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INVESTIGATION OF THE CAUSAL AGENT(S) OF TOBACCO LEAF CURL DISEASE IN SOUTH AFRICA

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Submitted to the School of Biology, University of the Witwatersrand, 1 Jan Smuts Ave., Johannesburg, in partial fulfillment of the requirements for the degree M. Sc. (Biotechnology).

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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.

_____

27th day of February, 1990.

LIST OF ABBREVIATIONS

ACMV – African cassava mosaic virus

BGMV – bean golden mosaic virus

bp – base pair(s)

BSA – bovine serum albumin

CsCl – caesium chloride

ddH_2O – double distilled water

dH_2O – distilled water

DNA – deoxyribonucleic acid

dsDNA – double stranded deoxyribonucleic acid

Na EDTA – ethylenediamine tetra-acetic acid (disodium salt)

ELISA – enzyme linked immunosorbent assay

EM – electron microscopy

EtBr – ethidium bromide

GLAD – gold labelled antibody decoration

h – hour(s)

HCl – hydrochloric acid

H_2O – water

ISEM – immune specific electron microscopy

kd – kilodalton(s)

KH_2PO_4 – potassium dihydrogen orthophosphate

K_2HPO_4 – dipotassium hydrogen orthophosphate

M – molar

$MgCl_2$ – magnesium chloride

$MgSO_4$ – magnesium sulphate

min – minute(s)

ml – millilitre(s)

mM – millimolar

MSV – maize streak virus

MW – molecular weight

MYMV – mungbean yellow mosaic virus

Na – sodium

NC – nitrocellulose

NaCl – sodium chloride

$NaClO_4$ – sodium perchlorate

NaOH – sodium hydroxide

nm – nanometres

PAGE – polyacrylamide gel electrophoresis

PBS – phosphate buffered saline

PEG – polyethylene glycol

PNP – p – nitrophenol

PVP – polyvinyl pyrrolidine

PVY – potato virus Y

rpm – revolutions per minute

SDS – sodium dodecyl lauryl sulphate

ssDNA – single – stranded deoxyribonucleic acid

TAE – Tris acetic EDTA

TBE – Tris boric EDTA

TE – Tris EDTA

TEMED – tetramethyl ethylene diamine

TGMV – tomato golden mosaic virus

TLC – tobacco leaf curl

TLCV – tobacco leaf curl virus

TmLCD – tomato leaf curl disease

UV – ultraviolet

v – volume

$ZnCl_2$ – zinc chloride

ABSTRACT

Tobacco leaf curl (TLC) is a serious disease in tropical areas including Zimbabwe and the Transvaal. TLC is thought to be caused by several virus strains inducing mild to severe symptoms. TLC is whitefly and graft transmissible but all mechanical and dodder transmissions have proved unsuccessful. Initial results indicate that the TLC disease agent may have been transmitted using a rapid electrotransmission method attempted in this study. Osaki and Inouye (1978) succeeded in isolating a geminivirus from infected tobacco in Japan, but in this study several attempts to purify a geminivirus from plants infected with TLC have failed, as have attempts to visualize a geminivirus by TEM. These results indicate that a geminivirus may not be involved in the TLC syndrome in South Africa. A small novel dsDNA molecule was isolated from leaves with a mild form of TLC and it is suggested that this DNA is associated with the TLC syndrome. This DNA could be a part of a geminivirus genome, or it could have a "satellite virus-like" function or be a part of a multipartite virus complex although there is no evidence for this. TEM of leaves with severe TLC revealed pinwheel inclusion bodies and potyvirus particles. Together with serological evidence, this indicates that a potyvirus may be involved in at least one of the strains of TLC disease in South Africa.

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1 INTRODUCTION

Tobacco leaf curl (TLC) disease, also called "kroepoek", "crinkly dwarf", "gilah", "curly leaf", "enroscamiento del cogollo", "tobacco cabbaging", "tobacco curly leaf" and "tobacco frenching" was first recorded in the Netherlands East Indies by Peters and Schwarz in 1912 (Lucas, 1974; Osaki and Inouye, 1981). Its cause was unknown until 1931, when it was independently announced by Storey in Tanganyika and Thung in Java that leaf curl is induced by a virus, known as tobacco leaf curl virus (TLCV) and transmitted by Bemisia tabaci Gen., a species of whitefly (Lucas, 1974; Osaki and Inouye, 1981). The disease is widespread in many tropical areas and some temperate regions (Osaki and Inouye, 1981) and has been found in Africa, Australia, Brazil, Central America, Colombia, India, Japan, Madagascar, Mexico, Morocco, Panama, the Philippines, Puerto Rico, Russia, Sumatra, Switzerland, Taiwan and Venezuela (Lucas, 1974).

According to Lucas (1974), the disease is serious in Sumatra and at one time was one of the most destructive diseases of tobacco in East Africa, Zimbabwe (then Rhodesia) and the Transvaal. In some years, the disease may reach epidemic proportions causing serious crop losses and, in some cases, total crop failure in the Transvaal. In other years it is virtually negligible and only a few diseased plants are seen on any land (Hill, 1967). The severity of the leaf curl infection, resulting from favourable conditions for the breeding of whiteflies, has apparently prevented the cultivation of tobacco in certain areas of Pakistan (Lucas, 1974).

The leaf curl or "kroesblaar" disease in South Africa has been investigated by Moore (1933; 1934), McClean (1940), Hill (1967) and Thatcher (1976), although it was apparently reported for the first time by Lounsbury in 1902 (Hill, 1967). The first large outbreak occurred in the Rustenburg district in the 1928/29 season. In 1931/32 the disease was prevalent in both the eastern and western Transvaal. During this season, Moore (1934) states that it was "not unusual to see half the plants in a field severely affected. In some cases the entire crop was a failure and infected plants were even seen in the seedbeds." In 1935 the occurrence of the disease was negligible in the Rustenburg area while in the Northern Transvaal it was very severe. McClean (1940) mentions that, as a rule, only a small percentage of infection occurs before midseason while many more plants are infected after midseason. Infection is often high in autumn suckers, even when only a small number of plants show infection at the time of harvest.

Despite the severity of the disease in many countries, isolation and characterization of the causal agent of TLCV have only been reported in Japan. In 1978 in Japan, Osaki and Inouye purified predominantly geminate icosahedral particles 18 -- 19 nm in diameter from leaf curl diseased tobacco and indicated the association of a geminivirus with this disease. Studies by the same group on ultracytopathology of leaf curl diseased tobacco showed nuclear abnormalities which are very similar to those caused by the whitefly transmitted geminiviruses. These observations, together with the fact that TLCV is transmitted by whiteflies, led to the tentative classification of TLCV into the geminivirus group by Osaki and Inouye (1978). In 1987, Ikegami, Osaki

- and Inouye found evidence that TLCV contains circular ssDNA of similar molecular weight to DNA of other geminiviruses, supporting the classification of TLCV as a member of the geminivirus group.

Apparently tobacco leaf curl is caused by several virus strains that cause mild to severe symptoms but the different strains or variants have not yet been fully characterised.

In this study, an investigation was made into the character of the TLC infectious agent and transmission of this agent.

The aims of the study were:

1. To isolate the infectious agent(s) causing the TLC syndrome.

2. To attempt to rapidly transmit these disease agent(s) by mechanical or other means in order to facilitate resistance breeding trials.

3. To characterize the disease agent(s) using nucleic acid, protein and biological assays.

4. To investigate the causal agent(s) of the disease using electron microscopy.

2 LITERATURE REVIEW

2.1 SYMPTOMATOLOGY AND HOST RANGE

Apparently tobacco leaf curl is caused by several virus strains that cause mild to severe symptoms. Five forms of South African leaf curl were recognised by McClean in 1940:- severe leaf curl, mild forms A, B and C and latent leaf curl, the latter identifiable with certainty only by transmission to Petunia. The typical or severe leaf curl syndrome described by McClean commences with a systematically induced, transient, yellow network coinciding with the smaller veins on the upper surface of young, expanding leaves. However, observations by Thatcher (1976) indicate that the vein clearing may be caused by a toxin exuded by the whitefly on beginning to feed on the plant. Subsequently formed leaves develop a downward curling of leaf margins and irregular grooves and pits in veins on the upper surface corresponding with dark green thickenings or occasional leaf-like enations on the lower surface. According to McClean (1940) the tobacco plant becomes stunted with the leaves more closely grouped. However, not all infected plants become stunted and some may attain normal height. The inflorescence is much condensed with the flowers falling prematurely and failing to set fruits. The veins of the calyx are thickened while the corolla is reduced in size and deformed. The mild and latent strains range from mild form A in which symptom severity is only slightly less than that described above, to a latent strain which produces occasional, small, downward bulges on the lower surface of some strains in the later stage of infection (Thatcher, 1976).

Other workers have also distinguished 5 different forms, some of which may be caused by mixtures of virus strains (Nariani, 1968; Pal and Tandon, 1937; Vasudeva and Raj, 1948; Yassin and Nour, 1965). It was concluded by Pal and Tandon (1937) that four of the five types are caused by distinct viruses, while the fifth is due to a mixture of two or more of these viruses. Extreme variability in symptom expression on tobacco was observed in Tanganyika and Rhodesia by Storey (1932) who suspected that both variation in the reaction of individual plants and the presence of a number of TLC strains of varying virulence were involved. However, these forms have not yet been isolated. Recently in Thailand, Honda and co-workers (1986) obtained results suggesting that the causal agent of TmLCD (tomato leaf curl disease) is a strain of TLCV and other results indicate that yellow vein mosaic of honeysuckle in Japan is also caused by a strain of TLCV (Osaki et al, 1979).

Kiriyama (1972), however, observed that severe isolates from southern Japan and mild isolates from north-eastern Japan produced similar symptoms when graft transmitted under uniform glasshouse conditions. Kiriyama concluded that the difference in symptoms was therefore dependent on environmental conditions and not on virus strains.

The most characteristic symptoms of common kroepoek, or severe leaf curl, are the leafy outgrowths or enations from the veins on the lower surface of the curled, twisted leaves which are much smaller than normal, the greening of the veins and the severe stunting of the plant (Lucas, 1974). In the curl form of the disease, which is common in Venezuela, the margin of the entire leaf is rolled downward, but the midrib and veins are not knotted and crooked. Flower parts are

curled and deformed and apical dominance is lost so that the plant assumes a broom-like appearance (Lucas, 1974). In transparent kroepoek the leaf margins roll upward and the smaller veins become transparent. Other mild strains of TLCV cause little stunting; the uppermost leaves of nearly-grown plants may be curled and twisted, whereas the older leaves look normal (Lucas, 1974).

Diseased tissues have more layers of densely aggregated spongy parenchyme cells with fewer intracellular spaces than normal. Common kroepoek is characterised by an increase of the primary phloem in the veins. The pericycle is enlarged, new woody vessels surrounded by a cambium are formed so that new stelae arise inside the old pericycle and leaf dorsiventrality is lost by the substitution of palisade for spongy parenchyma. In addition palisade parenchyma and stomata are formed in the lobed veins of affected leaflets (enations) in which the new stelae proceed in reverse orientation. The enations lie with the morphological underside against the underside of the old leaf (Lucas, 1974).

Transparent leaf curl produces in the leaf veins typical swelling of the ends of the xylem vessels and sieve tubes and enlargement of pericycle and cortical parenchyma cells. The sieve tubes are curved and the cell walls of the primary phloem and pericycle are irregularly swollen (Yassin and Nour, 1965).

Many other types of symptom in tobacco (McClean, 1940; Pal and Tandon, 1937), tomato (Vasudeva and Raj, 1948; Yassin and Nour, 1965) and pepper (Osaki and Inouye, 1981) are assumed to be caused by strains of the virus, although there is little serological evidence

that the causal viruses are related. Datura stramonium, when inoculated with TLCV by white fly, reacted very severely and showed vein clearing after 10 days of inoculation followed by curling and production of enations on the undersurface of the leaves (Seth and Dhanraj, 1972). Severity of symptom expression may depend on the strain or mixture of strains present.

The virus may be distinguished from two other whitefly-borne viruses with geminate particles – tomato yellow leaf curl (Cohen and Nitzany, 1966) and tomato golden mosaic (Russo et al, 1980) because tomato yellow leaf curl virus has a much longer period of latency (21 h at least) and progressively loses infectivity in the vector whitefly (Cohen and Nitzany, 1966), and tomato golden mosaic virus is transmissible by inoculation with sap (Costa, 1976). The virus may also be distinguished from other Bemisia tabaci-transmitted viruses that infect solanaceous plants, such as tomato yellow mosaic virus in Venezuela or India, because they cause mosaic in tomato and tobacco (De Uzcategui and Lastra, 1978; Verma, Srivastava and Mathur, 1975). However, information on the interrelationships of these viruses is still fragmentary and further work to compare them serologically and in other ways is desirable.

At least 14 plant families including 45 genera have been reported as hosts of TLCV (Lucas, 1974). With tobacco as an inoculum source, leaf curl has been induced in species of Asystasia, Brassica, Capsicum, Datura, Lycopersicon, Petunia, Physalis, Zinnia, Vigna, Solanum and Sida (Butter and Rataul, 1978; Olivares et al, 1972; Padmanabhan and Pathmanabhan, 1978; Shivanathan, 1982). Members of the Malvaceae, Euphorbiaceae and Leguminosae frequently serve as TLCV hosts. The

weed Rhynchosia minima DC is a common inoculum source in Puerto Rico (Lucas, 1974). Many species of Nicotiana are also susceptible to TLCV (Lucas, 1974). Leaf curl disease of chilli caused by TLCV is mainly responsible as a limiting factor in the cultivation of this crop (Seth and Dhanraj, 1972).

Diagnostic species include (Osaki and Inouye, 1981):

Datura stramonium – veinlets in young leaves are cleared followed by interveinal chlorosis of the lamina in the area between the secondary veins. Stunting of plants is accompanied by dwarfing, curling and spiral twisting of the leaves.

Lycopersicon esculentum (tomato) – Yellowing and curling of the young leaves is usually the first sign of infection. Leaflets become yellow but remain green along the veins; they are curled and puckered, and remain small. The plant becomes markedly stunted. Some isolates cause vein thickenings or enations on the veins (Vasudeva and Raj, 1945; Yassin and Nour, 1965).

Nicotiana glutinosa – Curling, crinkling and dwarfing of leaves occurs. Sometimes small enations or green thickenings may develop on the larger veins. Some isolates are not transmissible to N. glutinosa.

These three species as well as other susceptible solanaceous plants are used for propagation and assay of the virus, but no local lesion host is known (Osaki and Inouye, 1981).

2.2 TRANSMISSION

Besides whitefly transmission of TLC, grafting is the only other means whereby the disease has been transmitted (McClean, 1940; Pal and Tandon, 1937; Retuerma et al, 1976; Storey, 1931; Thatcher, 1976). Transmission of TLC through the seed of infected plants does not occur (McClean, 1940; Pal and Tandon, 1937) and actempts to transmit the disease by various mechanical means have all proved unsuccessful (Chen, 1972; Kiriyama, 1972; McClean, 1940; Pal and Tandon, 1937). Lack of vascular contacts between embryo and mother plants explains why viruses that cannot pass beyond the phloem (phloem-limited viruses, most of which, including TLCV, are transmitted by phloem-feeding insects in a persistent manner) have not proved to be seed-borne (Bos, 1983).

The localization of TLCV in the phloem is probably one reason why it is difficult to purify and almost impossible to transmit mechanically (Harrison, 1984; Jensen, 1969). The usual methods of mechanical transmission do not put these viruses into the same cells as the insect vectors do. The phloem tissue, being difficult to grind, is a poor source for virus purification (Brakke, 1988). However, Honda and co-workers (1983) in Thailand achieved mechanical transmission of the phloem restricted, whitefly borne geminivirus MYMV (mungbean yellow mosaic virus), but mechanical transmission could not be obtained with the Indian isolate of this same disease and it is assumed that the Indian isolate is a different strain of the same virus.

Pal and Tandon (1937) tried two methods of inoculation, viz., leaf pricking and rubbing of leaf homogenates onto leaves of healthy plants. In experiments performed by Dr. E. S. Moore in South Africa (reported by McClean, 1940), inoculations were attempted by numerous methods including leaf pricking, application of sap from diseased leaves to wounds in lamina and petioles, and injection of sap into test plants. McClean (1940) tried rubbing the sap from TLC-infected tobacco over the surface of healthy tobacco and Petunia plants. Chen (1972) and Kiriyama (1972) have also reported unsuccessful attempts to transmit TLC by mechanical means, but details concerning the techniques used are not given in the English abstracts of the respective Chinese and Japanese articles. In 1976, Thatcher also could not induce symptoms of TLC infection by injection of extracts, although a small necrotic lesion often developed at the site of injection. Thatcher (1976) was also the first to attempt transmission by dodder (Cuscuta campestris), but leaf curl symptoms did not develop during the observation period of two months in either of the two test plants parasitised by dodder trained from a TLC-infected plant.

Bos (1983) states that viruses that are stable in expressed sap and that occur in their hosts in high concentration are most readily transmitted in sap. Viruses that rapidly deteriorate outside host cells are hard to transmit. It is further claimed by Bos (1983) that it is impossible to transmit in this way most viruses that are naturally transmitted by phloem-feeding insects (such as whiteflies), and that appear to be phloem-limited within their host plants.

According to Thatcher (1976), the inability to transmit TLC

mechanically may be due to one or more of the following factors:

1) presence of TLC in very low concentration in the infected plant, which is likely as the virus is restricted to vascular tissue,
2) high instability of the agent in vitro,
3) extreme specificity or liability of the site at which the agent must be introduced in the plant.

The failure to achieve transmission by dodder carries little significance since some viral and mycoplasma-like diseases are transmitted by certain dodder species but not by others, and it thus remains a possibility that other species of Cuscuta are able to transmit leaf curl (Thatcher, 1976).

In transmissions using the vector, the experimental host range is rather narrow, comprising species from Solanaceae, Compositae and Caprifoliaceae but plants in five other families have been reported naturally infected. Natural hosts include Ageratum conyzoides, Eupatorium odoratum, Euphorbia hirta, Sida rhombifolia, Solanum nigrum and Vernonia cinerea (Pruthi and Samuel, 1942).

A new technique, electro-endosmosis, has been demonstrated to transmit maize steak virus (MSV) which, until this time, was transmissible only by leaf hoppers (Polson and Von Wechmar, 1980). Experiments on the effect of a direct current on plants showed that the movement of fluid into plants through their roots may be effected by passing an electric current from the roots to the stem. Plants could also take up fluid through passing an electric current through the damaged surface of a cut leaf (Polson and Von Wechmar, 1980).

This method appears to enable infections to be established with very low concentrations of virus particles and, more importantly, allows some viruses which are normally only transmitted by vectors to be experimentally transmitted to susceptible plants (Polson and Von Wechmar, 1980).

Another method of transmitting viruses is through infectious nucleic acids. Plasmids containing the complete genomes of the DNA viruses have often been shown to be infectious (Gardner et al, 1981; Hamilton et al, 1984; Stanley, 1983). The plasmids of the cDNA of ssRNA viruses appear not to be infectious.

Gierer and Schramm (1956) were the first to show that plant virus infectivity could be achieved by simply using RNA from tobacco mosaic virus. Isolated virus DNA from African cassava mosaic virus (ACMV), a geminivirus, is about 2% as infective as the DNA in virus particles (Bock and Harrison, 1985).

Cloned tomato golden mosaic virus (TGMV) ds DNA is infectious in plants, giving rise to progeny infectious virus indistinguishable from native TGMV, except for the presence in the latter of a subgenomic, possibly defective, DNA (Hamilton et al, 1983). This same group demonstrated that both parts of the genome are required to infect a plant.

It is probable that 3 or more viruses, or strains, are involved in the leaf-curl complex, all of which are transmitted by the whitefly. These are tobacco, cotton and yucca leaf-curl viruses. The last 2 will not infect tobacco and TLCV will not infect cotton or yucca

(Lucas, 1974). In addition there are several sub-strains of TLCV (Nariani, 1968; Pal and Tandon, 1937; Vasudeva and Raj, 1948).

2.3 HOST-PARASITE RELATIONS

Infective whiteflies insert the virus into phloem tissues by means of the stylets while feeding on the leaves (Pollard, 1955). The incubation period varies from 12 to 33 days. A number of factors such as temperature, leaf age and vigour probably condition the length of the incubation period (Valand and Desai, 1980). The disease is seldom seen in the plant bed, but usually appears 2 to 3 weeks after transplanting (Lucas, 1974).

Thatcher (1976) investigated the possibility that whiteflies secrete toxins that are harmful to plants, but could find no evidence for this, although it is known that a toxin secreted by the leafhopper Euscelis plebejus can induce virus-like histoid enations in clover on which the insects feed (Bos and Grancini, 1968).

From experiments crossing 4n leaf-curl susceptible N. tabacum with resistant N. glauca and N. plumbaginifolia it was concluded that leaf-curl resistance is either recessive or polygenic (Chen, 1971 in Lucas, 1974).

2.4 MEANS OF SPREAD OF THE PATHOGEN

The most important vector of TLCV is the tobacco whitefly, Bemisia tabaci Gennadius, a member of the family Aleyrodidae (Bird and Maramorosch, 1978; Pruthi and Samuel, 1941). The name B. gossypiperda Misra and Lamb, however, has been widely used instead (Lucas, 1974).

