumes in the Transvaal have rarely been identified. Occasional outbreaks of BCMV or BYMV have occured, although this documentation is based on symptoms only.
In 1977, an unknown virus disease affecting sugarbeans was recorded in the Koster district. The virus was thought to be transmitted by a sap-sucking leaf hopper population whose numbers increased rapidly, causing the entire field to be affected by the virus (Dry Bean Report, 1979). A virus causing necrosis has also been recorded (Boelema, 1981).

It appears that in the major bean growing areas of the Transvaal, non viral pathogens cause greater damage and yield losses than viruses. It is possible that the climate, strict control of vectors and the certification of seed have prevented viruses from becoming well established in this region.

Reliable identification of viruses is the cornerstone of efficient disease control (Bos, 1976). Viruses were initially known only by their infective and biological properties. Host range, symptoms, mode of transmission and the types of vectors involved were the first properties investigated for any unidentified virus. These properties were soon found to be unreliable as they depended on virus source, test plant type and other factors that could be influenced by external conditions. As a result, the identity of any virus described by these means became questionable and uncertain. With the ever increasing

numbers of viruses that were being discovered, a more reliable distinction, based on the actual characters of the virus itself and not on the host reaction, became essential.

Early workers attempted to determine the physio-chemical features, thought to be more representative of the virus itself. Significant advances in virus identification occured following the visualization of virus with the electron microscope. It has since become essential to chemically characterize any unknown virus before it may be described and to determine serological relationships with other viruses.

There are several basic procedures to follow when attempting to diagnose an unknown disease, presumed to be viral in origin (Bos, 1976; Hamilton et al., 1981). When an unknown virus disease is being investigated, two phases of research occur. In the initial diagnostic phase we examine those simple aspects of a virus which will enable us to classify it into a broad group. If the unknown virus is to be identified, the diagnostic phase passes into the descriptive phase in which several, more specific properties are investigated.
An outline of the basic procedures that can be used for virus diagnosis and description is presented in Table 4, some of the procedures being described in greater detail. It must be noted that to use most of these procedures, a virus must be transmissible in the laboratory and be able to be investigated outside the host. Diagnosis of a disease is therefore only complete once the causal agent has been isolated from the host, characterized and Koch's postulates have been fulfilled. It is only by using these procedures that an isolate can be identified as new or deviating little from an already described virus.

Table 4: An outline of the basic procedures used in identifying and describing an unknown virus

The information sought during both the diagnostic and descriptive phases can be divided into two sections:

A. What can be learned from the virus *in situ* or from crude preparations

B. What can be learned from purified virus preparations

A. Using virus preparations or virus *in situ*
   1. reproduction of the disease using the isolated virus
   2. host range studies and symptom expression
   3. mode of transmission
   4. determination of biological properties such as the thermal inactivation point i.e. '*in vitro*' properties
   5. electron microscopy
   6. simple serological tests i.e. reactions with likely antisera

B. Using purified preparations
   1. particle fine structure and sedimentation properties
   2. serological analysis
   3. analysis of protein component and nucleic acid properties

(adapted from Bos, 1976; Hamilton et al, 1981)

Most of the techniques used in the procedures for the isolation and characterization of an unknown virus are universal and are therefore only briefly described.

1. Inoculation of test plants

   Initially, inoculation of a series of indicator plants is essential to ensure that the causal virus has been isolated and that disease is not caused by a mixture of viruses. The isolate must always be re-inoculated to the original host which is then observed for typical symptoms of the disease. It is essential to be able to reproduce the disease using the isolated virus to fulfill Koch's postulates.

Inoculation of a specific host range can be carried out but is now considered not to be a reliable guide for virus identification. Exhaustive host-range tests are no longer appropriate (Hamilton et al., 1981) yet these preliminary investigations are useful for isolating the best laboratory hosts (Hollings, 1966).

2. Electron Microscopy

Electron microscopy of particles in negative stain is often very useful at the "crude extract" stage and sufficient diagnostic (although not fully descriptive) information can often be obtained. Often, a virus can be tentatively assigned to a group on the basis of particle morphology. More information, however, can be gained with the aid of immunoelectronmicroscopy, using antisera and purified virus samples. Cytopathological changes in host tissue, examined at the light and electron microscope levels, can also assist in identification of an unknown virus (Christie and Edwardson, 1977).

3. Serology

Rapid and accurate diagnosis of an unknown virus is often possible directly on crude preparations using precipitin tests, immunosorbent assays and various types of immunoelectronmicroscopy. Serological methods can obviously be applied to purified virus preparations as well.

4. Purification procedures

The choice of extraction buffers and additives depends on the virus being purified. Generally, methods are selected according to particle morphology and preliminary investigations of virus properties.

5. Electrophoresis

Determination of nucleic acid type and molecular weight by gel electrophoresis can assist in the assigning

of an unknown virus to a taxonomic group. More recently however, the sophisticated technologies of nucleic acid hybridization and amino acid sequence analysis have been used to establish the identity of unknown viruses as these methods are far more accurate.

6. Centrifugation

The sedimentation behaviour of viruses in various solutions using different types of ultracentrifugation has provided much detail about particle size and structure. Sedimentation coefficients and buoyant density values have become important taxonomic characteristics and should be determined for any unknown virus.

It is only as a result of the development and introduction of the sophisticated techniques mentioned above that much of the confusion of virus identity has been eliminated. In fact, only progressive methodology has enabled the distinction of those viruses affecting beans. By using methodology as it became available, more and more strains of a virus such as BCMV have been identified and described (Silbernagel, 1969; Kaiser and Mossahebi, 1974; Lockhart and Fisher, 1974; Gathuru and Corbett, 1977).

Once economically important viruses have been recognised, control measures can be introduced (Neergaard, 1979). In general, viruses can be controlled by

1. reduction and prevention of spread of established inoculum, including vector control
2. chemical control mechanisms
3. detection and elimination of infected material including seed indexing
4. reducing the susceptibility of plants to pathogens

One of the most effective methods is to limit the source of virus by quarantine restrictions and the distribution of healthy stock material. It is essential that plant material be tested for viral infections as once affected, planted fields cannot be cured. A crop must begin with clean stock, tested by various indexing methods. Conventional methods such as field trials have become unreliable and impractical. As a result, a new field of chemical indexing has been developed by workers such as Paul (1974). He carried out SDS - PAGE on small quantities of sap without extensive purification procedures. Viral bands could be detected. Serological tests have received even more attention as they are rapid and more specific than conventional tests. Tolin (1977) has proposed a diffusion test adapted to field conditions for identifying legume viruses.

For some viruses affecting beans, such as BCMV, infected seed is the primary source of infection (Hampton, 1975). Control of disease can only be achieved if infected seeds can be detected and eliminated.
To date, seeds have been treated in different ways to eliminate virus. These methods have proved ineffective as viruses are extremely resistant (Provvidenti and Braverman, 1976). Seed certification schemes employing serological and other techniques, appear to be the only reliable means of monitoring seed lots.

Due to the importation of large quantities of bean seed into South Africa, serological techniques should be exploited, as they are extremely sensitive, for the detection of seed borne viruses. The ELISA technique for example, has successfully detected virus in soy-bean seeds (Bossennec and Maury, 1977) and in lettuce seeds (Jafarpour et al, 1979).

Several modifications of the ELISA technique exist, for

example the direct and the indirect methods. The type of ELISA technique selected depends on the experiment. Koenig (1981) has suggested that the direct ELISA be used for the routine detection of virus in infected material while the indirect ELISA is more useful in the determination of serological relationships.

In conclusion, to effectively control viruses, a combination of all methods mentioned would be advisable, although seed certification schemes and breeding for resistance, if perfected in all major bean producing countries, would eliminate the need for other forms of control to be implemented.

In South Africa the following recommendations for the control of virus disease in beans have been made (Dry Bean Report, 1979):

1. Plant crops in September, that is, early summer. January plantings in the Highveld are highly susceptible to virus contamination due to increased insect populations.
2. For those diseases that are seed transmissible, only certified seed must be planted.
3. Resistant cultivars must be planted.
4. Pest control is essential. Once a disease is established, pest control procedures are worthless.

For efficient control in any country, knowledge of the different types of viruses and the existing strains must be accumulated. To date, indicator studies alone have been used in South Africa and few viruses have been detected. In view of the large numbers of viruses affecting beans and their wide distribution patterns, it is likely that additional viruses are present in local areas.

A study was therefore conducted in an attempt to determine what viruses could be detected in leaf samples collected from farms in the Transvaal.

1.6. The aims of the investigation were:

1. to survey local bean farms in the Transvaal for infected leaf material showing virus-like symptoms
2. to confirm the presence of any virus associated with the collected material by indicator studies
3. to attempt to purify the isolates
4. to identify one isolate using standard procedures
5. to prepare antiserum against this isolate
6. to establish if the enzyme linked immunosorbent assay (ELISA) technique could be used to detect the isolate in seed.

CHAPTER 2

Materials and Methods

All procedures used for the propagation, isolation and characterization of the virus in this study are described below.

2.1. Sampling sites and collection of leaf material

Leaf material showing symptoms was randomly collected from fields located in the major bean producing areas of the Transvaal. Farms from the following areas were included in the survey: Potchefstroom, Ermelo, Delmas, Nigel, Oogies, Bombardie, Witkop and Kendal. More than one sample was taken from those farms found to be producing different varieties of beans (Table 5). The farms in the Highveld were surveyed during February 1981 when the plants were approximately 5 weeks old. All leaf samples were placed in bags on ice and transported back to Johannesburg the same day. A freeze dried preparation of each sample, ground in 7% peptone/dextrose was made and the remaining portion was stored at -70°C until they were processed.

In addition, seeds suspected of being infected with virus were provided by Mr. Vermeulen. The seed was obtained from a trial carried out in Potchefstroom that developed mosaic type symptoms. The origin of the seeds (247 and 259) is outlined below:

Parents	Michelite x Great Northern
F_1	Result x Nep II
F_4	Result showing virus type symptoms

The F_4 generation aquired an infection apparently due to local contamination, the rows showing symptoms being known as 247 and 259. The presence of latent virus in the seed of one of the parents however, cannot be ruled out.

A further sample was a freeze-dried preparation of bean leaf sap provided by Roodeplaat Research Institute. The sap was obtained from bean plants found growing in a Pretoria garden in 1978 that showed apparent viral symptoms.

Table 5: Sampling sites

Area	Bean type	Symptoms
Nigel	Nep II	Leaves slightly yellow, small, slightly malformed, plants slightly stunted
Nigel	kidney	Leaves showed slight yellowing
Nigel	kidney	leaves showed slight yellowing
Nigel	kidney	leaves showed slight yellowing
Oogies	sugar	leaves showed slight yellowing
Oogies	brown haricot	leaves showed slight yellowing
Oogies	grootwit	leaves showed slight yellowing
Delmas 1	coffee	slight yellowing, curling, wilting of leaves
Delmas 2	kidney	slight yellowing of leaves with blotching
Delmas 3	haricot	slight yellowing of leaves, speckling, leaf curl, dark green prominent areas
Delmas 1	kidney	white patchy areas
Delmas 2	kidney	unusual leaf edges, malformation of leaves
Bombardie	kidney	vein thickening, leaf curling, some local lesions, single enlarged malformed leaves
Witkop	kidney	severe stunting, yellowing, small malformed leaves, slight mosaic, poor germination numbers

Area	Bean type	Symptoms
Kendal	kidney	slight yellowing of leaves
Potchefstroom sample 1	experimental crosses	slight yellowing of leaves
Potchefstroom sample 2		slight yellowing of leaves
Potchefstroom sample 3		slight yellowing of leaves
Potchefstroom sample 4		slight malformation of leaves
Potchefstroom sample 5		slight yellowing of leaves
Potchefstroom sample 6		slight yellowing of leaves
Potchefstroom sample 7		leaves showed mosaic symptoms
Potchefstroom sample 9		plants showing stunting, multiple branches, small leaves
Ermelo sample 8		leaves showing slight yellowing
Delmas sample 10		leaves showing slight yellowing

2.2. Indicator plants

The use of indicator plants has an established and important role in plant virus research. They are used primarily to confirm the presence of a virus in another plant which has vague or indeterminate symptoms (Smith, 1977). By means of indicator plants it is possible to gain considerable information about an unknown virus. The first necessity is that the virus be laboratory transmissible. This may be by sap or insect transmission. Insect transmission facilities were not available but it is fortunate that most viruses of beans can be transmitted by sap inoculation.

Four types of plant hosts are desirable in any investigation of an unknown virus (Gibbs and Harrison, 1976).

1. Plants in which characteristic symptoms are produced that can be used to aid detection and diagnosis of disease.
2. Plants in which a stock culture of the virus can be maintained for long periods.
3. A plant which is a source of large amounts of virus needed for purification.
4. Plants that can be used for assaying of infectivity preferably those that produce a visible primary infection e.g. local lesions on inoculated leaves that can be counted.

Different species were chosen to suit these different purposes. For example, hosts susceptible to a wide range of viruses were used in general screening of samples, whereas only varieties of Phaseolus vulgaris were used in more restricted studies such as strain determination.

Host plants were chosen on the basis of their susceptibility to general viral infection. Considerable evidence supports the view that "susceptible families" occur (Hollings, 1966), the families Leguminosae, Solanaceae, Labiatae, Chenopodiaceae and Curcubitaceae supporting many viruses.

Propogation hosts were selected in conjunction with purification procedures in order to obtain the greatest yield of undamaged and "clean" virus particles.

For several years new methods of inoculation and more drastic treatments of host plants before and after inoculation have been tested. These efforts have been aimed at increasing host susceptibility and reducing resistance to virus. Temperature, shading, humidity, plant nutrition and pre-inoculation dark treatments are some the factors that have been manipulated to markedly increase the susceptibility of plants to infection.

2.2.1. Mechanical inoculation procedures

All plants were subjected to a 24 hour pre-inoculation dark treatment. Plants were then inoculated following a routine method for any fairly infectious sap-transmissible virus. Leaf sap was mixed with a small amount of celite and 0,01 M phosphate buffer pH 7,6 and the inoculum was gently rubbed over the host leaf surface. Muslin or the forefinger was used for this purpose. After inoculation, leaves were gently rinsed under a stream of water to increase the points of entry for any virus present (Smith, 1977). Inoculated leaves were marked by making a small hole at the tip with a needle.

Any virus isolated from the collected leaf samples was inoculated in this way and maintained as a stock culture in virus-free sugarbeans provided by Dr. B. von Wechmar. For purification purposes however virus was propagated in Phaseolus vulgaris var. "Double Dutch Princess" provided by Plant and Seed Control. Systemically infected leaves were harvested three weeks after the inoculation of primary leaves.

a) Biological properties of virus

For infectivity assays and the determination of the biological properties, longevity in vitro and thermal inactivation point of the virus, only concentrated sap and celite may be used. In the first test, sap was maintained at room temperature and daily inoculations were made on replicates of 10 day old Phaseolus vulgaris vars. "Beka" and "Monroe" and 2 month old Chenopodium quinoa for 5 days. Symptoms, that is the presence of local lesions were recorded after 2 and 1 week respectively. To determine the thermal inactivation point, small amounts of virus infected sap were placed in thin-walled glass tubes. A separate tube (10 minutes) was subjected to the following temperatures: 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 waterbath. The temperature of the water was carefully monitored with a thermometer. Each sample was inoculated onto replicates of Phaseolus vulgaris vars. "Monroe" and "Beka" and Chenopodium quinoa. Symptoms were recorded after 2 and 1 week respectively.

The dilution end point of the virus was determined by diluting virus in sap ten-fold, that is, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} in 0,01 M phosphate buffer, pH 7,6. Each sample was inoculated onto replicates of Phaseolus vulgaris vars. "Monroe" and "Beka" and Chenopodium quinoa. Symptoms were recorded after 2 and 1 week respectively.

b) For the strain determination of the isolate, different varieties of Phaseolus vulgaris were used. Concentrated sap mixed with celite was applied on day 1 and again after 5 days (Drijfhout, 1978). Symptoms were recorded at weekly intervals for 4 weeks after the first inoculation.

c) Conditions for growth

Indicator plants	25° - 28°C
Propagation hosts	28°C
Infectivity assays	26° - 28°C
Strain determination	22° - 26°C

30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 70°C, 80°C by placing it in a waterbath. The temperature of the water was carefully monitored with a thermometer. Each sample was inoculated onto replicates of Phaseolus vulgaris vars. "Monroe" and "Beka" and Chenopodium quinoa. Symptoms were recorded after 2 and 1 week respectively.

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c) Conditions for growth

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Infectivity assays	26° - 28°C
Strain determination	22° - 26°C

Table 6: List of indicator plants used in preliminary investigations and determination of host range

Indicator plant	Family	Age	at time of Inoculation
Chenopodium quinoa Willd.	Chenopodiaceae	2 m	6 - 8 leaves
Chenopodium amaranticolor Coste and Reyn.	Chenopodiaceae	2 m	6 - 8 leaves
Pisum sativum L. "Greenfeast"	Leguminosae	2½ w	3 - 4 leaves
Nicotiana clevelandii Gray	Solanaceae	6 w	3 - 4 leaves
Nicotiana glutinosa L.	Solanaceae	6 w	3 - 4 leaves
Nicotiana tabacum "Samsun" L.	Solanaceae	5 w	3 - 4 leaves
Nicotiana tabacum "White Burley" L.	Solanaceae	5 w	3 - 4 leaves
Datura stromonium L.	Solanaceae	4 w	2 leaves
Petunia hybrida Vilm.	Solanaceae	5 w	3 - 4 leaves
Arachis hypogea L.	Leguminosae	3 w	4 leaves
Vicia faba L.	Leguminosae	10 d	primary leaves
Trifolium repens L.	Leguminosae	2 w	3 - 4 leaves
Glycine max (L.) Merr.	Leguminosae	2 w	2 leaves
Cucumis sativus L.	Curcubitaceae	9 d	cotyledons
Vigna senensis (L.) Endl	Leguminosae	10 d	2 leaves
Brassica chinensis L.	Cruciferae	3 w	4 leaves
Lycopersicum esculentum L.	Solanaceae	3-4	2 - 3 leaves
Phaseolus vulgaris L. "The Prince" "Bountiful" "Canadian Wonder" "Monroe" "Beka"	Leguminosae	10 d	first trifoliate leaves appearing, that is, 2 primary leaves are present

KEY m = months w = weeks d = days

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Nicotiana tabacum "White Burley" L.	Solanaceae	5 w	3 - 4 leaves
Datura stromonium L.	Solanaceae	4 w	2 leaves
Petunia hybrida Vilm.	Solanaceae	5 w	3 - 4 leaves
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Phaseolus vulgaris L. "The Prince" "Bountiful" "Canadian Wonder" "Monroe" "Beka"	Leguminosae	10 d	first trifoliate leaves appearing, that is, 2 primary leaves are present

KEY m = months w = weeks d = days

Table 7: Varieties of Phaseolus vulgaris used in the strain determination of BCMV

Host genotype	Varieties
1	Diacol calima, Canellini, Comptesse de Chambord
2	Bataaf, Redlands, Greenleaf C
3	Redlands, Greenleaf B, Great Northern 1140 Colombia Pinto
4	Sanilac, Pinto, UI III
5	Pinto 114
6	Monroe
8	Cornell 49 - 242, Nep II, Seafarer
9 a	Jubila, Peru 0257, Wintergreen
b	Topcrop
10	Amanda

2.3. Purification Procedures

The source of a virus is an important factor. Generally young plants showing recent systemic symptoms have higher virus concentrations and fewer pigments. Only leaves showing marked systemic and local lesion symptoms are selected for purification.

Purification involves the extraction and clarification of crude sap and the final isolation of virus. These are the basic procedures involved in any plant virus purification scheme (Guy, 1978). The object of purification is to produce a preparation containing only infective virus particles but this is rarely achieved as it is virtually impossible to remove the last traces of host constituents that sediment with centrifugation, even using appropriate chemicals. It is advisable to test diverse methods of purification depending on the properties of the virus so that the best yield and purity can be obtained.

In general, diseased leaves are ground in buffer to extract sap which is then clarified using low speed centrifugation or filtration aided by the addition of organic solvents or precipitating agents such as ammonium sulphate. Virus remains in the clarified sap suspension and is isolated using precipitating agents such as polyethylene glycol, or by gel filtration and centrifugation. Pure preparations are only obtained after subjection to further treatments such as differential centrifugation, density gradient centrifugation, column chromatography, ion exchange systems (Redgwell, 1980) or zone electrophoresis (von Regenmortel, 1964).

The purification procedures selected were specifically designed for the isolation of different legume viruses. For the centrifugation of all samples the following rotors were used:

SS 34 Sorvell rotor for speeds up to 20,000 RPM.
Ti 50 Beckman rotor for ultracentrifugation

2.3.1. Procedure 1

This was a modification of the method used by Maat (1975) for BYMV (Appendix I). Leaf tissue (10g) showing systemic symptoms were homogenized in a Virtis blender with 30 mls of TRIS-TGA buffer, pH 9,0. This extracted sap was mixed with a 1:4 ratio of equal volumes of carbon tetrachloride and chloroform, then centrifuged for 10 minutes at 8,000 RPM. The supernatent was centrifuged for 1½ hours at 15,000 RPM.
The pellet was resuspended in a small volume of TRIS-HCl buffer, pH 9,0.

2.3.2. Procedure 2

Several viruses have been isolated using this method promoted by Whitlock (1979). These include TMV, CMV, BBMV, EAKBMV, AMV and BBSV. The above viruses are all

spheres of between 25 nm and 50 nm, except for TMV which is a 300 nm rod, but all are capable of infecting beans.

Infected leaves (4 g) were homogenized in a Virtis blender with 4 mls of Phosphate-TGA buffer Ph 7,6 (Appendix I). The mixture was filtered through 2 layers of cheese cloth. Butanol, 8,5% (v/v) was added dropwise while the mixture was stirred for 1 hour at room temperature. The emulsion was centrifuged at 8,000 RPM for 30 minutes. The resultant supernatant was filtered through Whatman No. 1 paper, then centrifuged for 90 minutes at 27,000 RPM. The pellet was resuspended in M/30 phosphate buffer, pH 7,6 overnight. The preparation was clarified by centrifuging for 15 minutes at 1,000 RPM.

2.3.3. Procedure 3

Several spherical and elongated viruses have been isolated using this method also promoted by Whitlock (1979). These include BCMV, PSV, EAKBMV, BBSV, CoMV, BPMV and AMV.

Infected leaves were macerated in a 4:1 (w/v) ratio in a buffer containing chloroform and butanol with a few drops of ascorbic acid (Appendix I). The mixture was sedimented at 3,000 RPM for 20 minutes. The resultant supernatant was left overnight at 4°C, then centrifuged at 8,000 RPM for 10 minutes. The supernatant was centrifuged at 27,000 RPM for 2 hours. The pellet was resuspended in 0,03 M phosphate buffer, pH 7,6.

2.3.4. Procedure 4

The procedure for virus purification was similar to that previously used for tobacco etch virus and other members of the potyvirus group (Jafarpour et al, 1979). Leaf tissue was homogenized with a Virtis or Waring blender in cold phosphate-mercaptoethanol buffer pH 7,2 (Appendix I). The ratio used was 1 gram of fresh tissue per 1,2 ml of buffer. Crude extracts were filtered through 2

layers of cheesecloth and clarified by stirring for 5 minutes with an equal volume of chloroform. The emulsion was centrifuged at 8,000 RPM for 30 minutes. Virus was precipitated from the collected supernatent by addition of polyethylene glycol 6000 to a concentration of 8% (w/v) and NaCl to a concentration of 0,5% (w/v). Stirring was continued for ½ an hour 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 8,000 RPM and the pellets were resuspended for 4 hours in 1/10 of the original volume of resuspension buffer (Appendix I). The virus was then subjected to 2 cycles of differential centrifugation, a low speed spin of 8,000 RPM for 30 minutes followed by a high speed spin of 27,000 RPM for 90 minutes. Pellets were resuspended in small volumes of buffer (Appendix I).