Other genera of aleyrodids may serve as vectors. McClean (1940) stated that Trialeurodes natalensis Cobb [now known to be conspecific with T. vaporariorum (Hill, 1967)], or greenhouse whitefly, is the vector in the Transvaal. However, Hill in 1967 could not transmit TLCV with T. vaporariorum and claimed that B. tabaci is the only vector of tobacco leaf curl in the Transvaal. These results were confirmed in 1976 when both Thatcher in the Transvaal and Yamauchi and colleagues in Japan found that no leaf curl symptoms developed in plants on which T. vaporariorum were released. It has also been reported that B. tuberculata Bondar and Aleurotracnelus socialis Bondar are associated with leaf curl in Venezuela, as is Venezaleurodes pisonia (Lucas, 1974).

Species of Bemisia vary in their host range and different physiologic races vary in the ease with which they transmit TLCV. The agents of whitefly-transmitted disease appear to be at least semi-persistent in the bodies of their vectors since all undergo an incubation period, generally of a few hours, before transmission occurs (Varma, 1963). In 1958, Bird (in Lucas, 1974) found that a minimum acquisition period of 15 min to 2 hrs on infected weed hosts was required and a minimum feeding period of 10 min on healthy plants for virus

transmission. The virus persisted in the vector for at least 6 days. Conflicting results have since been reported, however, where it is claimed that vector whiteflies can transmit the disease after a 60 min inoculation access period (Osaki, Kobatake and Inouye, 1977 in Osaki and Inouye, 1981). These workers also reported that the minimum latent period in the vector is between 4 and 9 h, depending on the length of the acquisition access period. There is no evidence that the virus multiplies in the vector (Osaki and Inouye, 1981).

There is no evidence to date that insects other than aleyrodids serve as vectors for TLCV.

2.5 THE LIFE CYCLE OF THE VECTOR

The minute (0.2 mm) oval, whitish eggs are laid on the underside of the leaves of the host plants to which they are fastened by a short stalk. The eggs hatch in a few days, the larvae crawl about and then begin to feed by sucking the sap from the leaf tissue. Once they begin feeding the larvae do not move again but develop into flat, oval, white objects (0.3 mm long) closely attached to the leaf by their sucking mouth parts. The larvae moult 4 times to form the pupal stage. The tiny adults (1.2 mm long) that emerge from the pupae have yellow bodies. The wings are covered with white mealy or dust-like scales, hence the name "white fly". The adults also feed on the host but live only a few days. Wind is a potent factor in the spread of these insects as they are unable to fly more than a few metres in a single flight. The average life cycle is about 18 days but may require 107 days in the cooler months. As many as 12 generations a year are produced, although the broods overlap.

2.6 EPIDEMIOLOGY

Whiteflies are more abundant and active in the dry season of the tropics than in the rainy season, therefore leaf curl is more prevalent during dry weather. Disease development has also been reported, by Munshi and Choudry in 1964, to be positively correlated with increases in temperature (Lucas, 1974), although temperatures above 46 - 47 ^{o}C are lethal to the white fly. Pruthi and Samuel (1941) found that temperature has an important influence on the length of the incubation period of TLC in tobacco and other host plants, the shortest incubation periods occurring usually during the warmer months.

Flue-cured seedlings are easily infected in the greenhouse but mature plants are not. Tobacco seedlings are almost devoid of epidermal hairs and the sticky gums found on older plants, and leaf curl affected plants are much more pubescent than healthy mature plants. The epidermal hairs and gum on the mature plant leaves may impede whiteflies from properly feeding and subsequently acquiring or transmitting the virus. On the other hand, tobacco plants may become more resistant as they grow older (Lucas, 1974).

In South Africa, Hill (1968) observed that the whiteflies overwintered on ratoon tobacco, on which breeding continued slowly, and also on vegetables including tomatoes. Hill (1968) found that leaf curl epidemics occurred when conditions favoured overwintering and rapid development of whiteflies.

Thatcher (1976) found that vein-clearing could be transmitted through the egg of B. tabaci and suggested that the typical or severe form of leaf curl may be caused by more than one component of a virus complex, or possibly by two or more distinct viruses, and that only one member of the complex is transmitted through the egg of B. tabaci.

2.7 CONTROL

Leaf-curl control is largely dependent on whitefly control. Susceptible weed and cultivated hosts near seed beds or tobacco fields should be destroyed and seed beds or tobacco fields should not be located near alternate hosts. White flies should be kept out of seed beds with insecticides or use of mulches. Only healthy transplants should be used. As soon as harvest is completed the tobacco crop should be destroyed to prevent overwintering. Infected plants in the field should be pulled (if not too numerous) and destroyed.

It has been found that alternate sprays of insecticide and oil are effective in reducing B. tabaci, and thus the incidence of TLC, in the field (Singh et al, 1975).

Patel and co-workers in 1976 found that N. glauca and N. gossei were toxic and attractive to B. tabaci and suggested that these species might be used for the extraction of a biochemical substance to control B. tabaci and for breeding cultivars resistant to TLCV. N. gossei might also be planted around the edge of a field of other tobacco species to attract and kill invading whiteflies.

2.8 PURIFICATION OF PLANT VIRUSES

Before the biophysical and biochemical properties of virus particles can be described, the particles have to be purified. The optimum purification procedure differs from virus to virus, but once the particles are purified, the methodology for determining the biophysical and biochemical properties is the same for most viruses (Hull, 1985).

Various basic features and useful facts can be used in designing methods for virus purification.

Ideally one should have a systemic host in which to grow the virus and a local lesion host for assaying various stages of purification. Choice of a systemic host should take account of the amount of virus produced, the ease of extraction of the virus, the ease of growing the plant and the possibility of contamination with other viruses. The local lesion host should be used to ascertain the time of maximum virus content in the propagation host and also to check various stages in virus purification (Hull, 1985).

In most cases, however, it may not be possible to satisfy many of the above criteria. One often cannot find a suitable local lesion host. The technique of dot blotting can be used to replace local lesion assays.

To extract virus particles from the plant, the cell walls have to be broken and the cell contents released. This is usually done in the presence of a buffer to control the pH and of additives to prevent

the released enzyme activities from damaging the virus particles. The action of enzymes is often slowed by extracting in the cold (Hull, 1985).

Breakage of the plant cells can be increased by prior freezing of the tissue, though in some cases this will reduce virus yield (Hull, 1985). For viruses which are limited to vascular tissue, as is tobacco leaf curl disease-causing virus, it has been suggested that the leaf tissue should be initially disrupted with cellulases and pectinases (Hull, 1985).

The choice of extraction buffer can greatly affect the outcome of purification attempts. Viruses with elongated particles (eg. potyviruses) which tend to aggregate or to be absorbed onto cellular debris are best extracted in alkaline buffers (pH 8 - 9) of moderate ionic strength (eg. 0.1M); however some rod-shaped viruses [eg. tobacco mosaic virus (TMV)] can be damaged by alkaline buffers (Hull, 1985).

The most common additives are reducing agents to prevent the action of polyphenol oxidases "tanning" the viral protein coat. Commonly used reducing agents are 10mM ascorbic acid, 20mM sodium ascorbate, 0.5% mercaptoethanol, 20 - 40mM sodium sulphite, 10mM sodium thioglycollate or 10 - 20mM sodium diethyldithiocarbamate.

Chelating compounds are also used to reduce enzymic activity and to dissociate ribosomes; 5 - 50mM sodium ethylene diamine tetraacetic acid (EDTA) is the most commonly used (Hull, 1985).

Separation of the virus particles from all the other constituents of the host cytoplasm (eg. organelles, membranes, ribosomes and proteins) is achieved through a process known as clarification. Agents which are effective in removing the various contaminants include EDTA, NaCl, butanol, chloroform, heat, bentonite, butanol\chloroform, low pH, organic solvents and non-ionic detergents. Precipitated contaminants are then usually removed by low-speed centrifugation.

The virus is usually concentrated by precipitation using polyethylene glycol (PEG) or by high-speed centrifugation, or a combination of these methods. It is important to have sufficient salt present in the preparation to effect precipitation and NaCl is commonly used. This is usually followed by a low-speed centrifugation and then a high-speed centrifugation so that the virus is pure enough for biophysical and biochemical techniques (Hull, 1985).

Although tobacco leaf curl disease was attributed to a virus by Storey as early as 1931, only four attempts to extract a virus appear to have been reported (Kiriyama, 1972; Osaki and Inouye, 1978; Sharp and Wolf, 1951; Thatcher, 1976;). Of these, only the extraction by Osaki and Inouye in 1978 was successful and this group has since done further work using this technique to purify TLCV (Ikegami et al, 1987).

In the study of Sharp and Wolf (1951), performed in North Carolina, U. S. A., conventional differential centrifugation was applied to formolised sap received from Venezuela, where the crude extracts had been prepared from field-grown, TLC-infected tobacco plants shown to

be free of TMV.

Formaldehyde was added to preserve the suspected virus during transfer of the sample by air. However, evidence produced by Thatcher (1976) indicated that the "virus" recovered by Sharp and Wolf (1951) was probably a ribosomal fraction and not a pure, small, isometric virus as claimed by these workers.

The second purification study, published in Japanese with an English summary (Kiriyama, 1972), reported the isolation of isometric 30nm particles from TLC-infected tobacco leaves following butanol clarification and differential centrifugation of expressed sap. Although infectivity of the particles could not be tested, similar particles were not recovered from healthy material whilst healthy extract reacted only very weakly with antiserum homologous to the particles.

Thatcher (1976) performed nine extractions based on the differential centrifugation procedure reported by Sharp and Wolf (1951). The experiments varied with respect to initial buffering and storage of the crude sap. The pellets were resuspended in 0.06M potassium phosphate buffer pH 7.2. However, these extractions were unsuccessful. UV spectra obtained from extracts from healthy plants indicated that host nucleoproteins were not eliminated by the procedure.

A further three experiments were performed by Thatcher (1976) using the extraction procedure described by Kiriyama (1972) to extract TLCV. The procedure of Kiriyama (1972) comprised overnight freezing

of TLC-infected tobacco leaves, extraction in 0.1M phosphate buffer pH 7.0 containing 0.1% thioglycollic acid, butanol clarification, and at least four cycles of differential centrifugation. Purified preparations exhibited a single peak during analytical centrifugation and displayed UV absorption maxima at 260nm and minima at 238nm, with characteristic nucleoprotein extinction ratios of approximately 1,5. Although it was not possible to demonstrate infectivity of these extracts Kiriyama (1972) derived from them antiserum having titres of 1/64 against homologous extract and 1/4 against comparable healthy tobacco extract. The UV absorption spectra (220 - 300nm) obtained from extracts produced by Thatcher (1976) using the method of Kiriyama (1972) showed only slight variation between TLC-infected and healthy plant extracts within each experiment, and the ratio (260/280nm) of only 1,25 indicated that it was unlikely that these extracts contained purified TLC virus.

Several other purification procedures have been applied by Kiriyama (1973, pers. comm. with Thatcher, 1976) in attempts to extract the TLC virus in greater concentration, but all proved to be unsatisfactory. These methods included column separation (ion exchange cellulose or sephadex), polyethylene glycol precipitation, polymer two phase systems, pretreatment with chloroform or butanol / chloroform mixtures, and density gradient centrifugation of expressed sap.

According to Kiriyama (1974, pers. comm. with Thatcher, 1976) the selection of a suitable propagating host for the TLC virus as well as the growing conditions of the plants are most important in obtaining adequate virus yields. The choice of TLC-infected material used in

the experiments by Thatcher (1976) may have reduced the chance of recovering adequate yields of the TLC virus.

Kiriyama (1974, pers. comm. with Thatcher, 1976) reported that the concentration of the small, isometric TLC virus (Kiriyama, 1972) in infected tobacco plants is very low.

Thatcher (1976) investigated the possibility of a viroid causal agent of tobacco leaf curl. The main objective of these experiments was to ascertain whether infective nucleic acid could be recovered from TLC-infected host tissue. Infection was not achieved with any of the extracts tested , but in view of the consistent failure of all other attempts to bring about mechanical transmission of TLC this result alone could not be interpreted to exclude a proteinless virus as the causal agent of this disease. Nevertheless, the properties of the causal agent of TLC are markedly different to those of known infectious, proteinless virus strains or viroids, all of which are mechanically transmissible after phenol extractions and none is transmitted by a species of whitefly (Thatcher, 1976). The later work of Osaki and Inouye (1978) appears to preclude the possibility of a viroid causal agent of TLC in Japan.

Osaki and Inouye extracted tobacco leaf curl virus in 0.2 M borate buffer (pH 8.5) containing 0.1% mercaptoethanol and 1% Antifoam A emulsion (2 ml buffer / g tissue) (Osaki and Inouye, 1978). The sap was clarified by adding n-butanol to 10% and centrifuging at low speed. 4% PEG, followed by a low-speed centrifugation, was used to precipitate the tobacco leaf curl virus in the method of Osaki and Inouye (1978). The virus was purified by a high-speed centrifugation

in a sucrose gradient and the sediment resuspended.

2.8 a) *Stability in sap*

No information is available as to the stability of tobacco leaf curl virus in sap (Osaki and Inouye, 1981), although most geminiviruses survive more than one day in sap and have a TIP (thermal inactivation point) of about 60 °C (Hill, 1984).

In 1958, Bird heated infected Jatrophia gossypifolia (L) stems for 10 min at 50 °C and did not inactivate the virus (in Lucas, 1974).

De Uzcategui and Lastra (1978) reported that tomato yellow mosaic, a related virus transmitted by the tobacco white fly, lost infectivity in sap within 15 minutes if kept at room temperature after expression from infected leaves.

2.9 BIOPHYSICAL CHARACTERISATION

The biophysical properties of viruses are studied to provide information on the size and shape of the virus particles, on their molecular weight and on the structure (Hull, 1985).

2.9 a) Spectrophotometric readings

The majority of simple viruses comprise mainly protein and nucleic acid each of which has a characteristic absorption spectrum in the ultraviolet. The absorption spectrum of the virus particles is a combination of both nucleic acid and protein. However, since the nucleic acid has a much higher specific absorption at its maximum ($\sim$260 nm) (RNA $E^{0.1\%}_{260nm}$ = 25; DNA $E^{0.1\%}_{260nm}$ = 20) than does protein ($E^{0.1\%}_{260nm}$ = about 0.5) the nucleic acid spectrum dominates. In the extraction performed by Kiriyama (1972) purified preparations of TLCV displayed UV absorption maxima at 260nm and minima at 238nm, with characteristic nucleoprotein extinction ratios of approximately 1,5.

The absorption spectrum can also be used to measure virus concentration (Hull, 1985).

2.9 b) Electron microscopy

Electron microscopy is one of the most useful methods for determining the shape and structure of virus particles (Hull, 1985).

The main method for visualising particles is negative staining. While

phoephotungstic acid is suitable for many viruses, the particles of some viruses are destroyed or damaged in this stain. An alternative stain is uranyl acetate but this can also damage some viruses. Less damaging stains include ammonium molybdate, sodium acetate and methylamine tungstate (Hull, 1985).

The technique of thin sectioning from fixed and embedded plant tissues is important in cell ultrastructure showing the location of viruses and inclusion bodies in infected cells (Horne, 1985). Inclusion bodies were associated with virus diseases long before infectious particles were identified (Brakke, 1988). The function of many of these inclusion bodies, including the pinwheel-inclusion bodies associated with potyviruses, has remained speculative. However, potyviruses have been classified partly on the basis of the ultrastructure of these inclusion bodies (Brakke, 1988).

The particles of the tobacco leaf curl virus disintegrate in negative stains unless previously fixed with 2% glutaraldehyde (Osaki and Inouye, 1981).

Sharp and Wolf (1951) isolated a particulate material from the formalin-treated sap of leaf-curl affected tobacco plants by ultracentrifugation. The spherical particles had a mean diameter of 39 nm. Two decades later, in 1972, Kiriyama partially purified TLCV from the sap of infected tobacco leaves by butanol clarification and differential centrifugation. His results indicated the virus particles to be approximately 30 nm in diameter (Lucas, 1974).

It has since been determined that the tobacco leaf curl virus consists of "geminate", isometric particles measuring about 15 - 20 x 25 - 30 nm (Osaki and Inouye, 1981). They consist of two incomplete T = 1 icosahedral structures, with one shared subunit.

Electron microscopy of the tobacco leaf curl virus nucleic acid revealed circular molecules; this suggests that the virus particles, like those of some other geminiviruses, contain circular DNA molecules (Ikegami et al, 1987).

The intranuclear occurrence of virus particles is a distinctive feature of all geminiviruses investigated so far at the ultrastructural level (Bock et al, 1974; Francki et al, 1979; Kim et al, 1978). Intranuclear ring-shaped inclusions have been detected in association with infections by TLCV in Lonicera japonica (Russo et al, 1980). These structures were called "fibrillar rings" by Kim and colleagues (1978) who suggested that they might be a possible diagnostic feature for viruses with ssDNA, such as geminiviruses. Hypertrophated nuclei and aggregates of virus-like particles have also been associated with whitefly-borne viruses (Honda et al, 1983; Osaki and Inouye, 1978).

It has been found that phloem proliferation of tobacco leaf curl virus occurs in the stem apices, petioles, and leaflet veins and veinlets of infected plants (Kerling, 1933; Yassin and Nour, 1965). In ultrathin sections of infected tissues of tomato, Datura stramonium, Nicotiana glutinosa and Lonicera japonica, virus-like particles occur in close-packed arrays forming rigid rod structures

in some of the nuclei in phloem cells. The rods, each of which is about 25 nm wide, consist of a row of geminate particles . A distinctive abnormality of the nuclei is the splitting of the nucleoli into granular and fibrillar regions (Osaki and Inouye, 1981).

The inclusion bodies sometimes found in plant tissue infected with certain viruses are readily observed by light microscopy. Callose is deposited in phloem sieve tubes in abnormal quantities as a response to injury, toxins or infection with phloem-restricted viruses (Hill, 1984).

2.9 c) Serology

The outer coat or capsid of plant viruses consists of protein subunits of a type and in an an arrangement peculiar to each virus. The virus particle, therefore, because of its specific three-dimensional shape and its size, forms an ideal antigen which on injection into the blood system of a suitable animal stimulates the production of antibodies in the serum fraction of blood. Such antibodies react only with the stimulating virus or with very closely related viruses. The specificity of this antigen / antibody reaction can be used in a variety of in vitro tests to demonstrate the presence of a plant virus and to determine its character.

Tobacco leaf curl virus is moderately immunogenic. An antiserum prepared by intramuscular injection using purified virus had titres of 1/256 and 1/32 in microprecipitation and immunodiffusion tests respectively (Osaki and Inouye, 1981). No serological differences

were detected between Japanese isolates of the virus from tomato and tobacco propagated in the same host species. However, spurs formed in immunodiffusion tests between preparations of the same virus isolate propagated in different hosts (Osaki and Inouye, 1981).

Tomato isolates of the virus collected from Japan and Taiwan have been found to be serologically related (Osaki and Inouye, 1981). No serological relationship was found among four viruses that belong to the geminivirus group: tobacco leaf curl, maize streak, African cassava mosaic and bean golden mosaic (Osaki and Inouye, 1981). However, Roberts and co-workers (1984), using the technique of ISEM, were able to detect relationships between ACMV and TLCV. This was the first example of a relationship between sap transmissible and sap non-transmissible viruses.

Recently, nucleic acid hybridisation tests have been employed to detect relationships between viruses, and these are in some cases proving more sensitive than conventional serological tests, eg. nucleic acid hybridisation tests for relationships between ACMV strains parallel and extend those obtained in serological tests (Sequeira and Harrison, 1982).

Dot-blot detection of viruses involves the immobilisation of the nucleic acid of virus onto nitrocellulose and its detection by hybridisation to a probe of complementary homologous nucleic acid (Hull, 1985). There is no need to extract the nucleic acid from the virus particles prior to dot-blotting.

2.10 BIOCHEMICAL ANALYSES

Biochemical analyses are used to describe the type, proportion and sizes of nucleic acid in the virus particle as well as the number of species and molecular weights of the protein subunits making up the capsid (Hull, 1985).

2.10 a) Protein coat

Gel electrophoresis is the method of choice for determining the number of species and size of the protein coat of virus particles. No information is available on the structure and sizes of the protein coat of tobacco leaf curl virus.

Geminiviruses so far isolated have a capsid polypeptide species of MW of approximately 28 000 (Hamilton et al, 1983).

The preparation of TLC of Sharp and Wolf (1951) showed that almost all of the particulate material sedimented as a homogeneous component having a sedimentation coefficient of approximately 75S. However, about 10% formed a second, more diffuse boundary of about 113S. It was concluded that the two components were probably aspects of the same material, the faster component probably comprising dimer aggregations of the smaller particle. Because Sharp and Wolf failed to include a comparable extraction from healthy material their claim to have isolated the causal agent of TLC is questionable, particularly as infectivity could not be tested.

2.10 b) Nucleic acids

Viruses vary in the ease with which the nucleic acid can be extracted. It is advantageous to relax the protein-protein interactions if possible before removing the coat protein. This is usually effected by the use of alkaline pHs and chelating agents such as EDTA (Hull, 1985).

There have been tremendous advances in understanding the translation strategy and genome structure of plant viruses in the last decade, primarily because of powerful new techniques in molecular biology.