2.3.5. Methods for purifying viruses to homogeneity

For many experiments, a virus sample of high purity was required and therefore, samples had to be further purified using density gradient centrifugation or molecular permeation chromatography.

a) Density gradient centrifugation

Gradients of sucrose were prepared by layering 4 solutions of decreasing concentrations of sucrose (40 to 10%) in resuspension buffer (Appendix I). These were allowed to diffuse together to form a "smooth" gradient before the partially purified virus suspension was layered on top. For caesium chloride gradients, 14 mls of 30% (w/v) suspension of caesium chloride was prepared in 0,05 M potassium phosphate buffer, pH 7,5 (Morales, 1979). Partially purified virus (1 ml) was mixed with the caesium chloride and the gradient was centrifuged for 14 hours at 40,000 RPM. The visible virus zone was collected by fractionation of the contents

of the tube using an Isco fractionating system. Virus fractions obtained from both types of gradients, diluted in resuspension buffer (Appendix I) were centrifuged at 27,000 RPM for 90 minutes to concentrate the virus. Other ways of removing sucrose and caesium, such as dialysis, were also attempted.

b) Column chromatography

Barton (1977) devised and developed molecular permeation chromatography into a routine method for the purification of plant viruses. Dry CPG (of 70 nm pore size and 120 to 200 mesh, that is CPG 700) was suspended in 0,05 M sodium phosphate buffer, pH 7,0 and washed several times by decantation. It was then resuspended in buffer containing 1% (w/V) polyethylene glycol 20,000 and degassed for 15 minutes using a pump, while beads were kept suspended with a stirring magnet. CPG was then washed in sodium phosphate buffer, pH 7,0 by several decantations. A bed of CPG was packed into a 1 m by 15 mm glass column. Effectiveness of column packing was always checked before each virus separation by running a sample of blue dextran 2000 and monitoring the eluate for a compact profile. Up to 2 mls of virus sample(resuspended in 0,01 M phosphate buffer, pH 7,16) could be applied to the column. If more than one sample was run, the first sample was allowed to travel three quarters of the way down the column before the second sample was applied. Samples were eluted using 0,01 M phosphate buffer, pH 7,16, applied by peristaltic pump. Column eluates were monitored at 254 nm with an attached U.V. analyser and recorder. All chromatography was carried out at room temperature. Fractions containing suspected virus were concentrated by ultracentrifugation at 27,000 RPM for 90 mins.

2.4. Storage of virus preparations

When stored in suitable ways, virus preparations may retain full activity for long periods (Purcifull et al., 1975). The particles of most viruses are best kept in

suspension at 0°C to 4°C with an added antimicrobial compound such as a drop (0,05 mls) of sodium azide solution. Alternatively sap or purified preparation may be freeze dried or frozen. Little infectivity is lost when infected tissue or sap is frozen or freeze dried, while infectivity decreases considerably if virus is stored in the pure form.

For freeze drying, sap was mixed with 0,2 ml of a 7% peptone and 7% dextrose solution for protection during preservation and later during resuspension (Hollings and Stone, 1970). Frozen sap or purified samples were stored in an equal volume of 70% sorbitol. Partially dried, infected leaves were stored over calcium chloride according to the method of Bos and Benetti, (1979).

Wherever possible however, virus was purified and used immediately to avoid contamination and due to the limited time that particles remain intact.

2.5. Electron microscopy

2.5.1. Negative staining

The fastest and easiest way to assign an unknown virus to a structural group is the examination of negatively stained preparations by electron microscopy (Hamilton, 1981). Infectivity tests and other methods such as host range studies are indirect means of virus detection (Bos, 1976). Determination of mean particle length for rod shaped viruses or particle size for isometrics has aided their identification and classification. It is advantageous to make measurements on crude plant extracts as this avoids aggregation or breakage of particles that often occurs after purifications (Hitchborn and Hills, 1965). It is also the most rapid and simple method in use and the results help in the choice of any purification procedure. The examination of crude

extracts has been previously done with metal shadowing, yet negative staining is more simple and rapid, preserving finer particle detail..

The leaf dip method was first used by Brandes in 1957 (Ball, 1971). Sap is expressed from systemically infected leaves and is examined directly but may require clarification using low speed centrifugation if spherical particles are suspected that are easily obscured by sap debris. Grids are usually stained with 2% aqueous phosphotungstic (PTA) acid pH 6,5 or 2% aqueous uranyl acetate. Grids must be examined immediately after staining. PTA must be used with care as there are several documented cases of disruption of capsomere structure, isometric viruses being especially sensitive.

Measurements of particles are calculated from the magnification. As many particles as possible are measured and a histogram is usually drawn to determine the average particle length.

Dimensions of isometric and rod/flexuous virus forms can therefore be distinguished with negative staining. Negative staining, at high magnification, also provides information on capsome arrangement.

Copper grids (200 mesh) were prepared for transmission electron microscopy (TEM) by coating with diluted 0,2% formvar solution (Appendix II) and a thin layer of carbon. Grids were floated on drops of diluted sap suspension for 10 minutes. The side of the grid was touched on filter paper to remove excess sap. All grids were stained in drops of 2% aqueous uranyl acetate for 10 minutes. Grids were dried on filter paper then viewed immediately at 80 KV on the JEM 100S transmission electron microscope to detect the presence of virus.

2.5.2. Transmission electron microscopy

Until recently workers dealt primarily with the external effects of viruses on the host, that is, the host reaction (Bos, 1978). The emphasis of virology changed with the advent of the electron microscope. The techniques of fixation, embedding, staining and thin sectioning have enabled the study of virus *in situ*. This work has allowed one to gain insight into the position of viruses in cells, their synthesis and changes they may induce in host cells. These features have been found to remain constant and allow for the further characterization and subdivision of unknown viruses into groups (Martelli and Russo, 1977).

The yellow/green mosaic areas of infected leaves showing symptoms are the best source of material for inclusion bodies (Reysenbach, pers. comm.). Leaf tissue was cut and prepared for electron microscopy using the standard gluteraldehyde-osmium fixation technique (Appendix II) (Provvidenti and Cobb, 1975). Some modifications were introduced. Samples were treated with a mixture of 2% osmium and 0,5% buffered uranyl acetate prior to dehydration (Smith et al., 1976) in an attempt to preserve and stabilize the fine structure. After dehydrating in an ethanol series, leaf tissue was embedded in Spurr's resin (Spurr, 1969).

Blocks were sectioned on a Reichert ultramicrotome and sections of 70 to 150 nm were collected on 300 mesh copper grids. Sections were stained with 2% uranyl acetate and lead citrate (Venable and Cogglehall, 1965). Sections were viewed at 80 KV using the transmission electron microscope.

2.6. Serology

Serological techniques have a wide application in the diagnosis of plant virus diseases. Under suitable conditions,

the reactions of antibodies with antigen can be demonstrated in vitro, for example, precipitation (Bercks et al., 1972). Indeed most serological tests for plant viruses are a modification of the precipitation test (Ball, 1974). There is little agreement on the sensitivities of all available serological methods (von Regenmortel, 1966) and hence, where appropriate, a comparison of several standard techniques was made.

The recent combined use of the electron microscope and serology has enabled the visualisation of specific binding between antibody and antigen in fixed and sectioned material and more particularly in virus suspensions (Milne and Luisoni, 1975, 1977). The principles of serologically specific immune electron microscopy (SSIEM) are well established and routinely used, not only for virus identification but for trapping low concentrations of suspected virus in crude preparations (Derrick and Brlansky, 1976; Nakasone et al., 1978).

2.6.1. Antiserum production

Antiserum to purified BCMV antigen was raised in rabbits although several other laboratory animals have been suggested in recent studies (Bar-Joseph and Malkinson, 1980). An intravenous and intramuscular immunization scheme proposed by Whitlock (pers. comm.) was followed as it is more effective and only small quantities of antigen are required.

Virus was purified according to Procedure 4, documented in section 2.3.4. and column chromatography (2.3.5(b)). Virus was concentrated by ultracentrifugation and resuspended in 1 ml of physiological saline. The final concentration of virus was 1 mg/ml, estimated spectrophotometrically ($E_{260:280}$).

On day 1 of the immunization programme, 1 ml of virus in saline was injected into the left marginal ear vein. On day 7, 1 ml of virus emulsified with 1 ml of complete Freunds adjuvant was injected into the left thigh muscle. On days 14 and 21, 1 ml of virus and 1 ml of complete adjuvant was injected into the right thigh muscle. Between days 21 to 28, a small sample of blood was collected from the right ear to determine the titre using microprecipitin tests (2.6.3.). When this had reached a reasonable level, the rabbit was anaesthetized and about 60 mls of blood was obtained by cardiac puncture. The blood was kept at 30°C to clot for 1 hour then kept at 4°C overnight. The clot was removed and the remaining serum was centrifuged at 7,000 RPM for 30 minutes. The serum was divided into aliquots and frozen at -20°C until used. No preservatives were added for possible fear of reducing the low titre, therefore serum was filter sterilized before being used in any serological tests.

2.6.2. Immunoglobulin purification

Where necessary, specific IgG was extracted using the rivanol/saturated ammonium sulphate method (Appendix III). Purified IgG was dialysed against glass distilled water (at least 3 changes of 500 mls), and further purified by passage through a column of DEAE cellulose (Whatman Ltd.). Fractions with an E 278:250 of between 1,007 and 1,217 were collected. After adjusting the final concentration to 1mg/ml, IgG was stored in silicon treated glass tubes or as a freeze dried sample.

2.6.3. The microprecipitin test

In the initial determination of antibody titre, the microprecipitin test (Nocrdam, 1973) was used. The test was useful as only small quantities of reagents are required, yet precipitation can still be detected. To prepare the test, 2 rows of 9 small test tubes

were set up. The first row was used to perform doubling dilutions of purified antigen and the second row to perform similar dilutions of antiserum. In the first tube of both dilution series, 375 µl of PBS was added while 100 µl was added to all other tubes. A 1/4 dilution of purified virus (antigen) or antibody was made by adding 125 µl of the appropriate substance to the first tubes. A grid titration was performed in a petri dish divided by means of a wax pencil as shown below:

Diagramatically

		grid position antigen									
		1	2	3	4	5	6	7	8	9	10 PBS
grid position anti-body	1										
	2										
	3										
	4										
	5										
	6										
	7										
	8										
	9										
	10 PBS										

Healthy sap and phosphate buffered saline (PBS) were used as controls. In every square, 15 µl of each reagent was placed. The petri dishes were sealed with masking tape and incubated at room temperature. Observations were made after 3, 6 and 12 hours. Individual droplets were examined for evidence of precipitation using a dissecting microscope fitted with an incident light setting. Black paper was placed under the petri dish to make the precipitate more visible. The titre of the antiserum was taken as the highest dilution giving a positive reaction with antigen.

2.6.4. The tube precipitin test

The final antiserum titre was determined using this method. Greater quantities of reagents are required but precipitation is clearer. Virus preparations must be extremely pure or precipitates are obscured. Doubling dilutions of pure virus (1 mg/ml) were made in saline, the initial dilution being 1/2. Volumes (0,5 ml) of the virus dilutions were mixed with an equal volume of undiluted antiserum. Once the optimum antigen concentration was obtained, this was held constant and used in a test where doubling dilutions of the antibody were made. Controls consisted of

0,5 ml saline + 0,5 ml antiserum (concentrated)
0,5 ml saline + 0,5 ml virus suspension (1 mg/ml)
1,0 ml saline

All tubes were placed in a metal rack in a 37°C water bath such that the level of the water was half the level of the virus/antiserum/saline mixture, for ideal convection to occur.
Observations for the presence of precipitate were made at 15, 30 60 and 120 minutes, with intermittent swirling of the tubes. The antibody titre was taken as the last tube showing any sign of precipitate.

2.6.5. Passive haemagglutination

One of the advantages of this method is its great sensitivity for the detection of plant viruses (Rajeshwari et al., 1981) and therefore it is of use in confirming the titres of suspected low titre serum.

Sheep red blood cells were collected in Alsevers solution and stored for 1 day at 4°C. The cells were washed with an equal volume of PBS pH 7,2 by centrifugation at 1,200 RPM for 10 minutes. After 3 washings, 1,0 ml of packed cells was resuspended in 15,7 ml of PBS and treated with an equal volume of freshly prepared 0,01% tannic acid in PBS. At higher concentrations, tannic acid can

result in non-specific agglutinations. RBC were tanned by incubation for 20 minutes at 4°C with intermittent mixing. Excess tannic acid was removed by washing twice with equal volumes of PBS and resuspending the cells in PBS to give a 3,0% RBC suspension. The cells were sensitized by mixing serum diluted 1:5 with PBS to an equal quantity of RBC. The mixture was incubated at 37°C for 30 minutes. Excess globulin was removed by 2 washings in PBS and sensitized cells were resuspended to give a 3% suspension in 0,05 M phosphate buffer, pH 7,2, the diluent also employed for preparing the antigen samples

Purified virus (1 mg/ml) and partially purified virus samples (Rajeshwari et al., 1981) were diluted two-fold, the initial well containing a dilution of 1/4. Diluted antigen (250 µl) and 40 µl of antibody-sensitized cell suspension were added to each well. The plate was shaken gently until a uniform red blood cell suspension was observed and then it was left at room temperature for 2 hours before being placed at 4°C. Results were recorded after overnight incubation of the plate.

In the case of a positive reaction, RBC formed a smooth mat, frequently with serrated margins, on the bottom of the well. A negative reaction consisted of a discrete red ring at the base of the well.

The following controls were included for testing:

a) 0,05 M phosphate buffer, pH 7,2 and sensitized RBC
b) extracts from healthy leaves and sensitized RBC

2.6.6. Precipitation in semi-solid media

There are several advantages since the serological reaction is preceded by one or many fractionating steps such as diffusion. Resolving power is therefore greatly enhanced

(Bercks et al., 1972). More information may be gained on the purity, homogeneity and physical properties of reactants and on the occurence of non-specific reactions. In the past one serious limitation of semi-solid techniques was the poor diffusion of elongated rods. Degradation with detergents such as SDS or pH adjustments (Langenberg and Ball, 1972) have resulted in the greater acceptance of these methods for elongated plant viruses (Ball, 1974). Agar suitable for electrophoresis must be used, as often culture agars are too impure for double diffusion purposes (Hudson and Hay, 1980). Zimmer (1979) however, has suggested for example that the use of Bacto agar instead of Noble agar produced more distinct precipitation bands. Heavy metal impurities seem to contribute significantly in some unknown way to increase the sensitivity of immunodiffusion. Different concentrations of agar, azide and SDS can enhance or decrease precipitin lines.

Double diffusion in Ouchterlony plates

Agar plates were prepared by modification of methods proposed by Ziemiecki and Wood (1975), Purcifull and Batchelor (1977) and Whitlock (pers. comm.).

Ionagar # 2 or agarose (0,22 g) was added to 13 ml of glass distilled water in a large test tube. This was heated in a water bath until the agar was fully dissolved. Phosphate buffer, pH 7,2 (M/15, 13 mls) was then added and the agar was rehaeated. Sodium azide (0,1%) was added. One ml of hot agar was pipetted onto methanol cleaned slides marked with a wax pencil. The agar was allowed to set. Wells were cut out in several patterns (depending on the assay being performed), according to standard procedures (Hudson and Hay, 1980).

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for example:

i) O O O
O O O
to determine titre and optimum concentrations of reagents

ii) O O
O
O O
to determine titre

iii) O O
O O O
O O
to determine virus identity

(from Hudson and Hay, 1980)

Antiserum was used concentrated or diluted serially in M/15 phosphate buffered saline, pH 7,2. Purified virus or systemically infected leaves were used as the antigen source. Leaves were ground in a similar buffer, in a mortar and pestle. The resultant sap was pressed through cheesecloth and subjected to a low speed cycle of centrifugation. A few drops of 3% SDS were added to the sap solution before being placed in the wells (Ziemiecki and Wood, 1975). Volumes of 40 µl of the reactants were added to the appropriate well. The slides were placed in the humidity chamber overnight at room temperature, before inspection over light for bands of precipitation.

Slides were then washed for 36 hours in several changes of PBS to remove excess reagents and proteins that may result in non-specific staining.

Gels were stained according to the method of Whitlock

(pers. comm.) in 1% napthalene black in 7% glacial acetic acid for 90 seconds. Gels were destained in 7% glacial acetic acid with several changes of solution (Appendix IV), until the background was clear. After rinsing in distilled water, gels were dried to form a permanent record.

2.6.7. Immunoelectronmicroscopy (IEM)

The adhesion of virus particles to formvar coated grids, which is the basis of the 'leaf dip' method of analysis is a highly erratic phenomenon not suitable for quantitive measurements (Nicolaieff and von Regenmortel, 1980). Particles are easily "washed off" in staining and the distribution of particles seen does not reflect the quantity of particles in the suspension. The reproducibility of adhesion to grids can be greatly improved if a layer of antibodies specific for the virus is first adsorbed to the grid (Almeida et al, 1980).
Variations on the basic procedures proposed by Derrick (1973) and Ball (1974) were employed in the various experiments.

1. Method 1
   A drop of purified virus suspension was mixed with a drop of concentrated antiserum prepared against BCMV. The serum was provided from the World Reference Collection, Wageningen, Netherlands. The mixture was incubated at 37°C for 1/2 hour. A formvar coated grid was then floated on the surface for 10 minutes, drained and then stained in 2% uranyl acetate for 10 minutes.

2. Method 2
   Grids were floated onto serially diluted drops of the same serum, prepared against BCMV, for 2 hours. The grids were rinsed in PBS for 1 minute, dried, then floated onto purified preparations of suspected BCMV for 1/2 hour. This method was primarily for the identification of the unknown isolate as particles were easier to see.

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3. Method 3

A more recent solid phase phenomenon called "SMOG" (Almeida et al., 1980) was used to detect particles in survey samples suspected of containing low virus concentrations. A sandwich technique of captive (BCMV specific) antibody, virus and additional antibody was used. Grids were floated on drops of specific antiserum over a range of dilutions for 1/2 hour. After the adsorption period, they were rinsed with PBS briefly then inverted onto drops of virus suspension and left to react for 20 to 30 minutes. After the period of virus adsorption, grids were floated immediately on drops of specific antiserum again. This method was attempted because it was found that little difference in the numbers of particles attached to grids occured using previous methods. The SMOG effect makes the visualization of low concentrations of virus particles easier by drawing them together into clumps. Both supplied (BCMV specific) antiserum and raised antiserum were used in this technique.

Tests were performed using normal rabbit serum as a control in all of the above methods.

2.6.8. The ELISA technique

The limitations of conventional serological techniques have largely been overcome by the use of the microplate method, that is, the ELISA technique (Clark and Adams, 1977). The method has been applied for the detection and quantitative assay of several plant viruses in sap, purified preparations, seed, leaf discs and bulbs (Stein et al., 1979; Romaine et al., 1981).

For the routine detection of plant viruses, the direct double antibody sandwich form of the ELISA (Clark and Adams, 1977) has been extensively used. In this test, virus is selectively trapped by specific antibody adsorbed onto the microtitre plate surface. Immobilized virus is then reacted further with specific antibody to which an

enzyme has been linked. After washing, the complex is detected colourimetrically with the addition of the appropriate enzyme substrate.

There are several practical aspects to consider when carrying out the ELISA (Voller et al., 1979).

1. The solid phase

   Antigen and antibody are attached to the solid phase, usually polystyrene microtitre plates. The optimal conditions for coating the plate are determined using a chequerboard titration. These conditions are then adhered to in subsequent tests. Most IgG proteins readily coat at a concentration of 1 to 10 µg/ml in carbonate/bicarbonate buffer, pH 9,6, although in some cases different pH values may be required. Microtitre plates can be treated and then re-used if necessary (Bar-Joseph et al., 1979(a) and (b)).

2. Washing steps

   For the successful application of the ELISA, excess reactants, that is, antibody, antigen and conjugate must be removed from the plates by thorough washing of the wells with PBS-Tween. At least 3 washes are made between each reaction stage.

3. The test sample

   To avoid non-specific attachment to the solid phase, the antigen is usually made up in PBS containing a wetting agent, that is Tween 20. Quantitation of the sample being assayed is made on serial dilutions especially where a visually read endpoint is required.

4. Conjugates

   Alkaline phosphatase is often selected as the enzyme because of easy conjugation with a broad spectrum of substrates. A cross-linking agent such as gluteraldehyde is used (Avrameas, 1969) to conjugate the enzyme to the IgG.

5. Substrates

Chromogenic substrates, initially colourless, but yielding a strong colour reaction on degradation are used. The results of the test may be assessed subjectively by eye, or where equipment is available, a microreader can provide spectrophotometric measurements at 400 to 500 nm.

The standard procedure for carrying out the microplate method for virus detection was as follows:
(Appendix III)

1. 200 µl of purified IgG diluted serially in coating buffer was added to each well
2. incubation for 4 hours at 37°C
3. plates were then thoroughly washed at least 3 times with PBS-Tween
4. 200 µl of test antigen diluted serially in PBS-Tween was added to the wells
5. the test sample was incubated overnight at 6°C
6. plates were washed again
7. 200 µl of alkaline phosphatase labelled specific IgG (Appendix III diluted serially in PBS-Tween was added to the wells
8. plates were incubated for 4 hours at 37°C, then washed 3 times in PBS-Tween
9. 300 µl of p-nitrophenylphosphate substrate in diethanolamine buffer was added to each well and plates were incubated at room temperature for hours
10. the reaction was stopped by the addition of 50 µl of 3 N NaOH solution.

The results of the test were assessed visually as the required equipment was unavailable.

2.7. Electrophoresis

2.7.1. Immunoelectrophoresis

Where necessary the presence of IgG was confirmed by electrophoresis on 7% acrylamide gels. In addition, the prepared immunoglobulin was run on 2% agarose slides (Appendix IV) and subsequently reacted with specific anti-rabbit IgG (Merck).

2.7.2. Polyacrylamide gel electrophoresis for proteins

Microgram quantities (10 to 50 µg) of proteins from highly purified plant virus particles produce distinct bands using SDS-PAGE (Paul, 1974). The band positions are characteristic for a given virus and for groups of related viruses. SDS-PAGE is therefore extremely useful in determining the presence and possible identity of viruses in plants. Protein solubilizers are essential to disperse protein aggregates and render them more hydrophilic before electrophoresis. Methods for isolating viral protein must be chosen such that they disrupt tertiary virus structure yet maintain the sub-units in the native state (Tsugita and Hirashima, 1972). For electrophoresis the anionic detergent, SDS, has provided excellent separation of proteins that are globular, helical or rod shaped in the native state (Maizel, 1971). SDS is advantageous as it increases the resolving power of bands and prevents zone dispersion - two problems associated with ordinary PAGE (Chen and Chramback, 1980). The effects of SDS on denatured protein are not well understood; proteins are denatured by the reduction of disulfide bonds and conformational changes occur (Le Maire et al., 1980). In general, protein samples are treated with a mixture of urea, 2-mercaptoethanol and SDS before electrophoresis.

Electrophoresis in polyacrylamide gels in the presence of SDS detergent was therefore selected for the separation and identification of viral proteins.

Virus was propagated in Phaseolus vulgaris var. "Double Dutch Princess" and virus from systemically infected leaves was purified using procedure 4 (2.3.4.). After several cycles of differential centrifugation the virus band was separated by fractionation through a controlled pore bead column (2.3.5). Virus was concentrated by centrifugation at 27,000 RPM for 90 minutes and the concentration estimated spectrophotometrically. A sample containing approximately 1 mg of virus per ml of resuspension buffer (Appendix I) was used. Virus suspension (200 µl) was mixed with a few drops of splitting solution (Appendix IV) and heated in a boiling water bath for 5 minutes. A sample of 80 µl was applied to each well. Protein and standards were electrophoresed on 7% gels at 15 milliamps per gel for 3½ hours then stained in coomassie blue overnight (Appendix IV). After destaining, gels were scanned at 574 nm on a Beckman spectrophotometer, Model 25.

2.7.3. Polyacrylamide gel electrophoresis for RNA

Early studies on the electrophoretic properties of nucleic acids were carried out in homogenous solutions such as sucrose gradients (Olivera et al., 1964). The application of PAGE to the fractionation of viral RNA has added an indispensible tool to virology, replacing gradient sedimentation for the analysis of complex mixtures of RNA molecules, owing to its higher resolution (Richards et al., 1965; Adesnick, 1971). The constant charge to mass ratio of RNA severely limited the possibilities of resolving species different molecular weights by conventional electrophoretic methods (Struthers, 1973). Polyacrylamide gels have a simple sieving effect and pore size is chosen to suit the molecular dimensions of the migrating ions. The gel lattice then retards

molecules during electrophoresis according to their dimensions.