The genomes of more than a dozen plant viruses have been sequenced, including ssRNA, dsDNA and ssDNA genomes (Brakke, 1988). Reports of additional sequences are continually appearing. Knowledge of the nucleic acid sequence allows the prediction of a number of viral proteins and their amino acid sequences. Comparison with sequences of proteins of known function permits guesses about the function of the proteins. The nucleic acid sequences also allow the selection or construction of hybridisation probes for identification that are either highly selective (based on unique sequences) or that will detect related viruses (based on conserved sequences).

The replicative form (RF) of geminivirus DNA can be isolated by performing a whole plant dsDNA extraction.

The number of nucleic acid species in a virus preparation and their

molecular weights can be determined by gel electrophoresis (Hull, 1985).

Ikegami and colleagues (1987) were the first to isolate and characterise the nucleic acid from preparations of geminate particles of TLCV. They found the DNA to be single-stranded and examination of TLCV DNA under the electron microscope revealed that the molecule was circular and contained 2 482 nucleotides. This is unusual, as most geminiviruses so far isolated from dicotyledonous plants and transmitted by whiteflies have been shown to have a bipartite genome with two approximately equal sized DNA species (Stanley and Davies, 1985).

Typically, geminiviruses have a genome comprising single-stranded circular DNA of between 2000 and 3000 base pairs in size (Honda, 1986; Stanley and Davies, 1985). Nucleotide sequencing has shown the existence of two genome components (DNA-1 and DNA-2) of approximately equal size in several whitefly-transmitted geminiviruses including ACMV (Stanley and Gay, 1983), bean golden mosaic virus (BGMV) (Haber et al, 1981), and tomato golden mosaic virus (TGMV) (Hamilton et al, 1983). Geminiviruses transmitted by leafhoppers (including those which infect monocotyledonous hosts) appear to have monopartite genomes (Stanley and Davies, 1985), eg. MSV (maize streak virus) (Mullineaux et al, 1984). The two genomic DNA species of ACMV have different sequences except for a shared "common" region of 193 nucleotides (Stanley, 1983). Each geminate particle contains one molecule of either DNA-1 or DNA-2.

Some quasi-isometric particles of the type strain contain a smaller

circular DNA of about 1200 bp (Bock and Harrison, 1985) which shares nucleotide sequences with DNA-2 (Stanley, 1983). In addition to these circular molecules, preparations of virus DNA contain linear molecules which share nucleotide sequences with DNA-1 and DNA-2, and which are about 1900 bp in size (Bock and Harrison, 1985). A virus-specific circular dsDNA with a discontinuity in one strand has also been identified in BGMV-infected tissue and shown to contain the sequences of both types of genomic DNA (Ikegami et al, 1981). A further five novel species of virus-specific ss and ds DNAs were also isolated from TGMV by Hamilton and colleagues in 1982. Three of these molecules were ds circular DNA of about 3200 bp in size, while two others were ds and of high molecular weight (approximately 11000 bp and 5200 bp). The other DNA species was smaller (about 880 bp in size) and ss. The two large DNA species were probably concatamers composed of tandemly arranged viral genomes. The remaining three ds components were of unit genome length and represented the closed circular, relaxed circular and linear forms of ds TGMV DNA. However, the conformation and significance of the ssDNA was not described by Hamilton and co-workers (1982).

Cloned probes have been prepared to each of the ACMV strains (Stanley, 1983). Probes for either DNA species can be used in spot hybridisation tests to detect infection of N. benthamiana or cassava with isolates resembling the type strain or the Angolan defective isolates, but only DNA-1 probes react strongly in tests for the Kenya coast strain in these two plant species (Robinson et al, 1984). A nucleic acid hybridisation test has recently been developed by Polston and co-workers (1989) which can distinguish between virus strains having coat proteins that are not distinguishable by ELISA

and may be important in determining the relatedness of strains and whether mixed infections are present.

Sequence homologies have been detected between DNA-1 (larger genome part) of ACMV and the DNA of bean golden mosaic virus, tomato golden mosaic virus, tobacco leaf curl, tomato leaf curl and tomato yellow leaf curl viruses (Roberts et al, 1984). Some homology has also recently been detected between the genome of MSV and that of ACMV and TGMV (Mullineaux et al, 1985). The presence of a conserved 200-base region between the two DNAs of a single virus and between different viruses (ie. TGMV and ACMV) implies that this region is important in interacting with one or more host or viral proteins essential to the virus replication cycle (Hamilton et al, 1984).

Nucleic acid hybridisation tests performed by Robinson and colleagues (1984) on different ACMV strains indicated the possible presence of an isolate of ACMV which induces the production of substantial amounts of virus DNA but apparently no virus particles and very little virus particle protein. However, the failure to detect these new strains by ISEM or infectivity tests could be due to virus instability. Alternatively, the isolates could be defective for particle production.

2.11 POTYVIRUSES

The potato virus Y or potyvirus group is the largest known group of plant viral pathogens. There may be over 100 different members of this group (Dougherty and Carrington, 1988).

All members of the potyvirus group share common features. The virus particle or virion has a flexuous rod shape and is usually 700 - 900 nm in length and 12 - 15 nm in diameter. The genome of a potyvirus is contained in a single-stranded, infectious RNA molecule that is approximately 10000 nucleotides in length (Allison et al, 1986). The genome of all potyviruses encodes for a protein that aggregates in the cytoplasm during infection in the form of pinwheel- or scroll-shaped inclusion bodies (Dougherty and Carrington, 1988). Potyviruses can be mechanically transmitted and most members of this group are transmitted by aphids in a nonpersistent, noncirculative manner; transmission in seed or by mites and dodder has been reported (Dougherty and Carrington, 1988).

Capsid protein monomers of different potyviruses range in size from 30 - 45 kd (Dougherty and Carrington, 1988).

Inclusion body proteins are produced in about as high a concentration as capsid proteins in plants infected with potyviruses and other viruses with long, flexuous particles such as wheat streak mosaic virus and wheat spindle streak mosaic virus (Brakke et al, 1987; Carrington and Dougherty, 1987; Hiebert et al, 1971; Jensen et al, 1986). These proteins are as easy to purify by centrifugation and gel electrophoresis as virions and can be helpful in the diagnosis of

potyvirus-infected plants (Edwardson and Christie, 1978).

3 MATERIALS AND METHODS

3.1 VIRUS SOURCE AND MAINTENANCE

Nicotiana tabacum TL38 plants showing disease symptoms (see plates 1 and 2) were collected in the Rustenburg area at the beginning of June 1989 from the Tobacco and Cotton Research Institute near Kroondal, as well as from a farm in the district. Healthy seedlings grown in the Research Institute glasshouse were also collected. These were maintained in growth cabinets at the university of the Witwatersrand.



Plate 1: A healthy plant (right) and plant infected with TYLCV (left). Note stunting and curling of new leaves of infected plant.

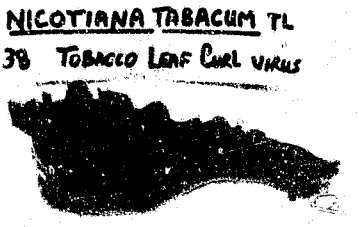


Plate 2: Symptoms of severe tobacco leaf curl infection. Diseased leaf from plant collected at Rustenburg showing enations on underside.

Diseased plants were again collected at the beginning of December 1989 from several farms in the Nelspruit / Hazyview area as well as from the Tobacco Research Institute at Nelspruit (see plate 3).

In both cases, these plants were established in the greenhouse and were used as virus source plants in subsequent studies.

Two of the plants collected from the research station at Kroondal were maintained in a controlled environment growth room, at a temperature of 25 ^{o}C.



Plate 3: Symptoms of severe tobacco leaf curl on plants co' 'cted from Hazyview. Leaf on left hand side shows severe leaf curl symptoms. Leaf on right hand side shows mild symptoms of curling leaf margin.

The plants were watered to saturation every two to three days. At least 200 ml of Long Ashton nutrient solution (appendix 1) was applied twice a week to the plants.

The plants were sprayed every ten days with Malasol, a systemic insecticide, in an attempt to prevent whitefly or other insects from feeding on the plants.

3.2 TRANSMISSION EXPERIMENTS

Transmissions were attempted using three different methods: mechanical inoculation, injection and electrotransmission as described by Polson and von Wechmar in 1980.

3.2 a) Mechanical inoculation and injection

Mechanical inoculation and injection were attempted on several species known to host TLCV. These were: Lycopersicon esculentum, Datura stramonium, Nicotiana glutinosa, Nicotiana tabacum (TL38) and Nicotiana benthamiana. The plants were grown up until the primary leaf, and, in some cases, the secondary leaf, had appeared. In a second attempt at mechanical inoculation and injection, Zinnia elegans, eggplant and squash were used in addition to the five plants described above.

Infected leaves were crushed in extraction buffer (appendix 2) at a ratio of 2:1 (vol buffer : mass leaves) until the leaves were in powder form. The homogenate was passed through one layer of cheesecloth pre-moistened with extraction buffer. The sap was used for rubbing onto leaves of healthy plants and injection into the vascular system of healthy plants.

Healthy leaves were also extracted as a control.

3.2 a) i) Rubbing of healthy leaves with inoculum

Primary (and secondary leaves if present) were brushed lightly with 600-mesh Carborundum. Sap was then gently applied with cheesecloth. After 20 - 30 min the leaves were washed with dH_2O.

3.2 a) ii) Injection

The stems, midribs and small veins of the primary leaves were injected with sap using the finest gauge needle. The needle was pushed through the stem or vascular tissue until a small droplet appeared on the other side, at which point the needle was drawn back through the stem or vein and a small amount of sap injected.

Half of the control plants for both inoculation and injection experiments were treated with sap extracted from healthy plants, while the other half were treated with dH_2O.

The plants were observed for symptom development for several weeks.

3.2 b) Electrotransmission

Electrotransmission as described by Polson and von Wechmar (1980) was attempted on ten plants of N. tabacum TL38 (see fig. 1 for experimental setup).

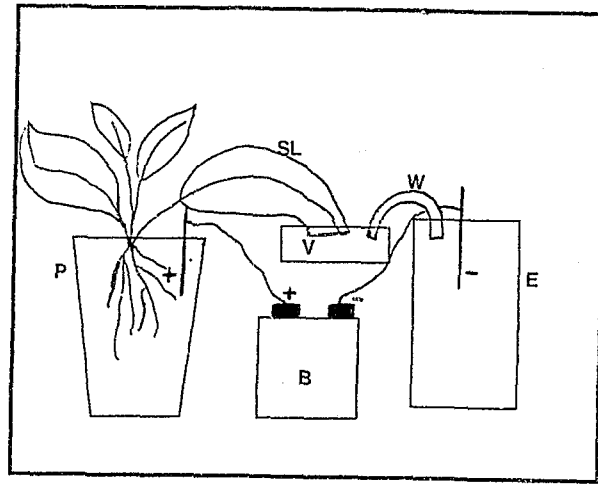


Fig. 1. Diagram illustrating how plants are electro-infected: P, the growth pot filled with well-watered soil; SL, a sliced leaf dipping into a dilute virus inoculum; V, a small Petri dish; W, a cotton or filter paper wick; E, an electrode vessel filled with the appropriate buffer; B, a 9V power source. The insulated electrical leads join each Ni electrode to a pole of the battery.

In the first attempt, infected leaves were ground up in buffer containing 0.5% sodium sulphite (appendix 2) and the sap was squeezed through a single layer of cheesecloth into a Petri dish. In later attempts, the sodium sulphite was excluded from the extraction buffer. The pot containing the plant was well watered and a young leaf was sliced at the tip and dipped into the virus inoculum. An electrode vessel containing 0.01M phosphate buffer pH 7.5 (appendix 4) · s prepared. A filter paper wick connected the buffer and the virus sap. A 9V power supply with positive pole attached near the roots of the plant and negative pole in the electrode buffer was set up and the experiments were run for 2 h, 4 h, 8 h and 24 h.

An initial experiment was performed using bromophenol blue dye to check whether the dye moved up the leaf with electric forces.

Electrotransmission was also performed on five N. tabacum TL38 control plants with sap from a healthy plant and five control plants with extraction buffer only.

Six week old N. benthamiana plants were also used for electrotransmission experiments, although only three plants were treated with infected sap and three with healthy sap.

Plants were maintained in a temperature controlled growth chamber, and later in a growth cabinet at 25 °C with light supplied for 16 h each day by a combination of fluorescent tubes and tungsten filament lamps. Plants were examined daily for symptoms.

3.3 VIRUS PURIFICATION

Six geminivirus and two potyvirus extractions were attempted. An extraction on a healthy plant was also performed with each method.

3.3 a) Geminivirus extractions

3.3 a) i) Adaptation of the method of Osaki and Inouye (1981) for the extraction of TLCV

94 g of fresh leaves infected with severe TLC and 77 g of healthy leaves were collected from plants obtained from Hazyview. These were frozen at -70 °C for two days. The leaves were then homogenized in liquid nitrogen with a pestle and mortar, before adding cold extraction buffer (0.2M borate buffer pH 8.5 containing 0.1% mercaptoethanol appendix 3) at a rate of 200 ml buffer per 100 g of plant material. This was mixed well for about 15 min before squeezing through a double layer of cheesecloth and clarifying by the addition of n-butanol to 10% (v/v) and stirring for 15 min. The homogenate was then centrifuged at 5000 rpm in a JA14 rotor in a Beckman J2-21 centrifuge. The supernatant was retained for further purification. A small aliquot was removed for immunobinding assays, PAGE and negative staining for observation under the EM. The virus was precipitated by the addition of 4% (w/v) PEG 6000 and 0.2M NaCl. The mixture was stirred for 3 h at 4 °C. The precipitate was collected by centrifugation in a JA14 rotor in a Beckman J2-21 centrifuge at 10000 rpm at 4 °C for 15 min. The pellet was resuspended overnight in 40 ml 0.01M potassium phosphate buffer pH 7.4 (appendix 4). The

suspension was clarified by centrifugation at 5000 rpm for 15 min in a JA14 rotor in a Beckman J2-21 centrifuge. The virus was collected by layering the supernatant over a 10% sucrose cushion made up in 0.01M potassium phosphate buffer pH 7.4 and centrifuging at 27000 rpm for 2 h in a SW28 rotor in a Beckman L8-55 ultracentrifuge at 4°C. The pellet was resuspended in 0.2 ml 5mM Tris-HCl pH 8 containing 2.5mM NaEDTA.

3.3 a) ii) Second adaptation of the method of Osaki and Inouye (1981) for the extraction of TLCV from infected plants

Homogenized leaves from the first extraction were suspended for three days at 4°C in extraction buffer to further extract the sap. The procedure described above was followed, but the supernatant was not spun on a sucrose gradient, but at 38000 rpm in a SW42.1 rotor in a Beckman L8-55 ultracentrifuge at 4°C. The pellet thus obtained was resuspended in 0.25 ml 5mM Tris-HCl pH 8 containing 2,5mM NaEDTA.

3.3 a) iii) Adaptation of the method of Sequeira and Harrison (1982) for the extraction of ACMV from infected cassava plants

This extraction was repeated twice. In the first attempt, 86 g of tobacco leaves from Rustenburg infected with a mild form of TLC were used and in the second, 114 g of leaves from Hazyview with enations were extracted. 68 g of healthy leaves were also extracted. The infected leaves were ground in a pestle and mortar in liquid nitrogen after being frozen at -70°C. The powder was further homogenized in a blender in cold extraction buffer (appendix 3) (2 to 3 ml

extraction buffer per gram leaf tissue). The mixture was stirred for 30 min. The sap was squeezed through a double layer of cheesecloth (pre-moistened with extraction buffer) and then stirred with one volume of chloroform for 15 min at 4°C, to clarify the sap. The mixture was then centrifuged for 10 min at 4°C at 11000 rpm in a JA14 rotor in a Beckman J2-21 centrifuge. The top phase was retained and 4% PEG 6000 and 0.2M NaCl were added. This was stirred overnight at 4°C. The sap was centrifuged at 12000 rpm for 10 min at 4°C in a JA14 rotor in a Beckman J2-21 centrifuge. The pellet was resuspended in 60 ml TE buffer pH 8 (appendix 4) by stirring at 4°C overnight. 1 ml of this semi-purified sap was removed and stored at 4°C for immunobinding assays, PAGE and negative staining for EM studies. The sap was clarified by centrifuging at 5000 rpm for 10 min at 4°C in a JA14 rotor in a Beckman J2-21 centrifuge. The supernatant containing the virus was concentrated by centrifugation at 38000 rpm for 2 h at 4°C in a SW 42.1 rotor in a Beckman L8-55 ultracentrifuge. The pellets were resuspended overnight in 0.8 ml of 5mM Tris-HCl pH 8 containing 2,5mM NaEDTA. The samples were stored in sealed Eppendorf tubes at 4°C.

3.3 a) iv) Modification of the method of Dollet and co-workers (1986) for the extraction of MSV Digitaria strain from infected plants

50 g of mildly infected leaves and 48 g of healthy leaves from Rustenburg were chopped up finely and frozen overnight at -70°C. The frozen leaves were then ground in a pestle and mortar using liquid nitrogen. Two volumes (v/w) cold extraction buffer (appendix 3) were added and the leaves were further crushed. 1% Triton X-100

was added to the mixture and the sap was squeezed through a double layer of cheesecloth pre-moistened with extraction buffer. The sap was clarified with 1/10 volume of chloroform / butanol (1:1 v/v). This was stirred for 15 min. The mixture was centrifuged at 5000 rpm for 15 min in a JA14 rotor in a Beckman J2-21 centrifuge. The supernatant was retained and the virus particles concentrated by the addition of 12% PEG 6000 and 0.2M NaCl to the supernatant. This was stirred overnight at 4 °C. The solution was centrifuged at 10000 rpm at 4 °C for 15 min in a JA14 rotor in a Beckman J2-21 centrifuge. The pellet was resuspended overnight in 2 ml TE buffer pH 8 (appendix 4). The suspension was centrifuged at 5000 rpm for 15 min to clarify and the supernatant was retained. An aliquot of this semi-purified preparation was removed for spectrophotometric readings, protein dot blots and negative staining.

The virus was collected by ultracentrifugation of the supernatant in a SW28 rotor in a Beckman L8-55 ultracentrifuge for 2 h at 27000 rpm in a 10% to 40% sucrose density gradient prepared in TE buffer pH 8. Since no light scattering bands were found after examination of the tube under UV light, 2 ml fractions were collected by hand and readings of each fraction at 260nm were taken on a Bausch and Lomb Spectronic 1001 spectrophotometer. Ultraviolet light absorbing fractions were pooled, diluted with TE buffer pH 8 (appendix 4) and ultracentrifuged at 38000 rpm for 2 h in a SW42.1 rotor in a Beckman L8-55 ultracentrifuge. The final pellet was dissolved in 500 ul TE buffer pH 8.

3.3 a) v) Method of Stein et al (1983) for extracting TGMV from infected tomato plants

100 g of infected leaf material with enations and 70 g of healthy leaf material from Hazyview was homogenized in 200 ml extraction buffer (appendix 3). The homogenate was made 2.5% (by volume) in Triton X-100 and stirred for 16 h at 4°C. The filtrate was centrifuged at 10000 rpm for 15 min in a JA14 rotor in a Beckman J2-21 centrifuge and the supernatant collected and centrifuged at 38000 rpm in a Beckman SW42.1 rotor in a Beckman L8-55 ultracentrifuge for 2 h. The pellets were resuspended in 12 ml CEM buffer (appendix 4) at 4°C overnight. The suspension was then divided into two aliquots. The first aliquot was overlaid onto a 16 ml cushion of 20% (w/v) sucrose in CEM buffer and centrifuged for 3 h at 25000 rpm in a Beckman SW28 rotor in a Beckman L8-55 ultracentrifuge. This was resuspended overnight in 2 ml CEM buffer before being further purified by centrifugation of 2 ml samples through 16 ml gradients of 10%- 50% (w/v) sucrose in CEM buffer for 16 h at 20000 rpm in a SW28 rotor in a Beckman L8-55 ultracentrifuge. The gradients were then examined for light scattering bands under UV light. Since no bands were found, the preparation was discarded. The second aliquot was centrifuged at 5000 rpm for 10 min in a JA14 rotor in a Beckman J2-21 centrifuge to clarify. An aliquot was removed for further analysis and the remainder was centrifuged for 2 h at 38000 rpm in a SW42.1 rotor in a Beckman L8-55 ultracentrifuge. The pellet was resuspended overnight in 3 ml CEM buffer.