Higher resolution with the acrylamide technique is derived from the ability to prepare gels of different pore sizes, each capable of fractionation within a limited range of molecular weights. This is acomplished by adjusting the concentration of acrylamide and the introduction of agarose or other compounds which also make the gels easier to handle, without affecting resolution. In those cases where electrophoresis of RNA samples on constant percentage gels does not resolve high and low molecular weight species on a single gel, a discontinuous gel system can be used (Petri, 1972). Low percentage gels are layered over high percentage gels with no disturbance of the interface.

Several methods have been proposed for the purification of RNA from viral components e.g. SDS pronase (Lambert and Daneholt, 1975), phenol (Mohamed and Thomas, 1980), glyoxal (Murant et al., 1981) and others. Whatever method is used, high concentrations of RNA are required for microanalysis and the extraction of RNA must therefore take place in small volumes. Once extracted and electrophoresed, RNA can then be detected by spectrophotometric scanning or the use of one of the many available staining procedures (Marcinka, 1975).

RNA was extracted from purified virus using the modified phenol method proposed by Perry et al., (1972) (Appendix IV). About 1 mg of virus was concentrated by ultracentrifugation and resuspended in 200 ul of NETS buffer (Appendix IV) in an eppendorf tube. An equal volume of phenol-chloroform cocktail was added and the mixture was vortexed then allowed to settle on ice for 10 to 15

minutes. RNA could be detected in the aqueous phase by scanning at 254 nm before the sample was precipitated with ether, sodium acetate and absolute alcohol (-70°C) (Appendix IV). The yield of RNA after extraction must be calculated to allow the gel to be correctly loaded. An A_{260} of 1,00 implies a concentration of about 50 µg of RNA. The precipitate was resuspended in 200 µl of E buffer and sucrose (Appendix IV).

The concentration of RNA was calculated using the following formula:

$$\text{Number of µg of RNA} = \underset{\text{(volume)}}{\text{Number of mls}} \times A_{260} \times \underset{\text{(conversion factor)}}{50}$$

Extracted RNA was electrophoresed on composite gels prepared according to the method of Petri (1972). A sample (80 µl) containing 10 to 100 µg of RNA was added to each well and gels were electrophoresed at 90 V or 6 milliamps per well for 3½ hours, or until the fast blue component of the tracking dye reached the gel end. Standards of commercial RNA from E. coli (20 µl) and purified RNA from E. coli (80 µl) were used with 20 µl of tracking dye also being applied to the gel.

Gels were stained in ethidium bromide or methylene blue for 1/2 hour and where necessary, excess stain was removed by destaining in water.

Bands of RNA were detected under U.V. light or by scanning at 254 nm or 540 nm.

Attempts were made to visualize the RNA obtained by the extraction procedure. A spreading formamide method was selected as it is recommended for the visualization of single stranded RNA molecules (Evenson, 1977). Grids were floated onto prepared nucleic acid (Appendix IV) then dried and rotary shadowed with platinum at an angle of 8° and a distance of 12 cms. Grids were viewed at 50,000 x magnification at 80 KV using the transmission electron microscope (JEM 100 S).

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CHAPTER 3

Results

3.1. Indicator studies

Indicator studies were performed to determine whether observed symptoms in the field could be attributed to viruses. Leaf material collected showed symptoms that may have been viral in origin, such as vein thickening, leaf curling, mottling, local lesions, mosaic, yellowing and malformations.

Mechanical inoculation on indicator hosts, using the techniques documented in section 2.2.1. however, gave rise to few symptoms. The different types of symptoms produced are summarized in Tables 8 and 9. In general, the only symptoms produced were local lesions (of variable size and type) and mild mosaic with the occasional malformation of leaves.

The only samples producing symptoms were some of those obtained from Pretoria, Witkop, Potchefstroom and Ermelo.

On the basis of symptomatology, samples could be subdivided into 4 distinct groups. Group I formed a very distinct group as the symptoms recorded for this pathogen, isolated from a sample collected in Pretoria in 1978, were unique (Figure 1).

The majority of the samples (247, 259, Potchefstroom 2, 5, 7, 9 and those obtained from the Ermelo and Witkop farms) were placed in Group II because of similarity in the indicator studies. In general, isolates produced local lesions on Chenopodium amaranticolor and Chenopodium quinoa. The symptoms were fairly consistent except for the Ermelo sample where symptoms were recorded on the

Nicotiana species. For the Ermelo sample, mosaic symptoms were recorded on Nicotiana clevelandii and Nicotiana tabacum var. "Samsun". This sample was included in the group however because of the local lesions produced on the Chenopodium species. Samples 2 and 7 from Potchefstroom also differed slightly from the remaining members of Group II in that they produced unusual local lesions on Phaseolus vulgaris var. "Prince", sample 7 causing concentric local lesions, sample 2 producing necrotic local lesions. These viruses may have been different strains of the form responsible for the mosaic symptoms caused on Phaseolus vulgaris var. "Prince" by the remaining members of Group II. In general, the symptoms recorded for Group II are similar to those documented for BCMV (Bos, 1976).

Potchefstroom samples 3 and 4 were placed in Groups III and IV respectively. Both samples failed to produce any symptoms on the Chenopodium species, thereby being similar in this respect. However, the samples were not grouped together as sample 4 produced local lesions on Nicotiana glutinosa and mosaic on Phaseolus vulgaris species, while sample 3 produced local lesions. In other respects, reactions on host plants were similar. It is possible that the causal virus in both samples is similar, but of a different strain or that a mixed infection occured in sample 4. Generally, symptoms in these two groups differed quite markedly from the other groups.

Host range studies therefore gave some indication of possible viral pathogens being present in the collected samples. It can be seen that the majority of the positive samples produced symptoms in a narrow host range, varieties of the genera Phaseolus and Chenopodium being primarily affected.

Table 8: Symptoms on indicator plants

Sample area	Indicator Host	Symptoms produced
Potchefstroom 247, 1980	Chenopodium amaranticolor	local lesions
	Pisum sativum "Greenfeast"	mosaic on inoculated new leaves
	Nicotiana clevelandii	no symptoms
	Nicotiana glutinosa	no symptoms
	Arachis hypogea	no symptoms
	Datura stromonium	no symptoms
	Vicia faba	mosaic
	Trifolium repens	mosaic
	Glycine max	mosaic
	Chenopodium quinoa	local lesions
	Phaseolus vulgaris "Prince"	mosaic
Potchefstroom 259, 1980	Chenopodium amaranticolor	local lesions
	Chenopodium quinoa	local lesions
	Pisum sativum "Greenfeast"	mosaic
	Nicotiana clevelandii	no symptoms
	Phaseolus vulgaris "Prince"	mosaic
Pretoria TNV, 1978	Chenopodium amaranticolor	galls on damaged stems and leaves
T.I.P. 85° to 95°C	Chenopodium quinoa	local lesions
D.E.P. 10^{-3} to 10^{-4}	Nicotiana glutinosa	local lesions
L.I.V. up to 24 days at 20°	Pisum sativum "Greenfeast"	no symptoms
	Phaseolus vulgaris "Bountiful"	mosaic
	Phaseolus vulgaris "Prince"	mosaic
	Nicotiana rustica	local lesions
	Nicotiana tabacum "Samsun"	local lesions
	Nicotiana tabacum "White Burley"	local lesions and mottle

Sample Area	Indicator Host	Symptoms produced
Pretoria TNV, 1978	Tetragonia expansa	local lesions
	Gomphrena globosa	local lesions
	Petunia hybrida	local lesions
	Vicia faba	local lesions
Nigel site 1 1981	Phaseolus vulgaris "Prince"	no symptoms
	Chenopodium amaranticolor	no symptoms
	Chenopodium quinoa	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms
	Petunia hybrida	no symptoms
Nigel site 2, 1981	Chenopodium amaranticolor	no symptoms
	Chenopodium quinoa	no symptoms
	Nicotiana clevelandii	no symptoms
	Nicotiana glutinosa	no symptoms
	Phaseolus vulgaris "Bountiful"	no symptoms
Nigel site 3, 1981	Chenopodium quinoa	no symptoms
	Chenopodium amaranticolor	no symptoms
	Petunia hybrida	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms
	Phaseolus vulgaris "Prince"	no symptoms
	Lycopersicum esculentum	no symptoms
Nigel site 4, 1981	Chenopodium quinoa	no symptoms
	Chenopodium amaranticolor	no symptoms
	Petunia hybrida	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms

Sample Area	Indicator Host	Symptoms produced
Oogies site 1, 1981	Chenopodium quinoa	no symptoms
	Chenopodium amaranticolor	no symptoms
	Petunia hybrida	no symptoms
	Lycopersicum esculentum	no symptoms
	Pisum sativum "Greenfeast"	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms
	Phaseolus vulgaris "Bountiful"	no symptoms
Oogies site 2, 1981	Chenopodium amaranticolor	no symptoms
	Petunia hybrida	no symptoms
	Chenopodium quinoa	no symptoms
	Lycopersicum esculentum	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms
	Phaseolus vulgaris "Prince"	no symptoms
Oogies site 3, 1981	Chenopodium amaranticolor	no symptoms
	Chenopodium quinoa	no symptoms
	Lycopersicum esculentum	no symptoms
	Petunia hybrida	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms
	Phaseolus vulgaris "Bountiful"	no symptoms
	Pisum sativum "Greenfeast"	no symptoms
Delmas site 1, 1981	Phaseolus vulgaris "Bountiful"	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms
	Pisum sativum "Greenfeast"	no symptoms
	Lycopersicum esculentum	no symptoms
	Petunia hybrida	no symptoms
	Chenopodium quinoa	no symptoms
	Chenopodium amaranticolor	no symptoms

Sample Area	Indicator Host	Symptoms produced
Delmas site 2, 1981	Phaseolus vulgaris "Bountiful"	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms
	Pisum sativum "Greenfeast"	no symptoms
	Lycopersicum esculentum	no symptoms
	Petunia hybrida	no symptoms
	Chenopodium quinoa	no symptoms
	Chenopodium amaranticolor	no symptoms
Delmas site 3, 1981	Phaseolus vulgaris "Bountiful"	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms
	Petunia hybrida	no symptoms
	Chenopodium amaranticolor	no symptoms
Delmas site 1, 1981	Phaseolus vulgaris "Prince"	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms
	Lycopersicum esculentum	no symptoms
	Petunia hybrida	no symptoms
	Chenopodium quinoa	no symptoms
	Chenopodium amaranticolor	no symptoms
Delmas site 2, 1981	Phaseolus vulgaris "Bountiful"	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms
	Pisum sativum "Greenfeast"	no symptoms
	Lycopersicum esculentum	no symptoms
	Petunia hybrida	no symptoms
	Chenopodium quinoa	no symptoms
	Chenopodium amaranticolor	no symptoms

Sample area	Indicator Host	Symptoms produced
Bombardie site 1, 1981	Phaseolus vulgaris "Prince"	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms
	Lycopersicum esculentum	no symptoms
	Petunia hybrida	no symptoms
	Chenopodium quinoa	no symptoms
	Chenopodium amaranticolor	no symptoms
Kendal site 1, 1981	Phaseolus vulgaris "Prince"	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms
	Pisum sativum "Greenfeast"	no symptoms
	Lycopersicum esculentum	no symptoms
	Petunia hybrida	no symptoms
	Chenopodium quinoa	no symptoms
	Chenopodium amaranticolor	no symptoms
Witkop site 1, 1981	Pisum sativum "Greenfeast"	mosaic
	Chenopodium amaranticolor	local lesions
	Phaseolus vulgaris "Bountiful"	mosaic
	Phaseolus vulgaris "Prince"	mosaic
	Nicotiana clevelandii	no symptoms
	Chenopodium quinoa	local lesions
Potchefstroom site 2, 1981	Chenopodium quinoa	local lesions
	Lycopersicum esculentum	no symptoms
	Phaseolus vulgaris "Prince"	necrotic local lesions
	Nicotiana tabacum "Samsun"	no symptoms
	Nicotiana clevelandii	no symptoms
	Datura stromonium	no symptoms
	Pisum sativum "Greenfeast"	mosaic
	Chenopodium amaranticolor	local lesions