3.3 a) vi) TLCV extraction according to the method of Osaki and Inouye (1978)

130 g severely infected leaf tissue and 80 g healthy tissue from Hazyview was frozen at -70°C overnight and ground in liquid nitrogen in a pestle and mortar. Two volumes extraction buffer (appendix 3) were added to the tissue and the tissue was further homogenized with a blender. The preparation was stirred for 1 h at 4°C. The homogenate was then squeezed through two layers of cheesecloth pre-moistened with extraction buffer. The extracts were emulsified with 10% n-butanol for 30 min and then centrifuged at 10000 rpm for 10 min in a JA14 rotor in a Beckman J2-21 centrifuge. The aqueous phase was adjusted to 6% PEG 6000 and 1% Triton X-100 and stored for 1 h at 4°C. This was then centrifuged at 12000 rpm for 20 min in a JA14 rotor in a Beckman J2-21 centrifuge. The pellet was resuspended in 0.1M potassium phosphate buffer pH 7.8 (appendix 4) overnight. The suspension was then centrifuged at 8000 rpm for 10 min, and the supernatant retained. An aliquot was removed for further analysis. The supernatant was layered on a 10% sucrose cushion made up in 0.1M potassium phosphate buffer pH 7.8 and centrifuged at 25000 rpm for 2 h at 4°C in a SW28 rotor in a Beckman L8-55 ultracentrifuge. The pellet was resuspended in 0.1M potassium phosphate buffer pH 7.8 overnight. An aliquot was removed for further analysis. The suspension was layered on top of a reverse concentration PEG solubility gradient (appendix 5) and centrifuged at 11000 rpm in a SW28 rotor in a Beckman L8-55 ultracentrifuge for 2 h. The interface between 0% and 4% PEG was extracted and spectrophotometric readings at 260nm were taken. Readings were also

taken of the other fractions. The interface and the other ultraviolet light absorbing fractions were then centrifuged separately at 38000 rpm for 2 h in a SW42.1 rotor in a Beckman L8-55 ultracentrifuge and the pellet was resuspended in TE buffer pH 8 (appendix 4) and stored at 4 °C.

3.3 b) Potyvirus extractions

3.3 b) i) Extraction according to the method of Jafarpour and co-workers (1979) for the isolation of BCMV from infected plants

This extraction was repeated twice. In both cases, leaf tissue from plants obtained from Hazyview and infected with severe leaf curl was used. In the first isolation procedure, 51 g of leaf tissue with enations was kept at 4 °C before extraction. In the second case, 60 g of fresh leaf tissue was used in the extraction. 50 g of healthy leaf tissue was also extracted.

The leaf tissue was homogenized with a blender in cold extraction buffer (appendix 3) (2:1, v/w). The extract was filtered through a double layer of cheesecloth pre-moistened with extraction buffer. The extract was clarified by shaking for 5 min with an equal volume of chloroform and then centrifuging at 8000 rpm for 25 min in a JA14 rotor in a Beckman J2-21 centrifuge. The virus was precipitated by adding PEG 6000 to a final concentration of 8% and NaCl to a final concentration of 0.5%. This was stirred for 1 h at 4 °C and then left to incubate overnight at 4 °C. The precipitate was collected by centrifugation for 25 min at 8000 rpm in a JA14 rotor in a Beckman

J2-21 centrifuge and the pellets were resuspended by shaking for 4 h at 4°C in about 15 ml 0.025M phosphate buffer pH 7.2 (appendix 4) containing 1M urea and 0.1% mercaptoethanol. The suspension was centrifuged at 5000 rpm for 10 min in a JA14 rotor in a Beckman J2-21 centrifuge to clarify. An aliquot was removed for protein dot blots, spectrophotometric readings and negative staining for EM studies. The supernatant was then centrifuged at 38000 rpm for 2 h in a SW42.1 rotor in a Beckman L8-55 ultracentrifuge. The pellet was resuspended in 1.5 ml TE pH 8 (appendix 4) containing a few grains of sodium azide and the sample was stored at 4°C.

3.3 b) ii) Potyvirus extraction according to Engelbrecht's (1990, pers. comm.) modification of Zee and colleagues' (1987) method for purifying closteroviruses from grapevines infected with leafroll disease

30 g of freshly harvested leaves from Hazyview infected with severe leaf curl and 50 g of healthy leaves were ground into a fine powder in liquid nitrogen with a pestle and mortar. The powder was transferred to a beaker containing extraction buffer (appendix 3) with 1.125% pectinase from Sigma and 0.375% Onozuka cellulase R-10. The mixture was shaken overnight at 20°C. The extract was then squeezed through four layers of pre-moistened cheesecloth in a funnel into a beaker kept in melted ice. The filtrate was centrifuged for 30 min at 2000 rpm at 4°C in a JA14 rotor in a Beckman J2-21 centrifuge. The supernatant was again poured through four layers of pre-moistened cheesecloth in a funnel into a beaker kept in melting ice, avoiding foaming. An aliquot was removed for further analysis.

The virus particles were sedimented by centrifugation of the supernatant on a 20% (w/v) sucrose cushion prepared in 0.1M Tris-HCl pH 8.2 in a SW28 rotor in a Beckman L8-55 ultracentrifuge at 25000 rpm for 2 h at 4 °C. Following centrifugation, the supernatant fluid from each tube was immediately discarded. The pellets were resuspended overnight in 10 ml chilled 0.1M Tris-HCl pH 8.2 containing 0.01M $MgCl_2$ on a shaker at 4 °C. The resuspended virus was centrifuged at 1000 rpm for 5 min at 4 °C in a JA14 rotor in a Beckman J2-21 centrifuge and the supernatant collected in a beaker kept in melting ice. An aliquot was removed for further analysis. The virus particles were sedimented by centrifugation of the supernatant at 38000 rpm for 2 h in a SW42.1 rotor at 4 °C in a Beckman L8-55 ultracentrifuge. The pellets were resuspended overnight with shaking at 4 °C in 0.1M Tris-HCl pH 8.2 containing 0.01M $MgCl_2$. The extract was stored at 4 °C.

3.3 c) Spectrophotometric readings

Spectrophotometric readings of 1/50 dilutions of each extraction were taken at 230nm, 260nm and 280nm on a Bausch and Lomb Spectronic 1001 spectrophotometer.

3.3 d) Protein dot blot immunobinding assays / enzyme linked immunosorbent assays (ELISAs)

Protein dot blots were made from each extraction as described below. Nitrocellulose (NC) paper was cut into squares about 4 cm x 4 cm in area. The NC paper was soaked in sterile dH_2O for 5 min and

airdried on Whatman No. 1 filter paper. Dilution series of sample were prepared in dH_2O and 5 ul samples dotted onto the NC paper. The NC paper was then baked at 37 °C for 1 h.

ELISAs were performed as described below against three different antisera: antiserum prepared against ACMV by John Stanley of the John Innes Research Institute, Norwich, England; antiserum against PVY from Gerhard Pietersen of Rietondale Research Station, Pretoria and a general potyvirus antiserum commercially obtainable from Agdia. A second batch of general potyvirus antiserum was obtained as a gift from John Hammond of Agdia Inc.

The prepared blots were blocked from binding non-specifically with 1% BSA in PBS (appendix 6) for 3 h at 37 °C with shaking. The blots were then washed in three changes of PBS-tween (appendix 6) for 5 - each. The antiserum was diluted in PBS as prescribed by suppliers, ie. 1:200 for ACMV, PVY and the first batch of commercial Agdia potyvirus antiserum; 1:1000 for host-absorbed PVY (see appendix 7 for method) and 1:5000 for the second batch of commercial Agdia potyvirus antiserum. 100 ul heparin per 100 ml PBS was added to the diluted antisera and the blots were incubated in this at 37 °C for 3 h. The blots were washed three times for 10 min each in PBS-tween, before incubation for 2 h at 37 °C in goat anti-rabbit alkaline phosphatase conjugate, diluted 1:1500 in AB buffer (appendix 6) containing 100 ul heparin per 100 ml AB buffer. The blots were washed three times for 15 min each in AB buffer, then twice for 20 min in AP buffer (appendix 6). The blots were then soaked in substrate solution (appendix 6) in the dark at room temperature. Once the colour had

developed (about 10 min) the reaction was stopped by the addition of 10mM Tris pH 7.5 containing 5mM NaEDTA to the blots.

The blots were photographed with back lighting using a blue filter.

3.3 d) i) Precipitation of virus from crude extracts of plants for protein dot blots

One leaf was collected from each of seven severely infected plants, one from each of three mildly infected plants and one leaf from each of two healthy plants. (All plants collected at Hazyview). Each leaf was homogenized separately in potyvirus extraction buffer as described by Jafarpour and co-workers (1979) (appendix 3) and extracts were squeezed through a single layer of cheesecloth pre-moistened with extraction buffer. Each preparation was clarified with an approximately equal volume of chloroform by mixing well and then centrifuging in a microfuge for 2 min. The upper phase was retained. 8% (w/v) PEG 6000 and 5% (w/v) NaCl was added to each Eppendorf tube, dissolved well and left to stand overnight to precipitate the virus. The extracts were then spun in the microfuge for 15 min, before resuspension of the pellet in 100 ul TE buffer pH 8 (appendix 4). Protein dot blots were made as described above and ELISAs were performed against PVA antiserum and commercial potyvirus antiserum from Agdia.

3.3 e) Polyacrylamide gel electrophoresis

Electrophoresis of denatured virus polypeptides was done in 10% or 12,5% SDS-polyacrylamide gels according to Laemmli (1970) (appendix 8). The gels were loaded with 10 ul MW markers and sample lanes contained 15 ul sample. Prior to loading, the samples were mixed with an equal volume of splitting solution (appendix 8) and boiled for 5 min. Gels were run at 151V and 40mA at room temperature, for 3-4 h.

Marker proteins included L-lactalbumin, soybean trypsin inhibitor, carbolic anhydrase, ovalbumin and BSA (see appendix 8 for molecular weights).

3.3 e) i) Staining of polyacrylamide gels

Gels were fixed and stained using Amersham's silver staining kit (appendix 9).

The first gel run was fixed and stained with Coomassie Brilliant Blue: GCI 42655) (appendix 9) followed by Amersham's silver stain.

Gels were stored in dH_2O at 4°C.

3.3 e) ii) Photography

Gels were photographed with back illumination using a blue filter.

3.4 ELECTRON MICROSCOPY

All grids used in EM studies were first coated with a mixture of 2% colloidon and colloidon in amyl acetate and then coated with a thin layer of carbon (appendix 10).

3.4 a) Negative staining for TEM

Semi-purified and purified samples were taken from all extractions for negative staining and observation on a JEM S-100 transmission electron microscope (JEOL).

A 10 ul drop of sample was placed on a piece of Parafilm and the colloidon-carbon-coated grid placed upside-down on this for 30 min. The grid was then turned upwards for 10 min before sucking off excess sample with filter paper and washing the grid well in dH_2O. The grid was once again dried and the sample was fixed by placing the grid on a drop of 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.2 for 5 min. The grid was dried and washed well in dH_2O before staining for 30 min on a drop of 1 or 2% ammonium molybdate made up in dH_2O. The grid was again washed and then dried and stored on filter paper before observing under the EM.

3.4 b) Leaf dips

Leaf dips were made from both infected and healthy leaves which were chopped up and then frozen overnight at -70°C. The leaves were further crushed in liquid nitrogen and 1 ml TE buffer pH 8 (appendix 4) was added to each leaf. The liquid was placed in an Eppendorf tube and frozen at -70°C for 2 h. The liquid was then thawed, and the tube spun in an Eppendorf centrifuge for 5 - 10 min. The pellet was resuspended in 200 ul of TE buffer pH 3, and the supernatant was also retained. Colloidon-carbon-coated copper grids were prepared and stained negatively before examination under a JEM100S (JEOL) electron microscope.

3.4 b) i) Leaf dips using bacitracin

An infected leaf was crushed in a pestle and mortar with 10mM Tris pH 8 containing 5mM Na EDTA and 0.05% bacitracin (2 vol extraction buffer : 1 g leaf tissue). The extract was placed in an Eppendorf and frozen for 2 h at -70°C before thawing. The extract was vortexed for 1 min before being microfuged for 10 min. The pellet and supernatant were retained separately, and the pellet was resuspended in 100 ul TE pH 8 (appendix 4). Colloidon-carbon-coated copper grids were prepared and negatively stained before examination under the electron microscope.

3.4 c) Thin sectioning of leaves

3.4 c) i) LR White resin embedding of leaves

Leaves from Hazyview infected with severe leaf curl and mild leaf curl as well as healthy leaves were embedded in LR White resin for sectioning and viewing under the EM. Sections were taken from the laminar, veins and midrib of leaves. Separate sections were taken from the enations in the case of severely infected leaves.

Leaf parts were chopped into pieces less than 1 mm^2 in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.2. The pieces were then fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.2 for 3 h at 4 °C. The sections were then washed in five changes of 0.1M sodium cacodylate buffer pH 7.2 for 15 min each. Pieces were post-fixed in 1% osmium tetroxide prepared in 0.1M sodium cacodylate buffer pH 7.2 for 2 h at room temperature, before washing again as before. Dehydration was performed in an ethanol series of 30%, 50%, 70%, 95% and absolute (twice) for 10 min each. The sections were left in LR White resin overnight at 4 °C. The LR White resin was replaced with fresh LR White resin and left for 2 h at 4 °C. Sections were then embedded in gelatin capsules and left to polymerize at 60 °C for 20 h.

3.4 c) ii) Sectioning

Ultrathin sections were cut from resin blocks with a glass knife in a Reichert OMU3 ultramicrotome. Sections were collected onto collodion-carbon-coated copper grids.

3.4 c) iii) Staining sections

Grids containing sections were stained in 1% uranyl acetate for 15 - 20 min. The grids were then washed well in ddH O before being placed on lead citrate (appendix 10) for 5 - 10 min. The grids were washed well once again and dried before observation under the electron microscope.

3.4 d) Gold labelled antibody decoration (GLAD)

GLAD was performed on sections cut from material with enations and placed on collodion-carbon-coated gold and nickel grids (appendix 10). The grids were placed on a 1% BSA solution in 0.05M Tris pH 7.2 for 1 h at room temperature to block non-specific binding reactions. They were then washed well in Beasley buffer (appendix 10). The antibody was diluted in PBS (appendix 6) as follows: the first batch of commercially obtained antiserum to potyviruses from Agdia - 1:100; antiserum to PVY - 1:200 and the grids were placed on this for 1 - 2 h at room temperature. The grids were washed well in Beasley buffer before being placed on goat anti-rabbit gold (1:50 dilution in Beasley buffer) for 1 - 2 h at room temperature. The grids were

washed well in dH_2O. The grids were then stained for 10 min on 1 - 2% uranyl acetate, washed well in ddH_2O and stained for 2 min on lead citrate (appendix 10). The grids were again washed well before observation in the electron microscope.

Control grids of sections on nickel were made as described above, omitting the antibody and floating the grids on PBS only.

3.4 e) Immune specific electron microscopic tests (ISEM) (from the method of Roberts and Harrison, 1979)

Collodion-carbon-coated nickel grids (appendix 10) were floated for 20 min on a drop of antiserum diluted in PBS (appendix 6). Antisera were diluted as described above in section 3.4 d). The grids were then rinsed in 20 consecutive drops of PBS before incubation for 50 min on a drop of sample extract. The grids were then stained as described above in section 3.4 a) (negative staining), before examination with the electron microscope.

3.5 DS DNA

Two attempts were made to extract the ds replicative form (RF) of geminiviruses from infected leaves.

3.5 a) ds DNA extraction

Three separate extractions were performed: on 36 g mildly infected leaf material from Rustenburg, 57 g severely infected leaf material from Hazyview and 8 g healthy leaf material from Rustenburg. The material was frozen overnight at -70 °C. It was then ground into a fine powder in a pestle and mortar in liquid nitrogen. The powder was suspended in an approximately equal volume to weight ratio of grinding buffer (appendix 11) to plant material. The mixture was stirred by hand for 30 min. The homogenate was squeezed through a single layer of cheesecloth (pre-moistened with grinding buffer) and centrifuged at 10000 rpm for 20 min at 4 °C in a JA14 rotor in a Beckman J2-21 centrifuge. 100 ug/ml RNase (appendix 11) was added to the supernatant. The supernatant containing RNase was incubated at 60 °C for 15 min. 100 ug/ml proteinase K (appendix 11) was added to the supernatant and the resulting suspension was incubated at 37 °C for 10 min. The solution was then phenol / chloroform (8:2 v/v) extracted twice (appendix 11). An equal volume of chloroform was added to the mixture, which was spun at 5000 rpm for 5 min in a JA14 rotor in a Beckman J2-21 centrifuge. The upper phase was retained. The DNA was precipitated by the addition of a 10% volume of 5M NaClO$_4$ and an equal volume of isopropanol to the upper phase. The

mixture was shaken up and left to stand for 1 h at -70 °C. The mixture was then thawed, before centrifugation at 10000 rpm for 10 min in a JA14 rotor in a Beckman J2-21 centrifuge. The supernatant was poured off and the pellet dried at 37 °C for 10 min to allow the isopropanol to evaporate. The pellet was resuspended in 4 ml TE buffer pH 8 (appendix 4) overnight.

0.36 g CsCl (ie. 0.9 g / ml resuspended DNA) and 0.8 ml ethidium bromide (from a 10 ug/ml stock) was added to 4 ml of resuspended DNA. The mixture was placed in a Beckman Quickseal centrifuge tube and the tubes balanced to 0.03 g using TE containing CsCl . The tubes were then sealed and spun at 45000 rpm at 4 °C for 16 h in a VTi 65.2 rotor in a Beckman L7-55 ultracentrifuge.

A fluorescing band of plant chromosomal DNA was found about two thirds of the way down the tube when it was observed in ultraviolet light.

200 ul fractions were collected dropwise from beneath the plant chromosomal band by piercing the tubes at the top and the bottom with a sterile wide gauge needle and allowing 4 - 5 drops of fluid to flow into sterile Eppendorf tubes.

The ethidium bromide was washed out of the fractions by adding an equal volume of TE-saturated 2-butanol to each Eppendorf tube, shaking the mixture and centrifuging it in the microfuge for a few seconds. The butanol containing ethidium bromide rose to the top and was discarded. The process was repeated at least five times until the butanol became colourless.

DNA precipitation was carried out as follows. Two volumes of TE were added to each fraction. An equal volume of isopropanol was added, the samples mixed well and stored at -70 °C for 1 h. Samples were thawed and spun in the microfuge for 15 min. The TE and isopropanol were poured off, and the pellet dried at room temperature for 5 – 10 min before being resuspended in 100 ul TE and stored at 4 °C.

3.5 b) Agarose gels

"Seakem" agarose gels of different concentrations (appendix 12) were prepared in TE buffer pH 8 (appendix 4), TBE buffer pH 8 (appendix 12) or TAE buffer pH 8 (appendix 12). Ethidium bromide at a concentration of 1 ul/20 ml agarose was added to each gel. Running buffer was either TE, TBE or TAE depending on the buffer in which the gel was prepared.

Samples were prepared as follows: the first gels were run with 8 ul DNA sample and 2 ul tracking dye (appendix 12). Once the concentration of the DNA had been ascertained, 5 to 6 ul DNA sample was loaded. In cases where the DNA was very concentrated, the DNA was diluted by half. DNA molecular weight markers in predetermined quantities (appendix 12) were added to 2 ul tracking dye and loaded. 5 ul sugarcane mosaic virus DNA obtained from the Department of Microbiology of the University of Cape Town was loaded with 2 ul tracking dye.

Samples were run on polymerized agarose gels at 40V for about 2 h (or until the tracking dye was between half and three-quarters of the way down the gel).

3.5 b) raphy

Ge. examined on an Ultraviolet Products Inc. UV tra photographed with UV back illumination with a Polaroid CU-5 land camera.

3.5 c) Recovery of DNA from agarose

As some of the extrachromosomal DNA associated with infected samples was contaminated with chromosomal DNA, the "freeze-squeeze" method of DNA recovery from agarose gels (Tautz and Renz, 1983) was used to recover the bands of interest.

A 0.9% agarose gel containing ethidium bromide was run. The gel was briefly viewed on a long wave UV transilluminator before rapidly cutting out the bands of extrachromosomal DNA with a sterile blade. The bands were transferred to large Eppendorf(s) and a 10X volume of 0.3M sodium acetate pH 7 containing 1mM NaEDTA was added to the Eppendorf(s). The tube was kept in the dark for 15 - 45 min, inverting occasionally. A small (0.5 ml) Eppendorf tube was prepared by piercing a hole in the bottom with a wide-gauge needle, adding a small amount of siliconised glass wool and centrifuging quickly inside a large Eppendorf in a microfuge to pack down. Gel slices were transferred into the small Eppendorfs, the lids closed and the

Eppendorfs dropped into liquid nitrogen in an ice-bucket for about 5 min to freeze solid. The vials were removed with forceps, placed directly into large (drained) Eppendorfs and centrifuged in the microfuge for 10 - 15 min. The small Eppendorfs were discarded, the volume of the eluate measured and the DNA precipitated with 1/10 volume of 3M sodium acetate pH 5 and 2 volumes 90% ethanol. The tubes were frozen at -70°C for 1 h, then thawed and spun in a microfuge for 10 - 15 min. The supernatant was poured off and the pellet airdried before resuspension in 10 ul TE pH 8 (appendix 4).

3.5 d) Restriction enzyme analysis of extrachromosomal dsDNA

Single digestions were performed with several 6bp cutting restriction enzymes (ie. BamH1, Bgl1, BglII, EcoR1, MluII, PstI, PvuII, SacI and SalI) obtained from Boehringer Mannheim. Two enzymes recognizing 4bp sequences, Sau3A and HaeIII, were also tried.

5 ul DNA, 8.5 ul sterile ddH$_2$O and 1.5 ul of the buffer recommended for use with the particular enzyme were added to each tube before microfuging and adding the appropriate enzyme. These were then incubated at 37°C for 1-3 h before running on a 0.4% or 0.6% agarose gel against DNA molecular weight markers III from Boehringer Mannheim. 2 ul tracking dye was added to each sample before loading on the gel.