Sample Area	Indicator Host	Symptoms produced
Potchefstroom site 1, 1981	Chenopodium quinoa	no symptoms
	Chenopodium amaranticolor	no symptoms
	Pisum sativum "Greenfeast	no symptoms
	Nicotiana clevelandii	no symptoms
	Nicotiana glutinosa	no symptoms
Potchefstroom site 3, 1981	Phaseolus vulgaris "Prince"	local lesions
	Pisum sativum "Greenfeast"	no symptoms
	Datura stromonium	no symptoms
	Nicotiana tabacum "Samsun"	no symptoms
	Nicotiana glutinosa	no symptoms
	Nicotiana clevelandii	no symptoms
	Lycopersicum esculentum	no symptoms
	Chenopodium quinoa	no symptoms
	Chenopodium amaranticolor	no symptoms
Potchefstroom site 4, 1981	Chenopodium quinoa	no symptoms
	Phaseolus vulgaris "Prince"	mosaic
	Pisum sativum "Greenfeast"	no symptoms
	Chenopodium amaranticolor	no symptoms
	Nicotiana glutinosa	local lesions
	Phaseolus vulgaris "Bountiful"	mosaic
	Nicotiana clevelandii	no symptoms
Potchefstroom site 5, 1981	Nicotiana tabacum "Samsun"	no symptoms
	Nicotiana clevelandii	no symptoms
	Chenopodium amaranticolor	local lesions
	Lycopersicum esculentum	no symptoms
	Datura stromonium	no symptoms
	Chenopodium quinoa	local lesions
	Pisum sativum "Greenfeast"	mosaic

Sample Area	Indicator Host	Symptoms produced
Potchefstroom Site 6, 1981	Phaseolus vulgaris "Bountiful"	no symptoms
	Nicotiana tabacum "White Burley"	no symptoms
	Pisum sativum "Greenfeast"	no symptoms
	Lycopersicum esculentum	no symptoms
	Petunia hybrida	no symptoms
	Chenopodium quinoa	no symptoms
	Chenopodium amaranticolor	no symptoms
Potchefstroom site 7, 1981	Nicotiana clevelandii	no symptoms
	Nicotiana glutinosa	no symptoms
	Phaseolus vulgaris "Prince"	concentric local lesions
	Phaseolus vulgaris "Bountiful"	concentric local lesions
	Pisum sativum "Greenfeast"	mosaic
	Chenopodium amaranticolor	local lesions
	Chenopodium quinoa	local lesions
Ermelo site 8, 1981	Phaseolus vulgaris "Prince"	mosaic
	Chenopodium amaranticolor	local lesions
	Nicotiana clevelandii	mosaic
	Nicotiana tabacum "Samsun"	mosaic
	Datura stromonium	no symptoms
	Lycopersicum esculentum	no symptoms
	Chenopodium quinoa	local lesions
	Pisum sativum "Greenfeast"	mosaic

Sample Area	Indicator Host	Symptom produced
Potchefstroom site 9, 1981	Chenopodium quinoa	local lesions
	Chenopodium amaranticolor	local lesions
	Nicotiana glutinosa	no symptoms
	Nicotiana clevelandii	no symptoms
	Phaseolus vulgaris "Bountiful"	mosaic
Delmas site 10, 1981	Nicotiana tabacum "Samsun"	no symptoms
	Nicotiana clevelandii	no symptoms
	Pisum sativum "Greenfeast"	no symptoms
	Chenopodium amaranticolor	no symptoms
	Chenopodium quinoa	no symptoms
	Lycopersicum esculentum	no symptoms
	Datura stromonium	no symptoms
	Phaseolus vulgaris "Prince"	no symptoms

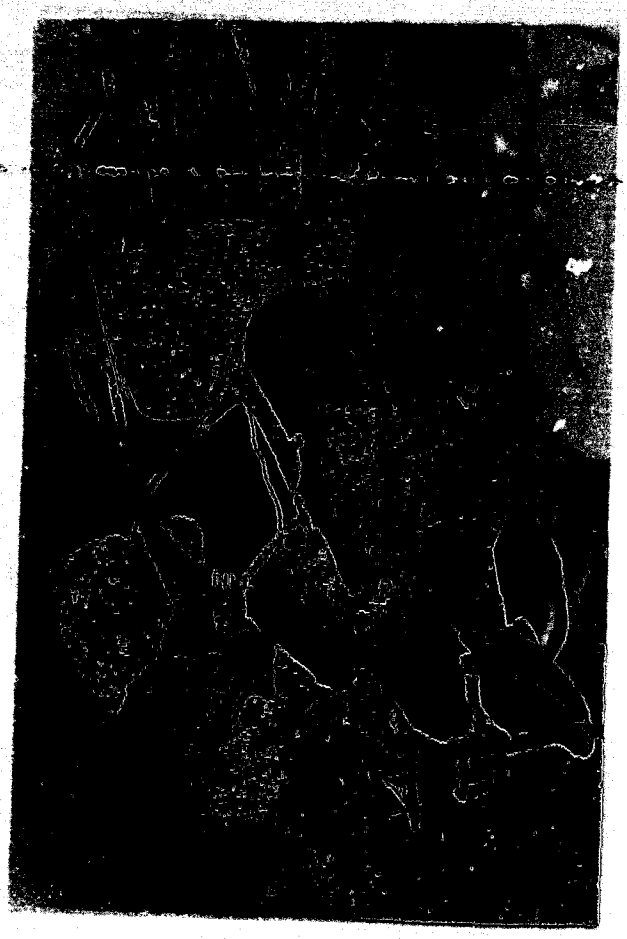

Figure 1: Symptoms induced by isolate suspected of being Tobacco necrosis virus on Chenopodium quinoa showing necrotic local lesions. Lesions (NLL) appeared only on inoculated leaves, after 4 days and gradually increased in size. Lesions eventually coalesced and turned brown, leading to the death of the inoculated leaves. These symptoms are typical of tobacco necrosis virus on members of the genus Chenopodium.

Table 9: A comparison of symptoms produced by positive samples on indicator host plants

Host Plant	Sample	PROPOSED GROUPS										
		Group I	Group II								Groups III	IV
		? TNV	247	259	P7	P2	P9	P5	W	E	P3	P4
Chenopodium amaranticolor		11,g	11	11	11	11	11	11	11	11	x	x
Chenopodium quinoa		11	11	11	11	11	11	11	11	11	x	x
Pisum sativum "Greenfeast"		x	m	m	m	m	m	m	m	m	x	x
Phaseolus vulgaris "Prince"		m	m	m	c11	n11	—	—	m	m	11	m
	"Bountiful"	m	—	—	c11	—	m	—	m	—	—	m
Vicia faba		11	m	—	—	—	—	—	—	—	—	—
Nicotiana clevelandii		11	x	x	x	x	x	x	x	m	x	x
Nicotiana glutinosa		11	x	x	x	—	x	—	—	—	x	11
Nicotiana rustica		11	—	—	—	—	—	—	—	—	—	—
Nicotiana tabacum "Samsun"		11	—	—	—	x	—	x	—	m	x	—
Nicotiana tabacum "White Burley"		11	—	—	—	—	—	—	—	—	—	—
Glycine max.		—	m	—	—	—	—	—	—	—	—	—
Datura stramonium		—	x	—	—	x	—	x	—	x	x	—
Trifolium repens		—	m	—	—	—	—	—	—	—	—	—
Arachis hypogea		—	x	—	—	—	—	—	—	—	—	—
Tetragonia expansa		11	—	—	—	—	—	—	—	—	—	—
Gomphrena globosa		11	—	—	—	—	—	—	—	—	—	—
Petunia hybrida		11	—	—	—	—	—	—	—	—	—	—
Lycopersicum esculentum		—	—	—	x	—	x	—	x	x	—	—

KEY:

x	no symptoms
ccl	concentric local lesion
E	Ermelo sample
g	galls
ll	local lesions
m	mosaic symptoms on leaves
nll	necrotic local lesions
P	Potchefstroom sample
-	not tested

GROUPS:

Group I	suspected TNV sample
Group II	samples 247, 259, Witkop, Ermelo, Potchefstroom 2, 5, 7 and 9
Group III	Potchefstroom sample 3
Group IV	Potchefstroom sample 4

Not all of the samples were inoculated onto all indicator plants due to restricted supply and satisfactory conditions for growing inoculated material. However attempts were made to use a similar range of hosts for collected samples displaying similar symptoms.

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3.2. Isolation and detection of individual viral particles

Attempts to purify viruses from infected leaf samples were made using the four procedures documented in 2.3. After the final ultracentrifugation cycle, each pellet was resuspended in a small volume of the appropriate buffer. Each sample was negatively stained and viewed under the electron microscope for the presence of viral particles. Sap samples of the host plants were also stained in this way. The results obtained are presented in Table 10 and photographs of the isolates are presented in Figures 1 to 4.

The purification procedures were selected on the basis of being able to detect either several viruses or a specific virus. The two specific methods chosen were for BCMV and BYMV, as syptoms on the field and indicator plants suggested that these viruses may be present. Procedure 4 (2.3.4.) is known to be a most successful method for isolating BCMV (Jafarpour et al., 1979) while procedure 1 (2.3.1.) selects specifically for BYMV (Maat, 1975). The remaining two procedures (2.3.2. and 2.3.3.) were selected as they are known to be the most successful methods for isolating a wide range of spherical and flexuous viruses that infect beans (Whitlock, 1979). The four procedures selected should have ensured the isolation of any virus present in the collected leaf samples.

The results indicate that procedures 2 and 4 were the most successful methods for the isolation of particles (Table 10).

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In terms of the quality of the final preparation however, procedure 4 resulted in a better separation of particles from the host material. Virus particles were less fragmented and the quantity of the particles that could

be isolated, greater (Figure 3).

Electron microscopy of infected sap from the Pretoria sample showed the presence of spherical particles, the mean diameter being 26 nm (Figure 2). From the symptoms produced on indicator plants, electron microscopy studies and biological properties (Table 8), the isolate is probably tobacco necrosis virus (TNV). The virus has been almost fully characterized (Kassanis, 1970) and for this reason further investigations were not carried out.

Electron microscopy showed the presence of flexuous virus particles in purified samples 247, 259, Potchefstroom 2, 3, 7 and the Ermelo and Witkop farms. Only in some instances could particles be seen in sap samples, this presumably being dependent on the initial host concentration (Table 10).

All particles isolated had similar dimensions but were present in low numbers. In each sample, about 20 particles were measured and the mean width and length were 15 nm and 750 nm respectively (Figures 3 to 5).

As the majority of the samples collected were grouped together on symptomatology (Group II) and as they showed similar particle morphology, one isolate in this group, namely 247, was selected for further characterization. The remainder were freeze dried and stored at 4°C. It is important to note that the most successful isolation procedure for this group of viruses was procedure 4 (2.3.4), a procedure recommended for BCMV (Jafarpour et al., 1979).

Table 10: Particles observed by electron microscopy

Sample	Crude sap	Virus purification procedure			
		1(2.3.1.)	2(2.3.2.)	3(2.3.3.)	4(2.3.4.)
247	flex virus	-	-	-	flex virus
259	flex virus	-	-	-	flex virus
Pretoria	spherical	-	-	-	-
Witkop	-	-	-	-	flex virus
Potchefstroom 2	-	-	-	-	flex virus
Potchefstroom 3	-	-	flex virus	-	flex virus
Potchefstroom 7	flex virus	-	-	-	flex virus
Ermelo 8	-	-	-	-	flex virus

KEY:

flex virus - flexuous virus particles

Figure 2: Electron micrograph of a suspected TNV particle (sv) in a leaf dip preparation of sap from infected Phaseolus vulgaris var. "Witsa" plants. (x 50,000; 2% uranyl acetate solution)

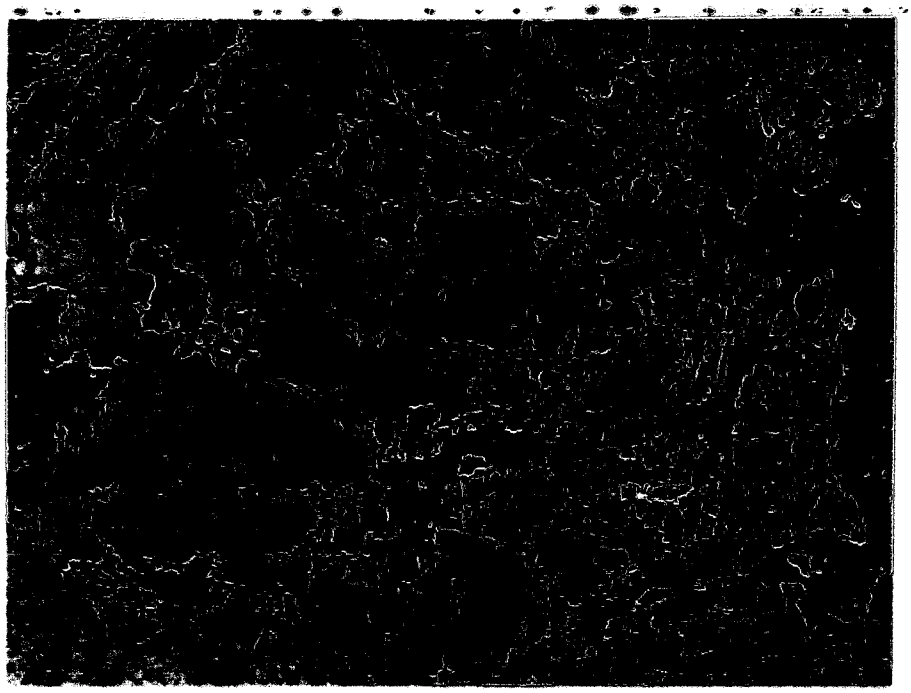

Figure 3: Potchefstroom sample 7. Several flexuous particles (flex virus) were isolated after purification using procedure 4 (2.3.4.). Particles measured an averaged 750 nm in length and 15 nm in width. (x 50,000; 2% uranyl acetate stain).

Figure 4: Electron micrograph of a flexuous virus (flex virus) isolated using the general procedure 2 (2.3.2.), from sample 3 (Potchefstroom). The average dimensions of the particles were 750 nm in length and 15 nm in width. (x 50,000; 2% uranyl acetate stain).

Figure 5: Electron micrograph showing a fragment of a flexuous particle (flex virus) isolated using procedure 4 (2.3.4.). The length of the particles could not be accurately measured as they were too fragmented yet the width consistently measured 15 nm. The particles were isolated from a sample obtained from Witkop. (x 50,000; 2% uranyl acetate solution).

3.3. Host range studies of the isolate

Having decided to study isolate 247 in detail, it was inoculated onto a wider range of indicator species to determine the host range. This aids the identification of any unknown virus. The previous indicator studies, the results of purification experiments and electron microscopy indicated that this (and possibly other isolates in Group II) could be BCMV.

Results (Table II) indicated that the isolate was only able to infect a limited number of hosts, the majority of which were confined to the family Leguminosae. The isolate was tentatively identified as BCMV and the possibility of it being one of several closely related viruses (such as BYMV) was ruled out. BCMV is easily distinguished from BYMV on the basis of symptomatology (Bird and Maramorosch, 1975).

Host range studies were also used to determine which variety of Phaseolus vulgaris was the most suitable for the propagation of large quantities of virus required for the biochemical and serological characterization of the isolate. It was found that the variety Phaseolus vulgaris "Double Dutch Princess" was the most suitable propagation host for the isolate. This variety is a recommended virus concentrating host for BCMV (Noordam, 1973). Symptoms appeared about 2 weeks after the inoculation of primary leaves (Figures 6 and 7). From the systemically infected leaves, high concentrations of virus could be extracted using procedure 4 described in 2.3.4.

Table 11: Extended host range studies on the 247 isolate

Indicator Host	Symptoms on leaves
Nicotiana tabacum var. "White Burley"	no symptoms
Nicotiana clevelandii	no symptoms
Nicotiana glutinosa	no symptoms
Chenopodium amaranticolor	small chlorotic lesions
Pisum sativum var. "Greenfeast"	mosaic
Cucumis sativus	slight mosaic
Datura stromonium	no symptoms
Arachis hypogea	no symptoms
Glycine max	slight mosaic
Petunia hybrida	no symptoms
Chenopodium quinoa	small chlorotic lesions
Vicia faba	mosaic
Trifolium repens	mosaic
Brassica chinensis	mosaic
Several varieties of Phaseolus vulgaris	mosaic, local lesions, veinal necrosis etc. depending on variety tested.

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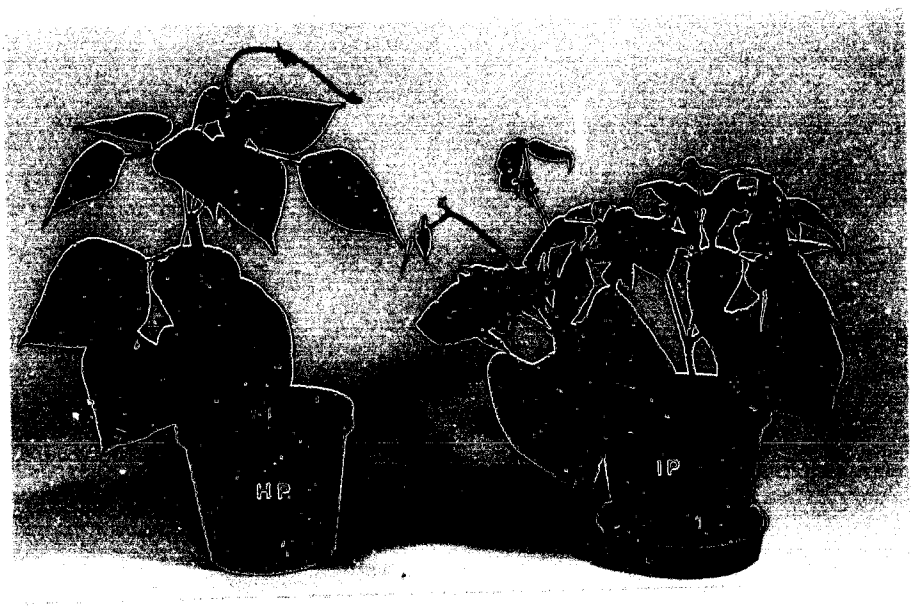


Figure 6: Phaseolus vulgaris var. "Double Dutch Princess" showing stunted and malformed leaves with mosaic in the infected host plant (I.P.). A healthy plant (H.P.) is provided for comparison.

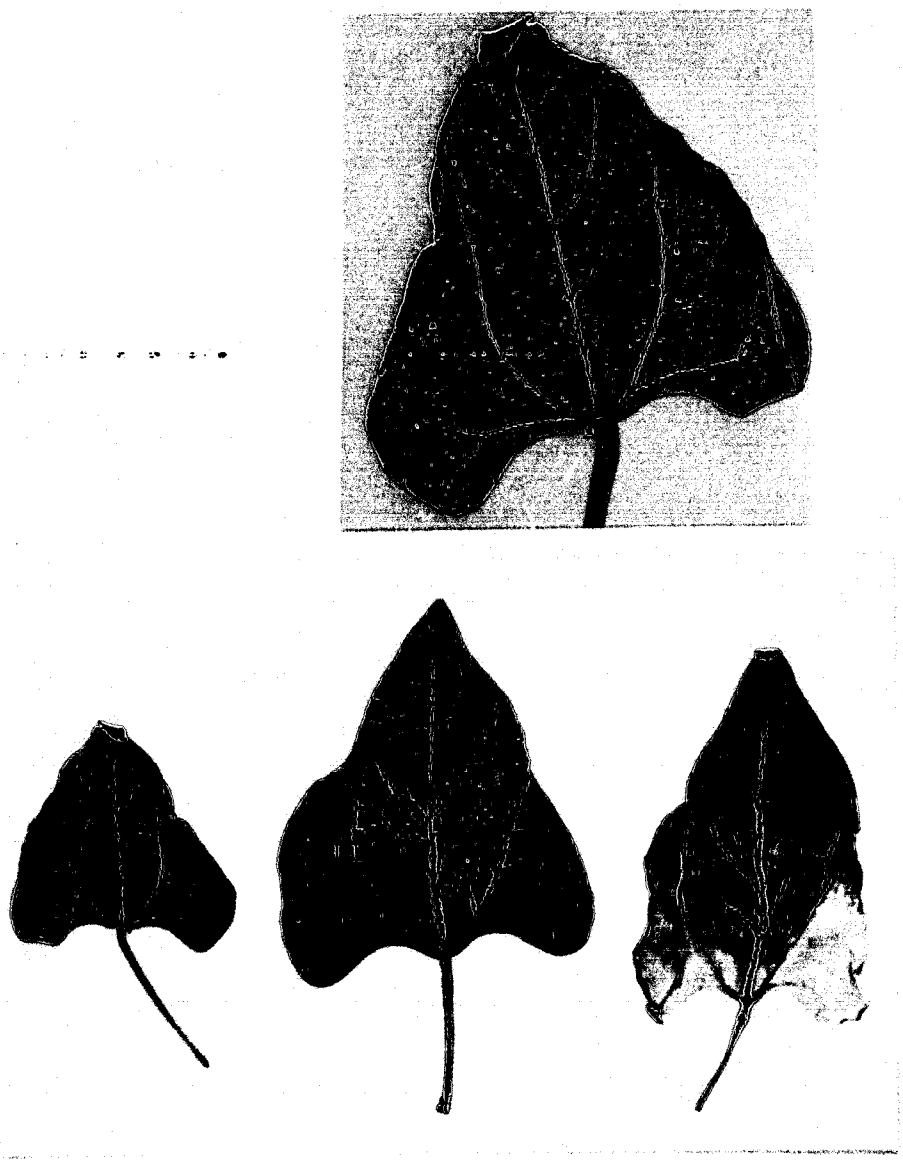
Figure 7: Infected Phaseolus vulgaris var. "Double Dutch Princess" plant with virus isolate suspected to be BCMV. Symptoms of mosaic (m) appeared 2 weeks after inoculation of the primary leaves. Infected leaves were collected 13 to 15 days after inoculation and purified immediately using procedure 4 described in 2.3.4.

3.4. Biological properties of the presumptive BCMV isolate

Inoculation of the isolate onto Phaseolus vulgaris vars. "Beka" and "Monroe" indicated that the dilution end point was 10^{-3} to 10^{-4}, the longevity in vitro was 1 to 2 days and the thermal inactivation point was 55°C to 60°C. A systemic reaction was produced on the "Beka" variety which is not ideal so the results were checked by inoculating the "Monroe" variety, a well known local lesion host for BCMV. The plants were assessed visually. No quantitative results on lesion numbers were recorded.

The detached leaf method for determination of biological properties using Chenopodium quinoa proved unsuccessful. The indicator host is well documented to be highly susceptible to BCMV infection producing local lesions that can be easily counted. It was however, found to be unsuitable as the leaves invariably began to turn yellow before symptom expression occurred. Results were therefore inconsistent and it was found that inoculation onto Phaseolus vulgaris var. "Monroe" produced even clearer lesions and a more accurate estimation of the biological properties of the virus was obtained.

Typical local lesion symptoms caused by the isolate on Phaseolus vulgaris var. "Monroe" can be seen in Figure 8. Lesions appeared about 10 days after the inoculation of primary leaves. As the plant aged, a limited local vein necrosis also occured, confined to the area around the lesions.



(a) (b) (c)

Figure 8: Local lesion symptoms on Phaseolus vulgaris var. "Monroe". In the photograph, a progression of symptoms can be seen, caused by inoculation of the 247 isolate on primary leaves. Small local lesions (LL) developed 10 days after inoculation of the seedlings (a). Lesions then increased in size, showing a characteristic ringspot appearance (b) and (c). Local vein necrosis (LVN) around the lesions occured some weeks after the initial inoculation (c). A concentrated sap preparation of isolate 247, suspected to be BCMV, was used to inoculate the plant.