Double digestions were then carried out using the following combinations: BglII and PvuII, BglI and PstI, BglI and SalI and SalI and PstI. The procedure described above was followed, but both

enzymes were added before incubation at 37°C for 3 h. These digestions were repeated, but 0.6% agarose gels made up in TAE buffer pH 8 (appendix 12) were only run after cleaning up the DNA (appendix 13) with phenol to remove the enzymes and buffer.

3.5 e) Characterisation of DNA

The size of the extrachromosomal DNA associated with infected plants was determined by comparison of this DNA to known standard MW markers run on agarose gels.

Four different procedures were carried out to determine the nature of the DNA: Thermal denaturation (Ikegami and Francki, 1966), Formaldehyde denaturation (Ikegami and Francki, 1966) and digestion with DNase and nuclease S1 (Ikegami et al, 1987).

The character of the DNA was determined after precipitation of the DNA as described above in section 3.5 a) and resuspension in the buffer appropriate for the characterization procedure to be performed. DNA isolated from phage lambda and obtained from Boehringer Mannheim was used as a control throughout.

In all cases, the blank consisted of the buffer without DNA, but containing enzymes or formaldehyde as required by the experiment.

3.5 e) i) Thermal denaturation of DNA

Thermal denaturation of DNA was carried out according to the method of Ikegami and Francki (1966).

Extrachromosomal ds DNA isolated from plants infected with tobacco leaf curl and phage lambda DNA (as a control) at a concentration of about 10 ug/ml was resuspended in 1 x SSC (appendix 14). The preparations were heated at a rate of 0.5 °C/min in a waterbath and spectrophotometric readings at 260nm on a Bausch and Lomb Spectronic 1001 spectrophotometer were taken at room temperature (22 °C), 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C and 90 °C.

A graph was plotted comparing the control readings to the experimental readings.

3.5 e) ii) Denaturation of nucleic acid with formaldehyde (according to Ikegami and Francki, 1966)

Extrachromosomal DNA isolated from infected plants and phage lambda DNA were resuspended in 500 ul of 1 x SSC (appendix 14) at a concentration of about 10 ug/ml. The absorbance was read at 200, 220, 240, 260, 280 and 300nm on a Bausch and Lomb Spectronic 1001 spectrophotometer. 1.8% formaldehyde was added to each suspension and the solutions incubated for 20 h at 37 °C before repeating the absorbance readings.

A graph was plotted once again comparing the readings from the control to those of the extrachromosomal DNA associated with infected plants.

3.5 e) iii) Digestion of DNA with deoxyribonuclease (according to Ikegami et al, 1987)

Extrachromosomal DNA isolated from infected plants and phage lambda DNA were suspended in 500 ul 10mM sodium acetate pH 5 containing 5mM $MgSO_4$. Readings were taken at 260nm on a Bausch and Lomb Spectronic 1001 spectrophotometer. 20 ul of Boehringer Mannheim DNase 1 (from a stock of 1 ug/ul) was added to each tube and readings were taken at 260nm at 5 min intervals over a period of 1 h.

A graph was plotted comparing the readings from the control DNA to those of the extrachromosomal DNA.

3.5 e) iv) Digestion of DNA with nuclease S1 (according to Ikegami et al, 1987)

Extrachromosomal DNA isolated from infected plants and phage lambda DNA were suspended in 500 ul 10mM sodium acetate containing 5mM $MgSO_4$ and 0.5M $ZnCl_2$ pH 5 at a concentration of about 10 ug/ml. Readings were taken at 260nm on a Bausch and Lomb Spectronic 1001 spectrophotometer before adding 2 ul of nuclease S1 (from a stock containing 1000 u/ul) to each tube. Readings were taken at 260nm at 5 min intervals over a period of 1 h.

A graph was plotted comparing the readings from the phage lambda DNA to those of the extrachromosomal DNA.

3.5 e) v) Tertiary structure of DNA

The extrachromosomal DNA obtained from infected plants was heated at 60 °C for 5 min before running on a 0.7% agarose gel to determine whether it had a complex tertiary structure.

3.5 f) Hybridization

3.5 f) i) Nucleic acid spot hybridization procedure

Plant samples were prepared for blotting according to the method of Maniatis and colleagues (1982). One leaf from each infected plant collected from Hazyview and one leaf from each of two healthy plants was ground in TE buffer pH 8 (appendix 4) at a ratio of 1:4 (w:v) and filtered through a single layer of cheesecloth pre-moistened with TE buffer. The extracts were incubated with NaOH to a concentration of 1N for 10 min at room temperature. The extracts were neutralized by the addition of 1/10 volume of 3M sodium acetate pH 5. Dilution series were made with TE buffer pH 8 (appendix 4).

Nitrocellulose paper was cut into squares about 4cm x 4cm large. The NC was presoaked in ddH $_2$O for 5 min. The NC was then soaked in 3.6M NaCl containing 0.2M sodium phosphate and 0.02M NaEDTA pH 7 for 15 min. The NC paper was dried at room temperature. 5 ul samples were spotted onto the NC paper and the blots were then baked at 80 °C in a vacuum oven for 2 h, or on the UV transilluminator for 5 min.

The crude DNA samples were tested for complementarity against labelled DNA prepared against the extrachromosomal DNA isolated from infected plants.

3.5 f) ii) Southern blotting

One 0.9% and one 0.8% gel were run and the DNA species from the gel transferred to nitrocellulose paper by Southern blotting according to the method of Southern (1975) (appendix 15). The transferred DNA was tested for complementarity with labelled DNA prepared against extrachromosomal DNA isolated from infected plants.

3.5 f) iii) DNA hybridization

A probe was prepared to the extrachromosomal DNA extracted from infected plants according to the instructions given in the Boehringer Mannheim nonradioactive DNA labelling and detection kit (appendix 15). The DNA was labelled by random primed incorporation of digoxigenin-labelled deoxyuridine triphosphate. The probe was used in spot hybridisation tests as described in the kit (appendix 15). Labelling of DNA and detection of hybridisation were performed using the Boehringer Mannheim nonradioactive DNA labelling and detection kit according to the instructions supplied with the kit (appendix 15).

After hybridization to the target DNA the hybrids were detected by enzyme-linked immunoassay using an antibody-conjugate and subsequent enzyme-catalyzed colour reaction with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium salt.

4 RESULTS

4.1 TRANSMISSION EXPERIMENTS

4.1 a) Mechanical inoculation and injection

After two weeks, no symptoms were observed on plants which had been mechanically inoculated or injected as described in section 3.2 a) above, although local lesions could be seen on some of the leaves which had been rubbed with Carborundum. However, the control plants exhibited the same lesions.

Small necrotic lesions often developed at the site of injection, but once again these were also found in the control plants injected with dH_2O.

4.1 b) Electrotransmission (plates 4 to 7)

Both experimental and control tobacco (N. tabacum) plants subjected to electrotransmission with sap extracted in buffer containing sodium sulphite (appendix 2) were observed daily for symptoms. In the plants treated with sap from infected plants, the brownish colour of the sap could be clearly seen following the vascular system of the dipped leaf. The plants were badly wilted in the second and third days after electrotransmission. No change was seen for the next four days. One week after electrotransmission, the dipped leaf developed yellow blotches in both experimental and control plants. After nine days, however, the plants became severely wilted and were dead within another two days.

The experiment was repeated, omitting the sodium sulphite from the extraction buffer, and subjecting the plants to a 9V potential difference for 2 h and 4 h.

One day after electrotransmission, the plants which had undergone electrotransmission for 2 h began to wilt and the leaves which had been dipped into sap began to blacken. The following day, the dipped leaves on some of the plants died, but the plants were otherwise healthy. After three weeks, these plants had developed no symptoms.

The plants which had undergone electrotransmission for 4 h showed the same pattern for the first three days as those which had been subjected to a 9V potential difference for 2 h. After four days, three of the ten plants had died. On the eighth day after electrotransmission, six of the seven remaining plants were showing early symptoms of TLC disease. The development of a yellow network on the upper surfaces of young leaves as described by McClean (1940) was not noticed. The margins of new leaves were curling downwards, and bulges could be seen on the older leaves. These symptoms were not observed on control plants. The leaves of both control and experimental plants developed mottling on their surfaces. Ten to twelve days after electrotransmission, the leaves on experimental plants were showing further bulging and the margins of young leaves were still curling downwards (plates 4 and 5). 21 days after electrotransmission, the symptoms were still present and experimental plants were markedly smaller than healthy plants. Six weeks after electrotransmission, no further symptoms had developed although the existing symptoms were more severe (plates 6 and 7). At this stage, observations were concluded and plants were kept for further

analysis.

Electrotransmission performed for 4 h on a single N. benthamiana plant produced similar initial symptoms to those of N. tabacum (ie. wilting and blackening of dipped leaves) although the leaves did not become mottled. After two days, the plant appeared healthy. One week after electrotransmission, bulges began to appear on a developing leaf. A week later, however, the plant was wilting badly and one week after that the plant was dead. The control plant began wilting severely two days after electrotransmission. However, after two weeks the plant appeared to be healthy, with no visible symptoms of TLC.



Plate 4. N. tabacum plant three weeks after electrotransmission. Bulges (B) are appearing on new leaves and the youngest leaf (arrow) has a margin curling upwards



Plate 5. a - Control plant treated by electrotransmission three weeks previously with buffer only. Note leaf mottling (m). b- Healthy plant of same age as treated plants. c - Plant treated with infected plant sap three weeks previously

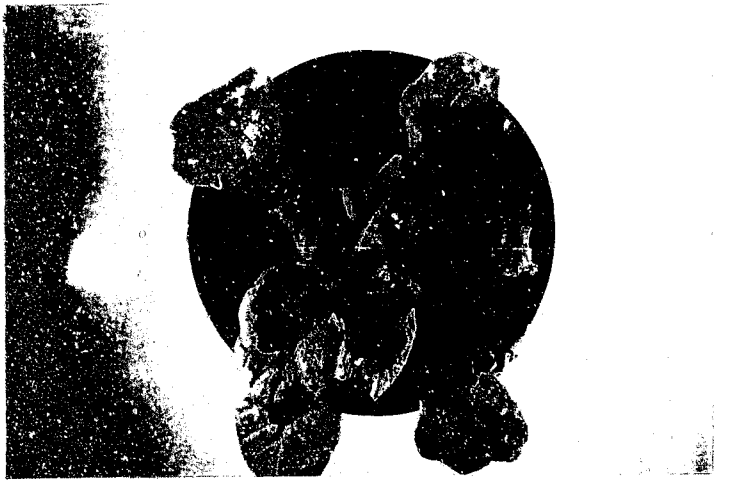


Plate 6. Six weeks after electrotransmission with infected sap, leaf margins were increasingly curly and bulges more pronounced.

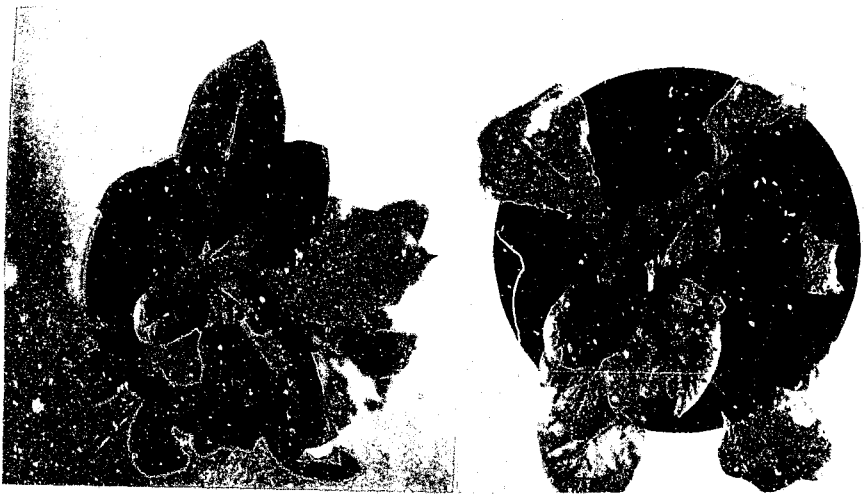


Plate 7. Comparison of plant treated with infected sap (right) and healthy plant of same age (left). The healthy plant is taller than the experimental plant.

4.2 VIRUS PURIFICATION

Intact virus particles were not isolated from any of the attempted extraction procedures.

4.2 a) Spectrophotometric readings (tables 1 and 2)

4.2 a) i) Geminivirus extractions (table 1)

The first geminivirus extraction from severely infected plants collected in Rustenburg (1 in table 1) (adaptation of Osaki and Inouye, 1978) gave no readings either at 260nm or at 280nm, indicating that no virus particles were isolated. No readings were obtained from the healthy samples.

The second extraction procedure using Osaki and Inouye's (1978) method (2 in table 1) with the same plants used in the first extraction gave positive absorbance readings from the semi-purified sample of 0.27 and 0.13 at 260 and 280nm respectively, with the reading at 260nm being higher than that at 280nm.

The healthy samples prepared from this method gave no absorbance readings at any of the wavelengths.

Similar results were obtained from the second extraction (3 in table 1) (modification of Sequeira and Harrison, 1982) from plants from Rustenburg showing mild symptoms. The absorbance readings at 260nm (0.22 for purified and 0.11 for semi-purified) were consistently

higher than those at 280nm (0.03 for purified and 0.08 for semi-purified), as expected. However, this was also the case with the healthy samples although these readings were slightly lower than those obtained from the infected samples.

The semi-purified sample from this extraction procedure was the only one in which a 260/280 ratio close to the expected ratio given by Osaki and Inouye (1978) of 1.4 (in this case, 1.38) was found.

On repeating this extraction with severely infected plants collected in Hazyview (4 in table 1), the absorbance readings at 260nm (purified - 0.24 and semi-purified - 0.41) were higher than those obtained at 230nm (purified - 0.17 and semi-purified - 0.29).
The third extraction (5 in table 1) (modification of method of Dollet et al, 1986) of mildly infected leaves from Rustenburg gave similar results to those of the previous extractions. The absorbance readings from healthy plant extractions were, again, lower than those from infected plant extractions.

The extraction carried out according to the method of Stein et al (1983) (6 in table 1) on severely infected plants from Hazyview yielded no absorbance readings at 260nm or 280nm for the purified preparation, although readings of 0.29 at 260nm and 0.13 at 280nm were obtained from the semi-purified preparation.

Table 1. UV absorbances (230, 260 and 280nm) and 260/280 ratios of purified (P) and semi-purified (S-P) samples (diluted 1/50) from geminivirus extraction procedures. (Healthy readings in brackets)

Extraction		Plant symptoms and origin	230nm	260nm	280nm	260/280
1	P	severe	0.00(0.00)	0.00(0.00)	0.00(0.00)	N/A (N/A)
	S-P	Rustenburg	0.00(0.00)	0.00(0.00)	0.00(0.00)	N/A (N/A)
2	P	severe	0.00(0.00)	0.00(0.00)	0.00(0.00)	N/A (N/A)
	S-P	Rustenburg	0.55(0.00)	0.27(0.00)	0.13(0.00)	2.02(N/A)
3	P	mild	1.16(0.19)	0.22(0.10)	0.03(0.00)	6.75(N/A)
	S-P	Rustenburg	0.25(0.40)	0.11(0.16)	0.08(0.00)	1.38(N/A)
4	P	severe	0.17(0.10)	0.24(0.03)	0.00(0.00)	N/A (N/A)
	S-P	Hazyview	0.29(0.18)	0.41(0.07)	0.10(0.04)	4.10(1.75
5	P	mild	0.09(0.08)	0.06(0.03)	0.00(0.00)	N/A (N/A)
	S-P	Rustenburg	0.54(0.36)	0.87(0.23)	0.36(0.19)	2.42(2.79
6	P	severe	0.47(0.10)	0.00(0.00)	0.00(0.00)	N/A (N/A)
	S-P	Hazyview	1.27(0.57)	0.29(0.28)	0.13(0.06)	2.10(4.67
7	P	severe	0.05(0.03)	0.26(0.02)	0.00(0.00)	N/A (N/A)
	S-P	Hazyview	0.14(0.14)	0.45(0.06)	0.00(0.00)	N/A (N/A)

The unmodified extraction method of Osaki and Inouye (1978) (7 in table 1) performed on severely infected plants from Hazyview gave no readings at 280nm in the purified or the semi-purified extracts. Readings from the interface between 0% and 4% PEG (from the reverse concentration PEG solubility gradient) were zero for all three wavelengths.

In the case of the semi-purified sample and the fraction purified from the remainder of the gradient, the readings at 260nm (semi-purified - 0.45; purified - 0.26) were higher than those at 230nm (semi-purified - 0.14; purified - 0.05).

4.2 a) ii) Potyvirus extractions (table 2)

In the first potyvirus extraction on leaves with enations from Hazyview (1 in table 2) (according to Jafarpour et al, 1979), it can be assumed that a peak would have been obtained at 260nm for both the purified and the semi-purified preparations had more data been available, since absorbance readings at 260nm (1.17 - purified and 0.47 - semi-purified) were higher than those obtained at 230nm (0.61 - purified; 0.23 - semi-purified). Surprisingly, the absorbance readings increased after purification - this was probably due to increased concentration of nucleoproteins after high speed centrifugation.

Table 2. UV absorbances (230, 260 and 280nm) and 260/280 ratios of purified (P) and semi-purified (S-P) samples (diluted 1/50) from potyvirus extraction procedures. (Healthy readings in brackets)

Extraction		Plant symptoms and origin	230nm	260nm	280nm	260/280
1	P	severe	0.61(0.57)	1.17(0.52)	0.55(0.39)	2.13(1.33
	S-P	Hazyview	0.23(0.12)	0.47(0.05)	0.07(0.00)	6.71(N/A)
2	P	severe	0.27(0.14)	0.00(0.00)	0.00(0.00)	N/A (N/A)
	S-P	Hazyview	0.35(0.03)	0.00(0.00)	0.00(0.00)	N/A (N/A)
3	P	severe	0.42(0.10)	0.36(0.08)	0.34(0.07)	1.06(1.14
	S-P	Hazyview	0.45(0.41)	0.93(0.28)	0.86(0.14)	1.08(2.00

On repeating the extraction of Jafarpour and colleagues (1979) (2 in table 2) on severely infected plants from Hazyview results were very different. No absorbance readings were obtained at either 260nm or 280nm for the semi-purified or purified samples.

The second method of extracting potyviruses (modification of Zee et al, 1987 by Engelbrecht pers. comm. 1990) (3 in table 2) on leaves with enations from plants collected at Hazyview yielded a peak at 260nm from the semi-purified suspension. However, this peak was lost after final purification although the readings were higher than those obtained from the healthy extraction.

Table 3. Serological relationships of infectious agent(s) of TLC in South Africa

Geminivirus extractions	ACMV	PVY	Agdia 1	Agdia 2
1 (severe - Rustenburg)	-	N/A	N/A	-
2 (severe - Rustenburg)	-	N/A	N/A	N/A
3 (mild - Rustenburg)	-	-	N/A	N/A
4 (severe - Hazyview)	-	N/A	N/A	N/A
5 (mild - Rustenburg)	-	N/A	N/A	N/A
6 (severe - Hazyview)	-	N/A	N/A	N/A
7 (severe - Hazyview)	-	N/A	N/A	N/A
Potyvirus extractions				
A (severe - Hazyview)	-	++	0	N/A
B (severe - Hazyview)	N/A	++	0	N/A
C (severe - Hazyview)	N/A	N/A	N/A	N/A

Key: h/a - host adsorbed
- - negative reaction
+ - positive reaction
++ - strongly positive reaction
N/A - not tested
0 - no reaction

Agdia 1 - 1st batch of general potyvirus antiserum
Agdia 2 - 2nd batch of general potyvirus antiserum

4.2 b) ELISAs

Indirect ELISA results are depicted in tables 3 and 4 and plates 8 and 9.

Immunobinding assays performed with antiserum against ACMV gave negative results with all extractions (see table 3 and plate 8).

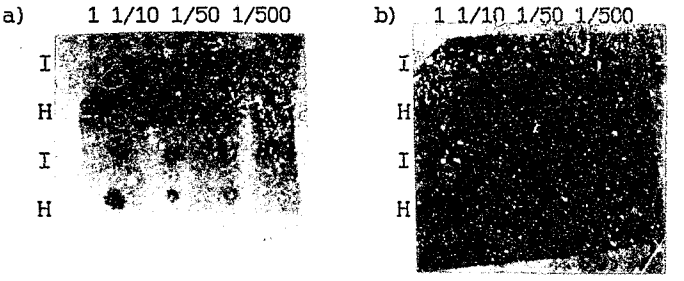


Plate 8. Spot ELISA tests with a) semi-purified extracts of TLC-infected (I) and healthy (H) N. tabacum using antiserum to ACMV and b) purified extracts of the same samples. Extraction procedure a modification of Sequeira and Harrison (1982) on severely infected leaves from plants collected from Hazyview. Each extract was tested undiluted (1) and at 1/10, 1/50 and 1/500 dilutions.

PVY antiserum also gave negative results against blots made from samples of mildly infected leaves from the modified method of Sequeira and Harrison (1982) (3 in table 3).

However, immunobinding assays performed against PVY antiserum, with blots made from potyvirus extractions performed by the method of Jafarpour and co-workers (1979) from leaves taken from plants from Hazyview showing severe leaf curl (A and B in table 3) gave positive results (see plate 9). The reaction was positive up to a dilution of 1/500 for both semi-purified and purified preparations. This experiment was repeated and positive results were again obtained.