3.5. Complete purification of the presumptive BCMV isolate

The purity of the virus preparation was affected by the final purification procedure used. With differential centrifugation and sucrose gradients (2.3.5.), the virus was always contaminated with cell debris and host components (Figures 9, 10 and 11). Caesium chloride yielded a better separation of virus as can be seen in the spectrophotometric scan (Figure 12) where the virus separated out as a single peak from the remaining host contaminants. With the electron microscope however, particles seemed somewhat altered in shape and structure (Figure 13). The procedure is extremely severe, entailing long periods of ultracentrifugation which could account ultimately for the atypical appearance of the virus and its fragmentation (Figure 13).
Of all the purification procedures attempted, separation of the virus was best achieved using the glass bead column apparatus (2.3.5.). Separation could be achieved with minumum treatment of the virus so that the effects of harsh chemicals and centrifugation were now eliminated. The glass bead column apparatus also enabled the separation of larger volumes of virus which is advantageous as other methods made use of small samples.

Electron microscopy of the virus peaks (Figures 14 and 15) indicated that cell debris was almost eliminated and particles were considerably less fragmented. When compared to the scans obtained from healthy samples treated in the same way, the virus peak could always be clearly distinguished (Figure 14). Owing to the success of this procedure, it was followed routinely whenever samples of high purity were required, for example, in electrophoresis and for raising antiserum.

As samples are diluted in 0,01 M phosphate buffer, pH 7,16 with column separation, they were ultracentrifuged at

27,000 RPM for 90 minutes to pellet the virus. Virus was resuspended in 0,025 M potassium phosphate buffer pH 7,2 (Appendix I). The final concentration of virus in purified samples was then determined spectrophotometrically. The E value for BCMV is not known, but Jafarpour et al., (1979) used a formula based on tobacco etch virus (which has similar dimensions and features to BCMV), to estimate the E value for BCMV. A similar value was used in the current study. No corrections for light scattering were made.

E^{o} = 2,4 mg/ml at 261 nm

(Jafarpour et al., 1979)

After purification the 260 nm absorbance values ranged between 0,2 and 2,6 implying a minimum concentration of

(0,2 / 2,4) x 1 = 0,08 mg/ml

and a maximum concentration of

(2,6 / 2,4) x 1 = 1,08 mg/ml

When required, virus concentrations were adjusted to approximately 1 mg/ml before use.

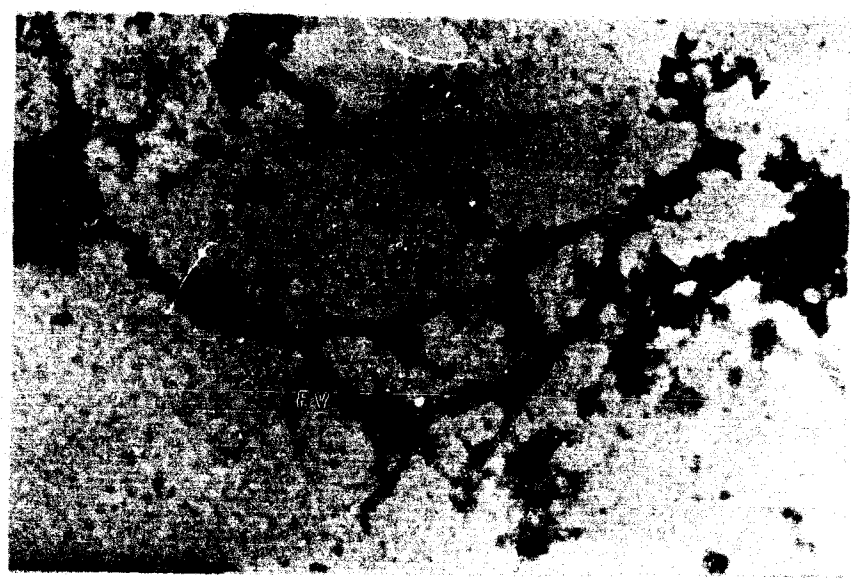
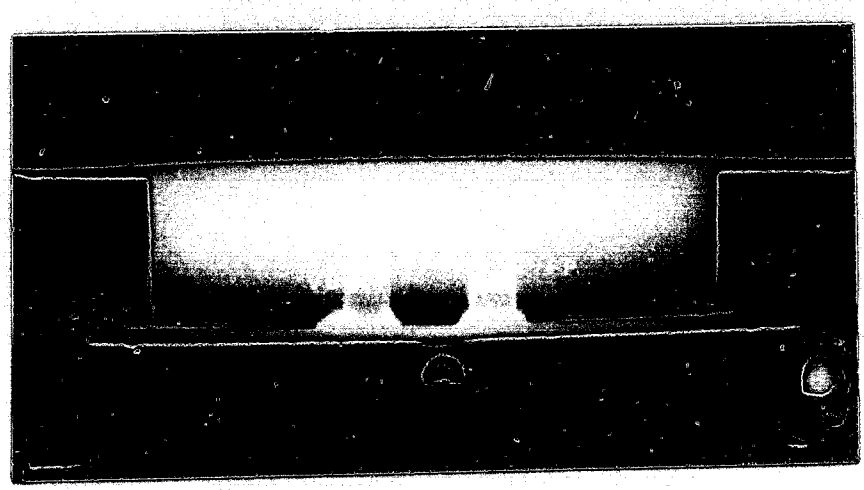


Figure 9: An electron micrograph of isolate 247 following purification by differential centrifugation only. Note the aggregation of flexuous particles (flex virus) with host debris. (x 50,000; 2% uranyl acetate st_ .n).



247 Sample 7

Figure 10: Separation of isolates by sucrose gradients. Isolates 247 and sample 7 (Potchefstroom) were found to separate in the 20% to 30% sucrose gradient zones. Host protein (Fr I) can be seen to separate just below the virus band (VB). As a result, contamination of the virus sample often occured and separation from host material was never complete. The virus bands were detected using an ultraviolet light source.

Figure 11: Electron micrograph of another isolate, 259, following separation on a 10% to 40% sucrose gradient. Flexuous particles (flex virus) were isolated from the gradient by fractionation. The particles occured in the 20% to 30% sucrose zone. Particles were concentrated by ultracentrifugation. Note the high degree of fragmentation of particles following their resuspension in 0,025 M phosphate buffer, pH 7,2. (x 50,000; 2% uranyl acetate stain).

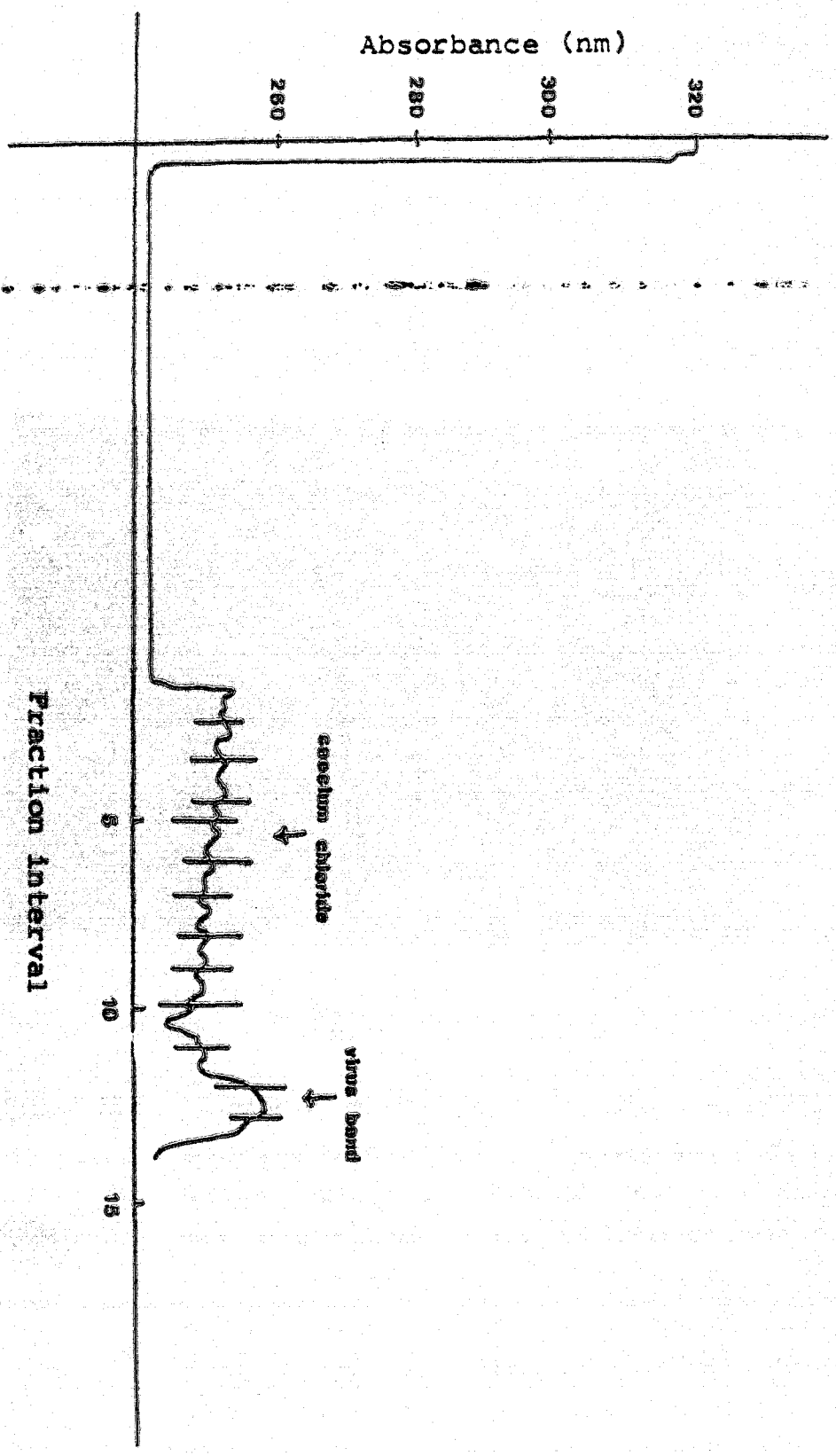


Figure 12:

A scan showing the fractionation of a 30% (w/v) caesium chloride gradient. The virus band always separated out as a single peak, distinct from the host contaminants. The virus band was concentrated by ultracentrifugation and resuspended in a minute volume of 0,025 M phosphate buffer, pH 7,2. Caesium was removed by dialysis or by alternating cycles of differential centrifugation.

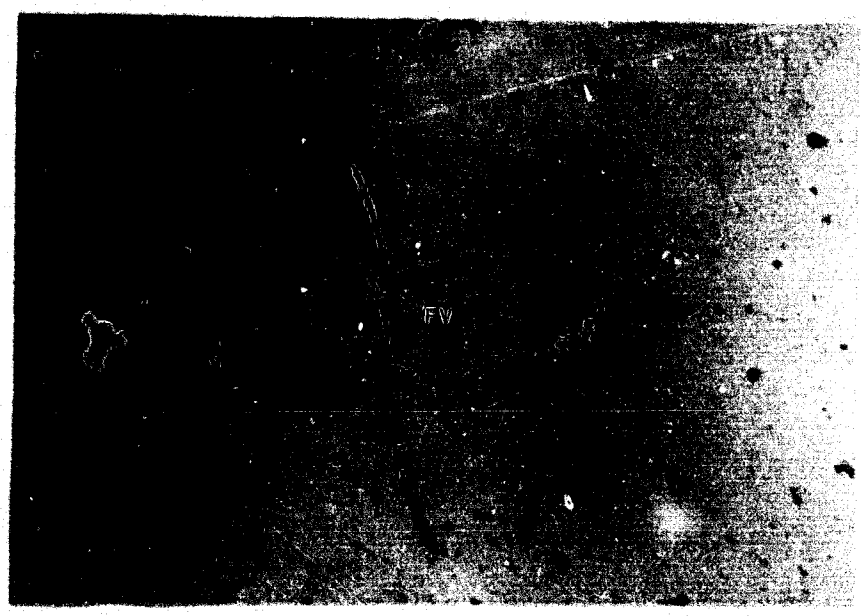


Figure 13: Electron micrograph of isolate 247 from a 30% (w/v) caesium chloride gradient. The virus band was concentrated by ultracentrifugation and resuspended in a minute volume of 0,025 M phosphate buffer, pH 7,2. Note the fragmentation of particles (flex virus) which also appear morphologically altered due to the severity of the separation procedure. Note however, the absence of contaminating material. (x40,000; 2% uranyl acetate solution).