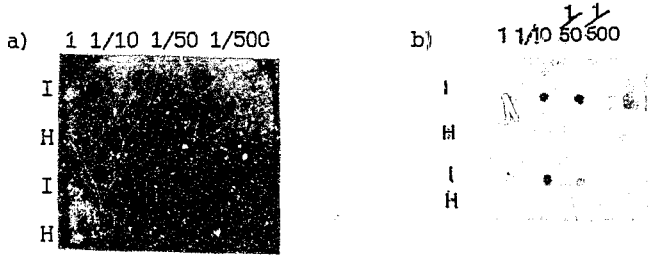


Plate 9. Spot ELISA tests with a) semi-purified extracts of severely TLC-infected (I) and healthy (H) N. tabacum using antiserum to PVY and b) purified extracts of the same samples. Extraction procedure of Jafarpour and co-workers (1979) on plants from Hazyview. Each extract was tested undiluted (1) and at 1/10, 1/50 and 1/500 dilutions.

These same blots (A and B in table 3) were tested against the general potyvirus antiserum obtained from Agdia. No reaction occurred between the antiserum and the protein dots. However, later a new batch of general potyvirus antiserum was received which reacted positively with blots prepared from crude extracts of leaves with enations (ie. severely infected leaves) and leaves from plants with mild symptoms

collected from Hazyview (see table 4) and it was suspected that the first batch was faulty.

Table 4. Serological relationships of crude preparations of N. tabacum plants infected with different forms of TLC

Type of infection	Antisera			
	PVY	PVY (h/a)	Agdia 1	Agdia 2
1 severe	-	-	N/A	+
2 severe	-	-	N/A	+
3 severe	-	-	N/A	+
4 severe	-	-	N/A	-
5 severe	-	-	N/A	+
6 severe	-	-	N/A	-
7 severe	-	-	N/A	-
8 mild	-	-	N/A	-
9 mild	-	-	N/A	-
10 mild	-	-	N/A	+

Key: H/a - host adsorbed

Agdia 1 - 1st batch of potyvirus antiserum

Agdia 2 - 2nd batch of potyvirus antiserum

N/A - not tested

PVY antiserum was tested against blots made from crude virus preparations of plants collected from Hazyview and infected with both the severe and mild forms of TLC (table 4). Results from these experiments were inconclusive and the antiserum was host-absorbed and

the blots tested again. Once again, results were negative.

A second batch of concentrated general potyvirus antiserum was obtained as a gift from John Hammond of Agdia. This was tested against the blots made from crude virus preparations and, although reactions with the antiserum were too weak to be photographically represented, results were positive on four of the seven severely infected plants and on one of the three samples extracted from plants with mild TLC (table 4).

4.2 c) Polyacrylamide gel electrophoresis (plates 10 to 12)

Initially, problems caused by carbohydrate contamination and problems with the silver staining kit hampered attempts at analyzing gels and only three gels gave satisfactory results. Running gel concentrations were increased from 10% to 12.5% since incomplete separation of the molecular weight markers was obtained on the 10% running gels.

A gel run with samples from a geminivirus extraction done on mildly infected leaves obtained from Rustenburg (according to a modification of Sequeira and Harrison, 1982) was initially stained with Coomassie Blue before silver staining. Resolution was fairly good, but no extra bands could be detected in the infected samples (plate 10).

An extra band was found at 26kd on a gel run with samples from the first potyvirus extraction (according to Jafarpour et al, 1979) from leaves with enations obtained from plants with severe leaf curl collected in Hazyview (plate 11).

Two extra bands were found at 59kd and 23kd on a gel run with samples from the last geminivirus extraction (according to Osaki and Inouye, 1978) of leaves from plants collected in Hazyview and infected with severe leaf curl (see plate 12).

A gel run for a shorter period of time than usual (and not shown here) had several low molecular weight peptide bands running faster than the tracking dye in the infected samples (both from potyvirus and geminivirus extractions) and not in the healthy samples.

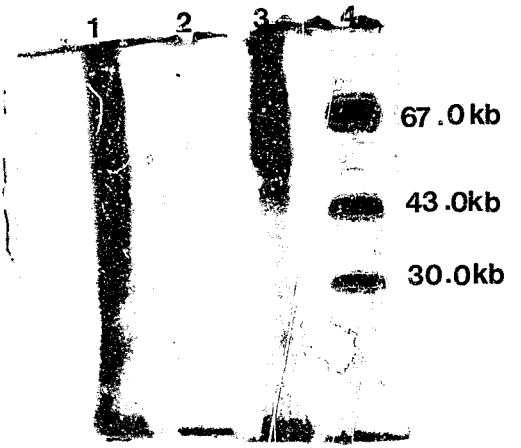


Plate 10. Polyacrylamide gel electrophoresis of polypeptides isolated from plants infected with mild TLC on a 10% running gel. Lanes: 1 purified infected sample; 2 semi-purified infected sample; 3 healthy sample; 4 molecular weight markers (MW shown down right hand side of gel). The gel was first stained with Coomassie Blue and then with Amersham silver stain kit.

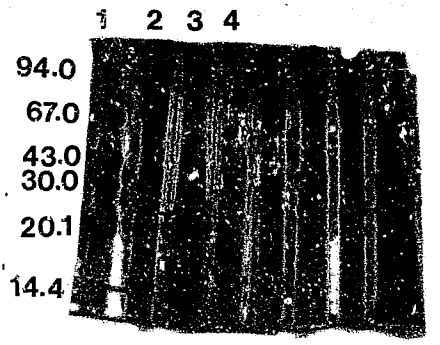


Plate 11. PAGE of polypeptides isolated from plants infected with severe TLC on a 10% running gel stained with Amersham's silver stain kit. Lanes: 1 MW markers; 2 semi-purified potyvirus sample; 3 purified potyvirus sample; 4 purified healthy sample.

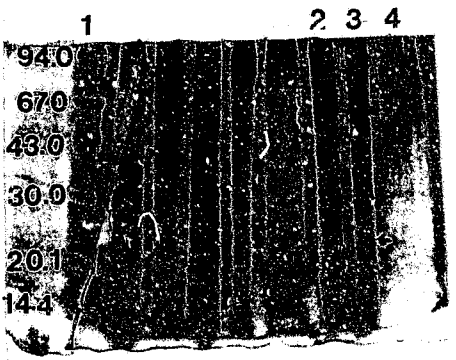


Plate 12. PAGE of polypeptides isolated from plants with severe TLC on a 12.5% running gel stained with Amersham's silver stain kit. Lanes: 1 MW markers; 2 healthy sample; 3 semi-purified geminivirus sample; 4 purified geminivirus sample.

4.3 ELECTRON MICROSCOPY

4.3 a) Negative staining

Virus particles could not be identified with certainty from any of the extractions even though glutaraldehyde fixation was carried out.

In the infected preparations made from the first extraction of severely infected leaves from Rustenburg plants (modification of Osaki and Inouye, 1978) crystalline protein, which may be associated with the virus, was found. Similar structures could not be found in healthy preparations. However, these proteins were not found in any of the preparations made from subsequent extractions.

Plant protein was commonly viewed on EM grids treated with the second potyvirus extract, but potyviruses were also not found in these preparations.

4.3 b) Thin sectioning (plates 13 - 17)

Thin sectioning from infected plant material from Rustenburg with leaf enations embedded in LR White resin has shown some interesting results. Unfortunately, due to time limitation, sections from plants collected in Hazyview were not viewed under the EM.

Geminiviruses could not be identified with certainty in the nuclei of infected companion cells in the phloem tissue from enations, as the nucleoplasmic material was densely packed. However, small dense round particles of between 20 and 10nm in diameter which appear similar to

those described by Osaki and Inouye (1978) were observed in these preparations (plate 13). Similar particles were not observed in healthy preparations of phloem tissue.

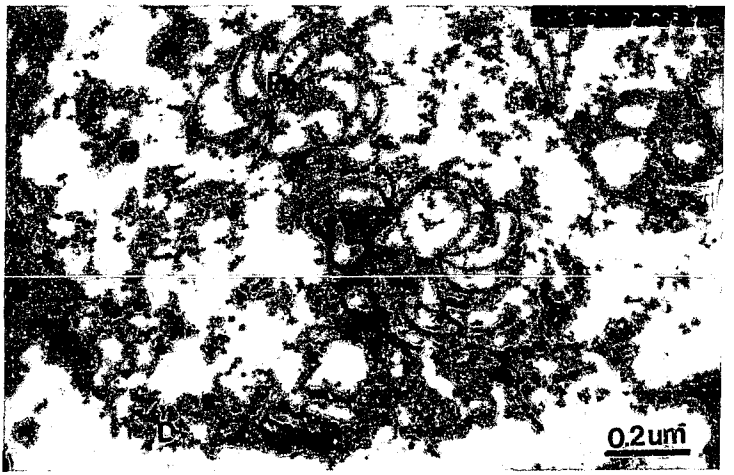


Plate 13. Potyvirus inclusion bodies (Pb) and small dense particles (D) in companion cell of phloem tissue from enations of plants with severe TLC disease. Magnification: 50 000

Fibrillar rings typically associated with geminivirus infections were also not found in these sections.

Chloroplasts in the phloem cells of enations were distorted and contained many starch grains, which could be an effect of the infection. The nuclear membranes were also broken down (plate 14), another possible effect of the virus infection.

Dense osmiophilic bodies were also identified in the phloem tissue of severely infected plants.

Potyvirus inclusion bodies of size 0.4 – 0.8 um (plates 13 to 15) and groups of potyvirus particles of about 600 nm in length (see plates 16 and 17) were found in the companion cells in the phloem parenchyma cells of enations from plants infected with severe leaf curl. Similar structures were not found in sections from plants infected with mild leaf curl, although further sectioning must be done.



Plate 14. Disintegrating nuclear membrane (NM) of companion cell from enation of infected plant. Potyvirus inclusion bodies (Pb) are also present. Magnification 5 000

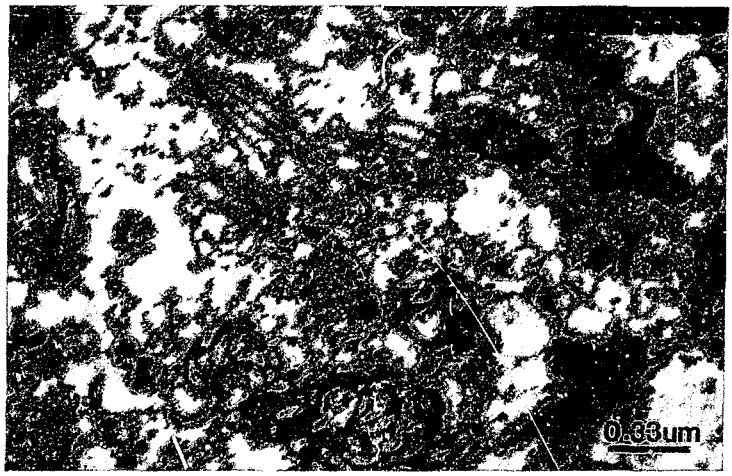


Plate 15. Potyvirus inclusion bodies in companion cell of phloem tissue from leaf with enations. Magnification 30 000



Plate 16. Potyvirus vircplasm (V) in companion cell of enation of plant infected with severe leaf curl. Magnification 40 000



Plate 17. Potyvirus viroplasm (V) in companion cell of enation of plant with severe TLC. Magnification 40 000

4.3 c) GLAD

No particles were observed using the GLAD technique from any of the extracted preparations from plants with either mild or severe symptoms.

4.3 d) ISEM

No virus particles were detected on the grids prepared using the technique of ISEM from plants with either severe or mild TLC symptoms.

4.4 DS DNA

4.4 a) ds DNA extraction

DNA obtained from either healthy or mildly TLC infected plant tissue was electrophoresed on a 0.9% agarose gel in the presence of ethidium bromide in order to detect discrete and possibly virus - related DNA species. As is evident in plate 18, preparations from infected plants contained a considerable amount of host DNA which migrated near the top of the well. A discrete band of DNA could be seen in the infected lanes (plate 18). However, a similar band was not found in extracts from plants infected with severe leaf curl or in healthy plants.

4.4 b) Recovery of DNA from agarose gels

About 70% of the extrachromosomal DNA was successfully recovered from bands contaminated with chromosomal DNA from the agarose gel using the freeze – squeeze method (plate 19) of Tautz and Renz (1983). This DNA was then transferred onto a NC membrane for hybridization studies.

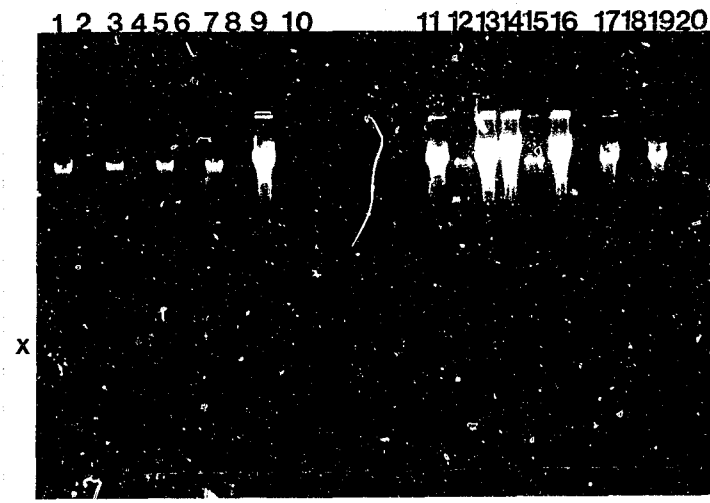


Plate 18. Electrophoresis of nucleic acids on a horizontal 0.9% agarose gel containing ethidium bromide. Lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 – dsDNA extracted from leaf tissue of healthy N. tabacum. Lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 – dsDNA extracted from leaf tissue of N. tabacum with mild TLC disease. An extra band (x) can be seen in lanes containing DNA from infected tissue.

4.4 c) Restriction analysis of extrachromosomal DNA associated with infected plants

No restrictions were obtained with any of the restriction enzymes recognizing six base pair sequences (ie. BamHI, BglI, BglII, EcoRI, MluII, PstI, PvuII, SacI and SalI). However, an uneven distribution of the DNA was obtained on the gels after the digestion period (see plate 20) and double digestions with BglII and PvuII, BglI and PstI, BglI and SalI and SalI and PstI were attempted to check that the DNA had not been cut only once by the enzymes. Once again, no restrictions were obtained.

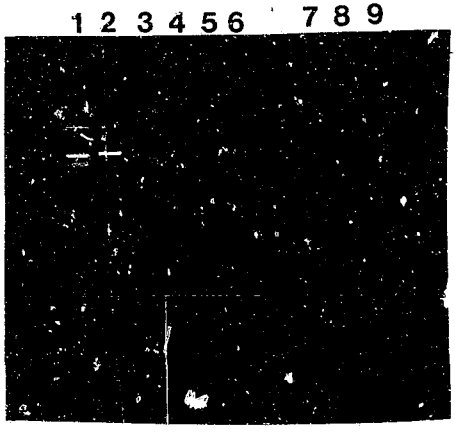


Plate 19. Electrophoresis of nucleic acids on a horizontal 0.9% agarose gel containing ethidium bromide. Lane 1: DNA MW markers II; Lanes 2 and 6: Plant chromosomal DNA (from healthy plant); Lanes 3 - 5: extrachromosomal DNA from infected plants; Lanes 7 - 10: DNA recovered from agarose gel using freeze squeeze method

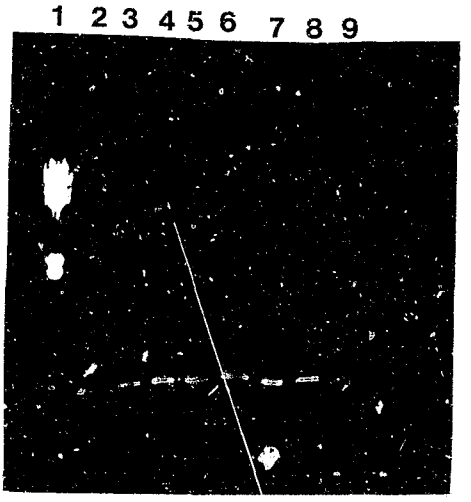


Plate 20. Electrophoresis of nucleic acids on a horizontal 0.7% agarose gel containing ethidium bromide. Lane 1: DNA MW II; Lane 2: Uncut DNA; Lane 3: BamH1 digestion; Lane 4: BglI digestion; Lane 5: BglII digestion; Lane 6: PstI digestion, Lane 7: PvuII digestion; Lane 8: SacI digestion; Lane 9: SalI digestion.

The uneven nature of the bands after digestion suggested an interference by buffer or enzyme and the double restrictions were repeated and, immediately after digestion, cleaned up with a phenol extraction. A 0.6% gel was run and the bands straightened, but again only a single band was observed (plate 21).

Since restrictions with enzymes recognizing six base pairs were unsuccessful, digestions were attempted with Sau3A and Hae III - two enzymes recognizing four base pair sequences. However, restriction was not obtained with either of these enzymes.

4.4 d) DNA characterization

4.4 d) i) Analysis of structure

After heating the extrachromosomal DNA from infected plants at 60 °C for 5 min and running it on a 0.7% agarose gel, only a single band was found in the same position as the unheated DNA.

4.4 d) ii) Sizing of DNA

Comparison to Boehringer Mannheim molecular weight markers III (plate 21) revealed the DNA species isolated from the infected plants to have a size of about 750bp (0.5 kd).

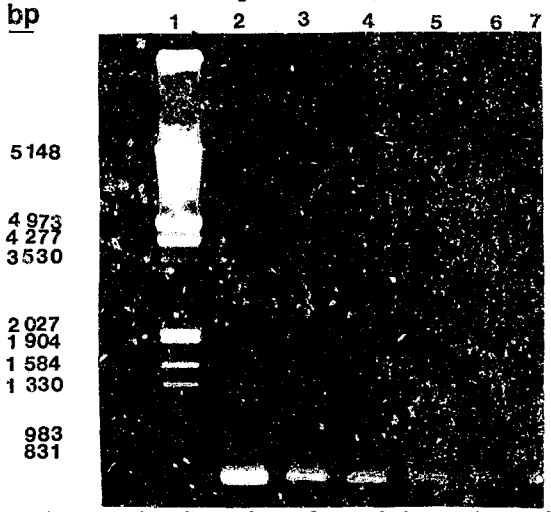


Plate 21. Electrophoresis of nucleic acids cleaned up with a phenol/chloroform extraction after a 3 h restriction on a horizontal 0.4% agarose gel containing ethidium bromide. Lane 1: DNA MW markers III; Lane 2:uncut DNA; lane 3: BglII digestion; Lane 4: PvuII digestion; Lane 5: BglII + PvuII double digestion; Lane 6: EcoRI digestion; Lane 7: MluII digestion.

4.4 d) iii) Thermal denaturation (see fig. 2)

Thermal denaturation of the extrachromosomal species of DNA extracted from the infected plant revealed double stranded characteristics when compared with ds DNA from lambda phage (fig. 2).

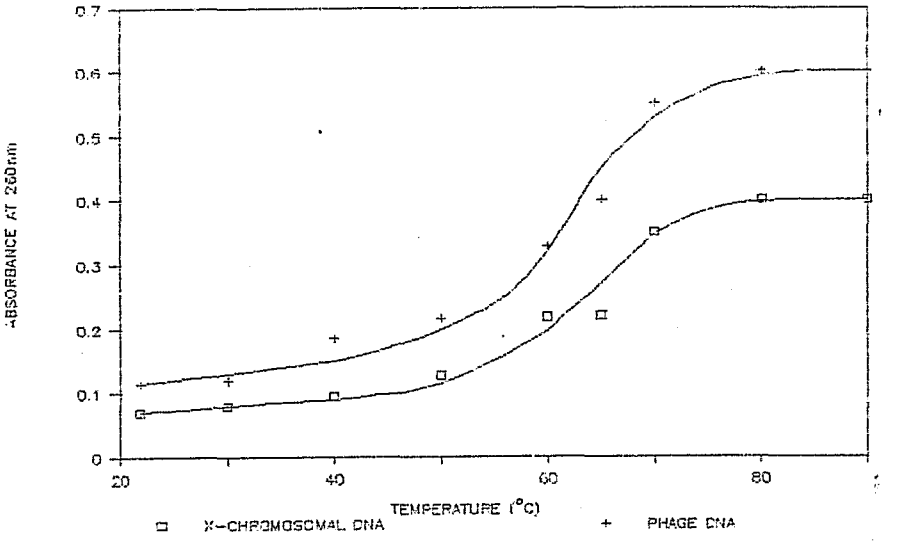


Fig. 2. Thermal denaturation kinetics of extrachromosomal DNA from infected plants (X-chromosomal DNA) and lambda phage DNA (phage DNA)

4.4 d) iv) Formaldehyde denaturation (fig. 3)

No conclusive evidence as to the nature of the DNA could be established from the denaturation with formaldehyde (fig. 3). While the DNA from lambda phage showed the expected increase in absorbance

after treatment with formaldehyde, the curve obtained before treatment with formaldehyde was not typical of DNA. The curve obtained with the extrachromosomal DNA from the plant infected with TLC was also not typical.

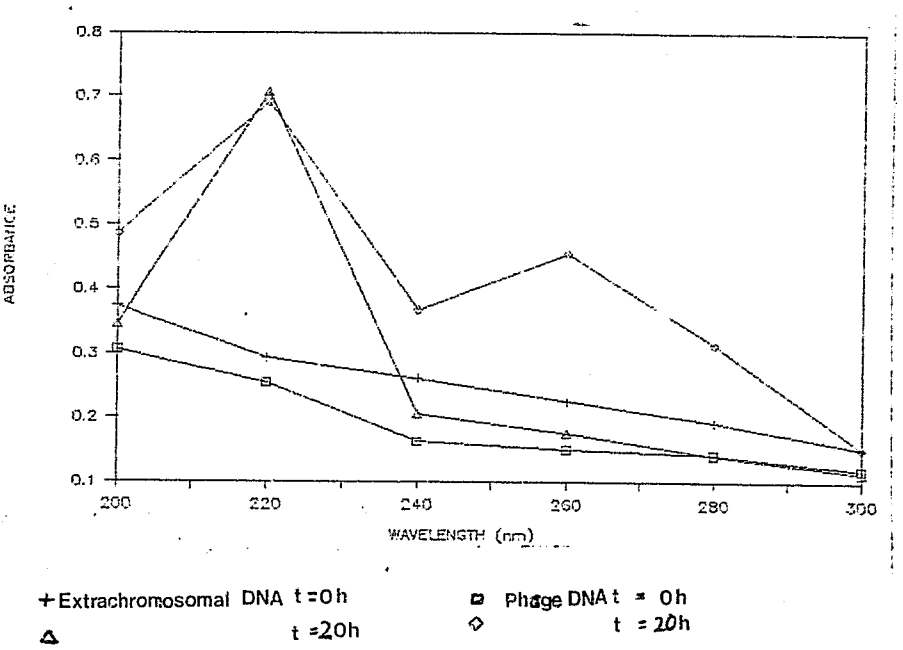


Fig. 3 Reaction of extrachromosomal DNA from infected plants and lambda phage DNA (phage DNA) to formaldehyde

4.4 d) v) Digestion of DNA with deoxyribonuclease (fig. 4)

The sensitivity of the extrachromosomal DNA from infected plants to DNase 1 when compared with that of lambda phage DNA also revealed double stranded character (fig. 4)

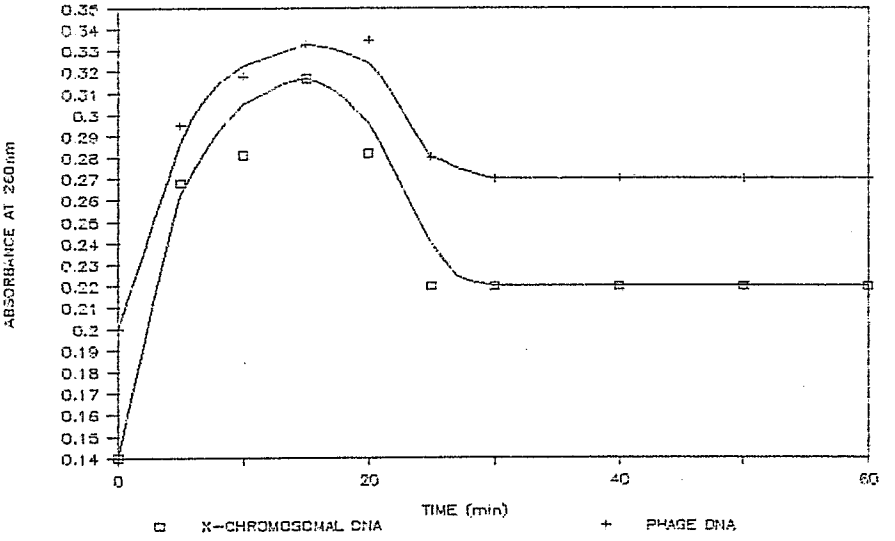


Fig. 4 Digestion of extrachromosomal nucleic acid from infected plants with DNase 1 compared to that of lambda phage DNA

4.4 d) vi) Digestion of DNA with nuclease S1 (fig. 5)

The extrachromosomal DNA was once again shown to have double stranded characteristics when treated with nuclease S1 and compared to DNA from lambda phage.

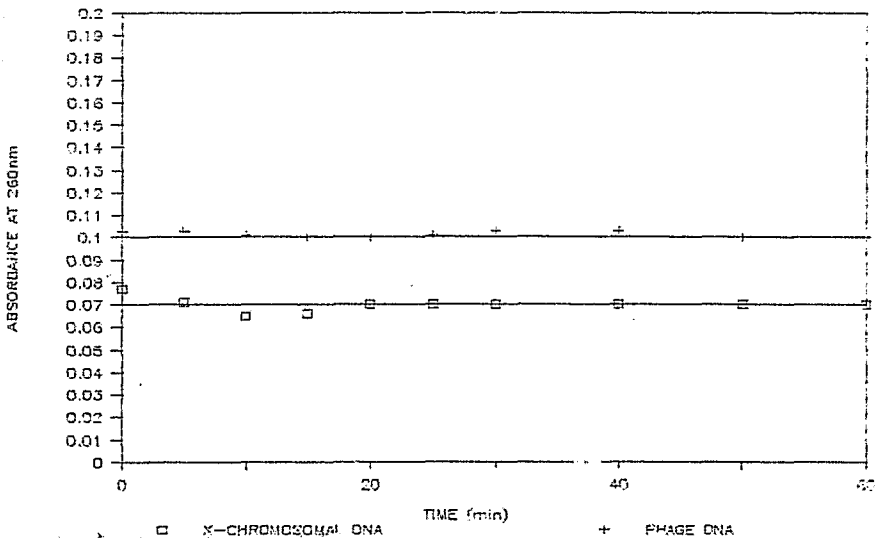


Fig. 5 Digestion of extrachromosomal DNA from infected plants with nuclease S1 compared to digestion of lambda phage DNA

4.4 e) Hybridization Analysis

4.4 e) i) Southern blot

Complete transfer of DNA was obtained from both gels run (plates 19 and 22) onto the nitrocellulose paper.

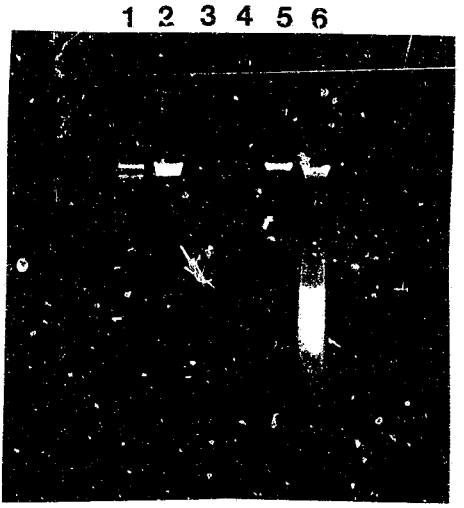


Plate 22. Electrophoresis of nucleic acids on a horizontal 0.8% gel containing ethidium bromide. Lane 1: MW markers III; Lane 2: MW markers II; Lanes 3 and 4: extrachromosomal DNA; Lane 5: chromosomal DNA from healthy plant; Lane 6: sugarcane mosaic virus DNA

The probe hybridized weakly to the DNA bands transferred from the first gel (plate 19) onto nitrocellulose by Southern blotting (plate 23).

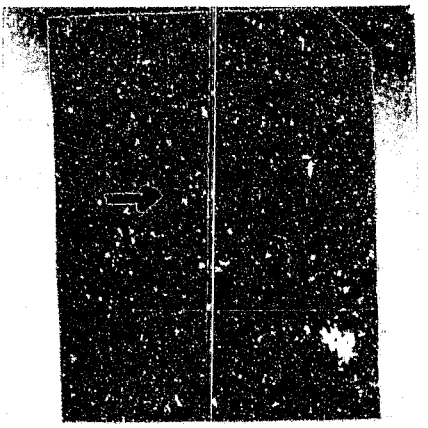


Plate 23. Southern blot 1: Hybridization of probe prepared from extrachromosomal DNA to the same DNA transferred from an agarose gel (plate 19) onto NC paper (arrow indicates point of binding)

Results obtained from the second Southern blot (plate 24), however, conflicted with those obtained with the previous Southern blot (plate 23). The probe bound strongly to the 2027 bp fragment of Boehringer MW markers II and III, very strongly to the chromosomal band obtained from the healthy DNA, and weakly to the chromosomal band of the sugarcane mosaic virus sample. The probe did not, however, bind to the extrachromosomal DNA extracted from the infected plant.

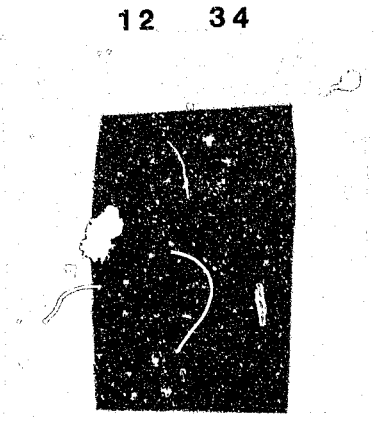


Plate 24. Southern blot 2: Hybridization of probe prepared from extrachromosomal DNA to: 1 and 2: 2027 bp fragment of DNA MW markers III and II; 3: chromosomal DNA from healthy plant; 4: chromosomal DNA from sugarcane

4.4 e) ii) Dot blot hybridisation analysis

The labelled DNA probe hybridized to all infected plant samples, both mild and severe, prepared from plants collected in Hazyview, but not to the healthy plant samples spotted onto nitrocellulose paper (plate 25).

Although the probe did not hybridize equally strongly to all infected samples, the lowest concentration detected was 1/100. The strength of detection was not related to the severity of the disease.

Since the blots were dirty and the reactions weak, the hybridisation was repeated.

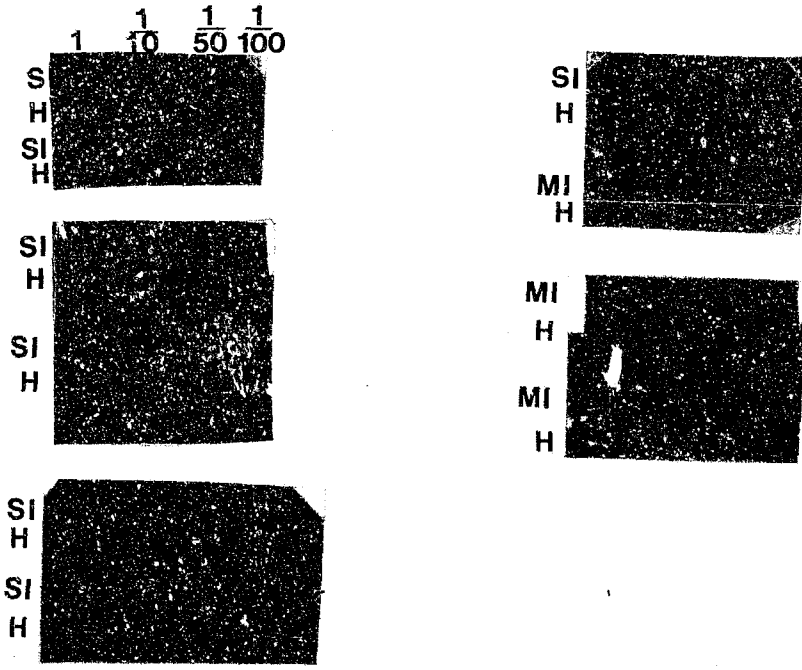


Plate 25. Dot blot hybridization showing specificity of probe prepared from extrachromosomal DNA associated with infected plant to crude isolates of infected plants (SI – severely infected plants; MI- mildly infected plants; H- healthy extracts). Each sample was tested undiluted (1) and at 1/10, 1/50, 1/100 dilutions

In the repeat of the experiment, hybridizations with the dot blots were again confirmed. The reactions of the probe against the samples were, however, still weak. Stronger binding of the probe was obtained when the samples from mild and severe forms were concentrated (plate 26). The dot blots showed equally strong hybridization for both severely and mildly infected plants. These blots also indicated strong binding of the probe to the control DNA from which the probe was prepared.

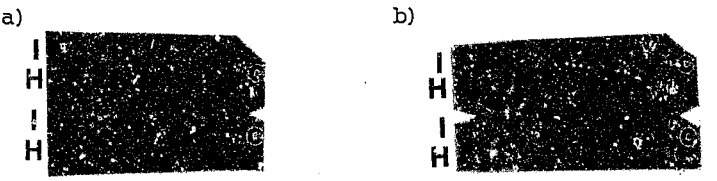


Plate 26. Dot blot hybridization showing stronger binding of probe with more concentrated infected samples [undiluted (1), 1/10 and 1/20]. a) I – extracts from severely infected plants, H – healthy extracts (concentrations of 1/10, 1/20 and 1/50) c – control DNA b) I– extracts from plants with mild TLC, H– healthy extracts (diluted as in a), c – control DNA

5 DISCUSSION

Several lines of evidence from the study reported here indicate the possible presence of a potyvirus in at least some of the strains of TLC in South Africa. These include:

1. The presence of potyvirus particles and inclusion bodies in thin sections made from enations of severely infected plants;

2. The reaction of both PVY antiserum and a general potyvirus antiserum with protein dot blots of severely infected plants in immunobinding assays.

The results presented in this study do not provide any evidence for or against the involvement of a geminivirus in TLC disease in South Africa. Although a geminivirus has not been isolated in association with the TLC syndrome in South Africa either by Thatcher (1976) or in this study, it has always been assumed that the disease in South Africa is caused by a geminivirus (Thatcher, 1976). This is supported by the whitefly transmission of the disease, the inability to transmit the disease mechanically and the discovery by Osaki and Inouye (1978) of a geminivirus as the causal agent of the TLC syndrome in Japan. This study, together with that of Thatcher (1976), however, presents little evidence at this stage for a geminivirus as the cause of TLC in South Africa. This proposal is supported by the failure to isolate geminivirus particles by any of the seven extraction procedures attempted and the failure to isolate the

double-stranded replicative form of geminivirus DNA from infected plants. However, geminivirus-like particles were observed in thin sections and the possible involvement of a geminivirus in the TLC syndrome cannot be excluded.

The development of early TLC symptoms on six of the ten plants treated with electrotransmission in this study was encouraging. Previously, this technique of virus transmission had only been proved to be successful in the transmission of MSV (Polson and von Wechmar, 1980) where 100% transmission was obtained, but only four plants were infected in the experiment reported by these workers. However, since the observations in this study could not be carried beyond six weeks the development of enations (which, according to McClean, 1940, is the only sure way of confirming TLC) could not be confirmed.

Death of the remaining four N. tabacum plants and the two N. benthamiana plants after electrotransmission could be ascribed either to the mechanical breakdown of the growth cabinet in which they were kept, and in which the temperature at one stage reached 40 °C, or to the electrotransmission treatment itself which almost always caused the plants to wilt within a few days after treatment.

The plants showing symptoms must still be further tested for the

presence of virus antigen.

Repeating this experiment would be useful as confirmation of the ability of this method to enable transmission of TLCV and optimization of this transmission system could be useful in large scale resistance breeding trials.

The failure in this study to achieve mechanical transmission of the infectious agent causing TLC disease confirms previous attempts by several workers, including Thatcher (1976), and Osaki and Inouye (1978) and could be explained in part by the same factors that make purification of phloem-limited viruses difficult and by the fact that the virus has to be introduced at a specific tissue in the plant. However, if the disease is caused wholly or in part by a potyvirus, the inability to transmit the virus mechanically is unusual even if the virus is phloem – limited.

The failure to isolate intact particles of either gemini- or potyviruses from infected plants using any of the techniques attempted could be attributed to several factors:

1. The low concentration of virus particles in the sap (Thatcher, 1976).

2. The limitation of the particles to phloem tissue, which is difficult to break down by conventional methods (Hull, 1985).

3. The instability of the virus particles in the sap once

isolated (Thatcher, 1976).

4. The possibility that no geminivirus particles are present in the infected plants.

An important factor in the isolation procedures attempted in this study is that the virus particles may have been lost during the final purification step. The results presented here indicate a drop in spectrophotometric readings at 260nm between the semi-purified and purified samples from most of the samples. However, this theory could not be confirmed as virus particles could not be identified in negatively stained preparations of either the semi-purified or the purified samples, although the concentration of virus particles in the semi-purified preparations would have been very low.

In most cases, extra nucleoprotein does appear to have been isolated from infected plants as the spectrophotometric readings from infected plant samples were almost always higher than those from healthy plant extractions.

The failure of extracted proteins to react positively with antiserum to ACMV in immunobinding assays performed in this study confirmed the results of Osaki and Inouye in 1981 and Sequeira and Harrison in 1982, who found that TLCV and ACMV were not serologically related. The positive reaction of PVY antiserum to protein dot blots made from the first potyvirus extraction carried out in this study was encouraging, as PVY is a potyvirus and this indicated the possible involvement of a potyvirus related to PVY in the TLC syndrome. This

assay was repeated with protein dot blots made from the second potyvirus extraction, and the results were confirmed.

The negative reaction of protein dot blots made from the third geminivirus extraction to PVY antiserum may possibly indicate that the potyvirus is only implicated in the severe leaf curl syndrome, as this extraction was done on leaves from plants with a mild form of TLC.

The failure of antiserum to PVY to react positively to the crude virus preparations from both healthy and infected plants indicated a possible cross-reaction between host plant protein and the antiserum. This could not be confirmed, however, as host-absorbed PVY antiserum also bound (although very weakly) to both healthy and infected samples.

The weak reaction of a general potyvirus antiserum obtained from Dr. J. Hammond (John Innes Research Institute, Norwich) to four of the seven crude samples prepared from severely infected plants and one of the three crude samples from plants with a mild form of TLC indicated the possible involvement of a potyvirus in both the mild and the severe forms of TLC infection in South Africa. Another possible explanation for the reaction of the mildly infected plant with the potyvirus antiserum could be that the plant had not yet developed the enations characteristic of the severe form of TLC, as it is very difficult, at the early stages of TLC disease, to distinguish between severe and mild forms (McClean, 1940). The antiserum was diluted to a very low concentration (1:5000) and this may have contributed to the

weak reaction obtained.

Unfortunately, there was insufficient time to repeat the dot blot immunobinding assay with the general potyvirus antiserum.

GLAD and ISEM were carried out using the first batch of general potyvirus antiserum from Agdia which was concluded to be faulty after it failed completely to react with protein dot blots and gave widely fluctuating results within the same samples in a well plate ELISA. This could also explain the absence of gold particles in the GLAD sections and of virus particles prepared by ISEM on grids examined in the EM.

No extra polypeptide bands corresponding exactly to the molecular weight of either a gemini- or a potyvirus were found on the polyacrylamide gels run in this study.

The extra polypeptide bands that were found on the polyacrylamide gels could represent one of several different proteins:

1) The extra proteins could be proteins induced by the infection. Cytoplasmic pinwheel inclusion bodies are typically of molecular weight 65 - 75kd (Dougherty and Carrington, 1988). The 59kd polypeptide band could correspond to one of

these inclusion bodies.

2) The 23kd and the 26kd bands could correspond to potyvirus proteins which, since they are composed of fairly long (700nm -900nm) flexuous particles, could have broken down during extraction to yield particles smaller in size than the expected 30 - 45kd proteins (Dougherty and Carrington, 1988). The bands noted in infected fractions running faster than the tracking dye could correspond to smaller fractions of the particles.

3) Alternatively, the 26kd band could represent the polypeptide coat of a geminivirus as the protein coats of most geminiviruses so far isolated have molecular weights of about 28kd (Hamilton et al, 1983). The molecular weight of the protein coat of the geminivirus isolated from plants infected with TLC in Japan has not yet been ascertained.

4) Finally, the bands at 23kd and 26kd could represent the same polypeptide species (this is particularly likely since the 26kd band was found on a 10% running gel, which was later found to give incomplete separation of proteins) and this band could be the protein coat of a small virus-like particle.

While results in this study indicate that the geminivirus isolated by Osaki and Inouye in 1978 as the causal agent of TLC disease in Japan may not be the infectious agent of the syndrome in South Africa, if indeed a geminivirus is implicated in the infection, then the reasons described above for the failure to isolate virus particles could also explain the inability to visualize particles in the EM using negative staining, ISEM and GLAD. However, a further problem with the technique of ISEM is that small subunit proteins or degraded capsids are not detectable, due both to their size and their lack of a characteristic shape. Excess free coat protein in the sap or purified preparations may also inhibit the trapping and / or "decoration" of virus particles on an EM grid (Rybicki and von Wechmar, 1985).

Micrographs taken by Osaki and Inouye (1978) of geminivirus particles negatively stained without fixation in glutaraldehyde did, however, show particles similar to some found in preparations made in this study. The particles may have broken down prior to fixation and staining, as the methods took between two and four days for particle isolation and purification and the semi-purified samples could usually only be fixed and stained at least two days after starting the extraction procedure.

Initially, the plant protein found in negatively stained preparations from potyvirus extractions were believed to be disintegrated potyvirus particles, but it was later found that these particles were too broad and not flexucus enough to be potyvirus particles.

The identification in this study of the pinwheel inclusion bodies

characteristic of potyvirus infections, and of viroplasms containing potyviruses in thin sections of the phloem tissue from enations of severely infected plants is the first indication that a virus of any kind may be associated with tobacco leaf curl disease in South Africa, since previous workers (Thatcher, 1978) have not established the presence of any causal agents for the disease. The potyvirus may be associated with another infectious agent in a dual infection to produce at least one of the five different forms of TLC in South Africa. Several workers have suggested that mixtures of virus strains may cause the different forms of TLC (Nariani, 1968; Pal and Tandon, 1937; Vasudeva and Raj, 1948; Yassin and Nour, 1965), but the evidence presented in this study is the first indication that another virus may indeed be involved in the TLC syndrome.

Since a novel dsDNA of 750 bp was isolated from infected plants and not from healthy plants, and hybridized only to infected plants in experiments performed during this study, it is suggested that another small DNA virus is involved in TLC disease in South Africa, but whether this dsDNA is associated with a geminivirus is still to be ascertained.

Mixed infections by plant viruses are very common but have been little studied (Matthews, 1981). Carr and Kim (1985) indicate that ultrastructural features in mixedly infected cells may be useful in discriminating related viruses or strains of the same virus. Carr and Kim (1985) working with mixed infections of bean leaves with BGMV and TMV found that BGMV in a mixed infection lost its tissue-specificity and was found in all cells of the plant, not just in the phloem.

In the research described here very few sections were investigated from plants infected with milder forms of TLC, and thus it cannot be stated with certainty that potyvirus particles are not implicated in other forms of tobacco leaf curl as well. However, as explained previously in this section, the results presented here of the reactions of protein dot blots with the general potyvirus antiserum from Agdia indicate the possibility that a potyvirus is involved with at least some occurrences of severe TLC and may be implicated in mild infections as well.

The problem of the viral nature of the disease is further exacerbated by the fact that no potyvirus particles were isolated by either extraction method. However, the presence of virus protein and potyvirus inclusion bodies in sections of enation tissue strongly suggests that a potyvirus may be associated with the TLC disease complex.

Further research into the causal agent(s) of the various strains of TLC disease is complicated by the difficulty in distinguishing between the milder forms of TLC disease and the virtual impossibility of identifying the latent form of TLC disease.

The failure to find geminivirus particles or structures such as fibrillar rings normally associated with geminivirus infections in thin sections could be attributed to the limited amount of time available for this study, which permitted only a small number of sections to be cut and examined. Thatcher (1976) was also unable to

find any particles or inclusion bodies in thin sections made from infected plants, supporting the proposal here that a geminivirus is not involved in the TLC syndrome in South Africa.

The possibility that the disease in South Africa is only caused by potyviruses has been considered but is unlikely since, unlike geminiviruses, potyviruses are not generally known to be whitefly transmitted. Furthermore, the inability to transmit the disease mechanically and the nature of the nucleic acid particle extracted from diseased plants in this study is not consistent with the characteristics of a disease caused solely by potyviruses.

Initially it was believed that the extrachromosomal band of DNA in infected samples found on the agarose gel on which DNA extracted from infected and healthy plants was run was the dsDNA of the geminivirus DNA. Initial estimates of size (based on Boehringer Mannheim's DNA molecular weight markers II) were about 2200 bp, which is in the 2000 - 3000 bp size range of the DNA so far isolated from geminiviruses. However, the inability to separate the DNA into two separate parts (to illustrate the bipartite nature of the genome associated with all whitefly borne viruses investigated so far, although not, as yet, with the genome of TLCV isolated by Ikegami and colleagues in 1987), the failure to restrict the DNA using restriction enzymes previously found to digest the DNA of other geminiviruses and the further investigation and comparison of this extrachromosomal DNA isolated from infected plants with Boehringer Mannheim molecular weight markers III revealing that the DNA consisted of only 750 bp (0.5 kd), indicated that this conclusion

was untimely.

The failure to isolate this same extrachromosomal DNA species from a second plant showing severe disease symptoms could indicate that the DNA was not in a replicative form at the time of isolation. This factor could also explain the failure to isolate a viral genome if a geminivirus is associated with the TLC syndrome, as the extraction was specific for ds replicative DNA.

Alternatively, the extrachromosomal species of DNA may have occurred only in very low concentrations in the severely infected plant as the probe hybridized to all infected plant samples.

The failure to restrict this DNA species using various restriction enzymes could be attributed to the fact that, since the DNA was so small (only 750 bp in size), and enzymes recognizing six base pair sequences will, on average, cut at only one out of every 4096 base pairs, no restriction sites could be found. However, an enzyme recognizing a four base pair sequence would cut at an average of one out of every 256 nucleotides and it is less likely that a recognition site would not have been found using Hae III or Sau3A. As the sequence of the DNA was not known, the ratio of GC:AT pairs could not be determined and appropriate enzymes could not be chosen for DNA restriction. Further work is being carried out in this regard.

Another possibility is that the DNA was in some way inappropriate for restriction, eg. phenol may have remained in the preparation from the extraction, or the restriction sites may have been modified (by, eg.

methylation or adenylation) preventing the enzymes from working on the recognition sites.

A further explanation for the lack of restriction could have been that the extracted DNA was single-stranded. However, thermal denaturation and digestion of the DNA with DNase and nuclease S1 showed, firstly, that the nucleic acid was DNA and not RNA and, secondly, that the DNA was double-stranded. Furthermore, the DNA was labelled by nick translation using enzymes which will only work on dsDNA.

The abnormal behaviour of the DNA during formaldehyde treatment could indicate that the DNA has a complicated tertiary structure which at this point is unknown and further experiments must be performed to verify the structure of the DNA.

Hybridization of the probe made from the extrachromosomal DNA isolated from the infected plant only to the infected samples and not to the healthy samples suggests that this DNA species is associated with the disease etiology. The reactions with plant chromosomal DNA and molecular weight markers in the second Southern blot indicate that a few sequences found in this novel dsDNA may be [illegible]on with the chromosomal DNA or that more stringent washing may [illegible]ecessary to reduce non-specific binding reactions when more strongly labelled probes are used.

Since the extrachromosomal DNA species is so small, it may have been destroyed by nucleases or been denatured after transfer to the NC paper in the second Southern blotting experiment, explaining the lack

of binding to this DNA by the probe. Another explanation for the lack of hybridization could be that the extrachromosomal DNA only bound weakly to the NC paper and was removed during washing.

However, the dot blot hybridization and the results of the first Southern blot do indicate that the DNA does appear to be associated with the disease and could be a virus-specific DNA species, possibly a replication intermediate, as described by Haber and co-workers in 1981. However, sequencing of the extrachromosomal DNA would be necessary to confirm this. Hamilton and co-workers (1982) described several novel species of virus-specific single- and double-stranded DNA isolated from cells infected with TGMV. The double-stranded forms were not found in DNA preparations obtained from purified virus and this suggested that these were replicative intermediates which exist in a free state in infected cells. However, these dsDNA species were all larger than the extrachromosomal DNA found in this study.

Alternatively, the extrachromosomal DNA isolated from infected plants in this study could represent a section of the virus genome which, through a faulty replication mechanism in the virus genome, is the only region of the genome replicating. However, this is unlikely as some normal non-mutant DNA should still be found in addition to the smaller piece of DNA.

Robinson and colleagues in 1984 suggested a similar theory for the previous inability to detect particles of an Angolan isolate of ACMV. They postulated the existence of a novel kind of isolate of geminiviruses which induces the production of substantial amounts of

viral DNA but apparently no virus particles and very little virus particle protein. Alternatively, the isolates may be defective for particle production in a similar manner to NM isolates of tobacco rattle virus (Robinson et al, 1984) and strain PM2 of tobacco mosaic virus (Siegel et al, 1962). The DNA in itself could maintain the symptoms of the infection (possibly in association with another virus, such as the potyvirus found in this study) while explaining the inability to isolate or visualize geminivirus particles.

In 1976, Thatcher investigated the possibility of a viroid causal agent of TLC. While he could find no evidence to exclude a proteinless virus as the causal agent of TLC disease, the properties of the causal agent are markedly different to those of known viroid and proteinless virus infections, all of which are mechanical transmissible after phenol extractions (which Thatcher was unable to achieve in 1976) and none of which is transmitted by a species of whitefly. Thatcher's findings were supported by this study in which it was established that the size of the isolated extrachromosomal DNA (750 bp) is too large to be a viroid or virusoid which, in any case, have thus far only been found to contain RNA.

The DNA could also represent part of a multipartite virus complex but there is no evidence for this at present.

If the extrachromosomal dsDNA associated with infected plants is not a subgenomic fragment of a geminivirus genome, it may have a "satellite virus-like" function in association with a helper virus such as the potyvirus found in this study. The nature of the nucleic

acid (dsDNA) precludes the possibility that a satellite virus such as those described thus far is implicated in the TLC infection since these all contain ssRNA. The size of the molecule (MW 0.5 X 10^6) is in the region of the MW of satellite viruses (0.08 - 0.6 X 10^6) ((Murant and Mayo, 1982). However, further study is needed to clarify the role of the extrachromosomal DNA isolated from plants infected with TLC disease.

6 SUMMARY

1. Several lines of evidence have indicated the involvement of a potyvirus in at least the severe form of tobacco leaf curl disease. These include:

1) The finding of pinwheel inclusion bodies and viroplasms of potyviruses in thin sections made from enations of severely infected plants;

2) The reaction of both PVY antiserum and a general potyvirus antiserum with protein dot blots of severely infected plants in immunobinding assays.

2. A novel extrachromosomal dsDNA species of 840 bp in size isolated from infected plants has been found to hybridize only to crude DNA extracts from plants infected with both the severe and the mild form of TLC and not to healthy plant extracts. This dsDNA may be associated with the TLC disease complex in South Africa, but whether this novel dsDNA is associated with a geminivirus must still be ascertained. The novel dsDNA may, in fact, indicate the possible involvement of another small DNA virus (as yet undescribed) in the TLC disease syndrome.

3. The possibility of a dual infection by a potyvirus and another unknown virus species in at least some of the forms of TLC disease is proposed from the results presented in this study.

The results presented in this study, as well as those of Thatcher (1976) do not show that a geminivirus is implicated in the TLC syndrome in South Africa, despite the whitefly transmission of the disease agent, the lack of mechanical transmission and the geminivirus particles extracted from TLC infected plants in Japan by Osaki and Inouye (1978). However, further studies must be done to confirm the nature of the disease agent, including the investigation of more infected plants with EM and the purification of geminiviruses and / or potyviruses and / or other viruses from infected tobacco and other indicator plants.

4. Results obtained from electrotransmission experiments are promising and analysis of the plants showing symptoms must be performed. Further investigations into the electrotransmission of TLC must be executed in order to extrapolate and confirm the results obtained so far in this study. Other methods of transmission of the virus must still be attempted, since time ran out in this study.

5. A dsDNA species associated with the infection has been isolated. However, its role has not yet been identified and further studies must be done to determine whether the DNA is circular or linear and whether it is related to the disease. The extrachromosomal dsDNA extracted from infected plants needs to be cloned, sequenced and its ability to infect plants with the TLC syndrome needs to be tested before any further conclusions can be made as to its origin and nature.

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8 APPENDICES

APPENDIX 1

LONG ASHTON MEDIUM

A number of stock solutions were made up at the following concentrations. The stock solutions were made up 100 times more concentrated.

Solution 1	$g.l^{-1}$
$MgSO_4.7H_2O$	36.9
$MnSO_4$	0.223
$CuSO_4.5H_2O$	0.024
$ZnSO_4.7H_2O$	0.0296
H_3BO_3	0.186
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.00352
$CoSO_4.7H_2O$	0.0028
NaCl	0.585

Solution 2	
KNO_3	101.108

Solution 3	
$Ca(NO_3)_2.4H_2O$	116.88

Solution 4

FeEDTA	6.0

Solution 5

$NaH_2PO_4.2H_2O$	20.8

Solution 6

$K_2HPO_4.3H_2O$	57.056

APPENDIX 2

Transmission extraction buffers

1. Mechanical inoculation and injection

0.1M Tris pH 8 containing 0.01M NaEDTA

2. Electrotransmission

a) 0.1M Tris pH 8 containing 0.1M NaEDTA and 0.05% sodium sulphite

b) 0.1M Tris pH 8 containing 0.1M NaEDTA

APPENDIX 3

Extraction buffers

Geminivirus extractions

1. Extraction 1 and 6 (Osaki and Inouye, 1978: modification - extraction 1, unmodified - extraction 6)

0,2M borate buffer: 500ml glass dH_2O was added to 6.183g Boric acid
500ml glass dH_2O was added to 19.06g Borax
($Na_2B_4O_7.10H_2O$.
The Borax solution was added to the Boric acid solution till a pH of 8.5 was reached.

0.1% mercaptoethanol was added to the buffer before using.

2. Extraction 3 (Adaptation of Sequeira and Harrison, 1982)

First extraction using this procedure:

0.1M Tris pH8 containing 0.01M NaEDTA, 0.01M NaDiECA, 0.001M mercaptobenzothiazole, 0.5% Na sulphite, 0.001M trypsin inhibitor and 1% mercaptoethanol dissolved in a few drops of ethanol.

Second extraction using this procedure:

0.1M Tris pH8 containing 0.1M NaEDTA, 0.01M NaDiECA, 0.2% Na sulphite and 0.01M ascorbate.

3. Extraction 4 (modification of Dollet et al, 1986)

0.1M Tris pH8 containing 0.01M NaEDTA, 0.01M NaDiECA and 0.02M Na sulphite.

4. Extraction 5 (according to Stein et al, 1983)

0.1M trisodium citrate containing 0.005M NaEDTA, 0.75% Na sulphite, 1% B-mercaptoethanol and 0.325% L-ascorbic acid was adjusted to pH7.5 with NaOH.

Potyvirus extractions

1. Extraction 1 (Jafarpour et al, 1979)

0.5M potassium phosphate buffer pH 7.2 was prepared by adding 13.6g KH_2PO_4 to 200ml glass dH_2O and 17.52g K_2HPO_4 to 200ml glass dH_2O. The two solutions were mixed, the volume measured and 0.5M urea, 0.05M NaEDTA and 1% sodium sulphite were added before adjusting

the pH of the solution to 7.2. 0.5% B-mercaptoethanol dissolved in a few drops of ethanol was then added to the solution.

2. Extraction 2 (according to Zee et al, 1987)

0.5M Tris-HCl pH8.2 was prepared and to this was added 4% water-insoluble polyvinyl pyrrolidine (PVP), 0.5% bentonite, 0.2% mercaptoethanol and 5% Triton-X100 as well as 1.125% pectinase (Sigma) and 0.375% Onozuka cellulase R-10.

APPENDIX 4

Other buffers used in extractions

1. 0.01M potassium phosphate buffer pH 7.4

500ml glass dH 0 was added to 0.88g K_2HPO_4
500ml glass dH 0 was added to 0.68g KH_2PO_4

When dissolved, the KH_2PO_4 solution was added to the K_2HPO_4 solution until a pH of 7.4 was reached.

2. 0.1M potassium phosphate buffer pH 7.8

200ml glass dH 0 was added to 3.5g K_2HPO_4
200ml glass dH 0 was added to 2.72g KH_2PO_4

When dissolved, the KH_2PO_4 solution was added to the K_2HPO_4 solution until a pH of 7.8 was reached.

3. 0.025M potassium phosphate buffer pH 7.2

500ml glass dH 0 was added to 1.7g KH_2PO_4
500ml glass dH o was added to 2.19g K_2HPO_4

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

4. TE buffer pH 8

0.01M Na EDTA was added to 0.01M Tris pH 8.

5. CEM buffer

0.01M trisodium citrate and 0.001M NaEDTA were dissolved in glass dH$_2$O and the pH adjusted to 7.5 with NaOH before adding 0.05% B-mercaptoethanol.

APPENDIX 5

Reverse concentration PEG solubility gradient

40% and 10% sucrose solutions were prepared in 0.1M potassium phosphate buffer pH 7.8.

PEG discontinuous reverse solubility gradients were prepared by layering 7ml of 40% sucrose at the bottom of a cellulose centrifuge tube. Another 7ml of 10% sucrose containing 4% PEG and 0.2M NaCl was layered on top of the 40% sucrose solution.

APPENDIX 6

Buffers and solutions used in ELISAs

1 buffered saline (PBS)

KH_2PO_4, 2.9g $Na_2HPO_4.12H_2O$ and 0.2g KCl were added
The pH was then adjusted to 7.4.

2. PBS-Tween

0.05% tween 20 was added to the PBS.

3. AB buffer

0.2% BSA and 2% PVP were added to PBS.

4. AP buffer

0.1M Tris, 0.1M NaCl and 0.005M $MgCl_2$ were solubilized in glass dH_2O and the pH adjusted to 9.5.

5. ELISA substrate solution

1mg nitrotetrazolium blue was dissolved in 3ml AP buffer by mixing vigorously for 1 - 2 min. The solution was then centrifuged for 5 min at 10000 rpm.

0.5mg 5-bromo-4-chloro-3-indoyl phosphate-p-toluidine was dissolved in 10ul N,N-dimethyl formamide by mixing with a vortex for 1 min. This solution was then added dropwise to the nitrotetrazolium blue solution.

APPENDIX 7

Host adsorption of antisera

Healthy tissue (10g for 200ul antiserum) was homogenized in PBS (Appendix 5) at a ratio of 1 : 1 (v : weight). The homogenate was stirred for 30 min before centrifuging at 5000 rpm for 15 min. The supernatant was then concentrated by adding an equal volume of saturated ammonium sulphate. The mixture was left to stand on ice for 60 min. The mixture was then centrifuged at 10000 rpm for 10 min. The pellet was resuspended in 1 ml PBS. The suspension was dialyzed overnight against PBS at 4°C. 1ml of the protein was added to 0.2ml of the antiserum in an Eppendorf and the mixture was incubated at 37°C for 1h, before leaving to stand overnight at 4°C. The solution was then centrifuged at 10000 rpm for 20 min and the supernatant retained as cross-adsorbed antiserum to be used in ELISAs.

APPENDIX 8

SDS - Polacrylamide gel electrophoresis (SDS-PAGE)

Solutions

1. 30% Acrylamide stock

30g acrylamide and 0.8g bis-acrylamide (methylene di-acrylamide) were dissolved in 100ml ddH$_2$O and the solution was then filtered through Whatman No. 1 filter paper. The solution was stored in a dark bottle at 4^{c}C.

2. 10% SDS

10g SDS was added to 100ml ddH$_2$O.

3. 1.5M Tris pH 8.8

18.2g Tris was added to 100ml ddH$_2$O and the pH adjusted to 8.8 with HCl.

4. 0.5M Tris pH 6.8

7.9g Tris-HCl was dissolved in 100ml ddH$_2$O and the pH adjusted to 6.8 with HCl.

5. Splitting solution

0.1M Tris-HCl pH 6.8, 2.5% SDS, 5% 2-mercaptoethanol and 5% glycerol were mixed together. 0.5mg bromophenol blue was added per 9ml of this solution.

6. Electrode buffer

14.4g glycine, 16.5ml 1.5M Tris pH8.8 and 10ml 10% SDS were added together and made up to 1l in dH_2O.

Making of gels

A 10% or 12.5% running gel (as described below) was poured between two glass gel plates and allowed to polymerize for 1h at 4 °C. A 5% stacking gel was then poured on top of the running gel and allowed to polymerize before loading and running the gel as described below.

1. 5% stacking gel

1.8ml 30% acrylamide stock, 2.5ml 0.5M Tris-HCl pH 6.8, 0.1ml 10% SDS and 5.4ml dH_2O were mixed together. Just before pouring the gel, 10% ammonium persulphate and 12ul TEMED were added.

2. 10% or 12.5% running gel

Quantities added for the different concentrations were the same, except where indicated in brackets.

8.4ml 30% acrylamide stock (6.67ml for 10% gel), 5ml 1.5M Tris pH8.8, 0.2ml 10% SDS and 6ml dH_2O (7.74ml for 10% gel) were added together. Shortly before pouring the gel, 0.4ml 10% ammonium persulphate and 15ul TEMED were added.

Loading and running the gel

10ul MW markers and 10ul splitting solution were loaded in one well. Wells loaded with samples contained 15ul sample and 10ul splitting solution. The samples with splitting solution were boiled for 5 – 8 min before being loaded onto the gels.

The gels were run in vertical apparatuses with electrode buffer at 40mA for about 4h.

Weights of MW markers

sucrose	14.4kb
L-lactalbumin	20.1kb
soybean trypsin inhibitor	30.0kb
carbolic anhydrase	43.0kb
ovalbumin	67.0kb
BSA	94.0kb

APPENDIX 9

Staining gels

1. Coomassie Blue staining

Gels were fixed and stained with coomassie brilliant blue (0.1% Coomassie Brilliant Blue: GCI 42655) in methanol : acetic acid : water, 5 :1 :4 (v/v/v) overnight with gentle rotation at 25 ^{o}C. The gels were destianed in methanol : acetic acid: water, 1 : 1 : 8 (v/v/v), with several changes and stored in distilled water at 4 ^{0}C.

2. Amersham silver staining kit

After electrophoresis, the gels were prefixed for at least 1h in a solution containing 25% propan-2-ol and 10% acetic acid in dH_2O.

All other solutions were supplied in the kit and components were not described.

The gels were then fixed overnight in fixative prepared according to instructions in the kit. The fixing solution was removed and the gel soaked with gentle shaking in dH_2O for 1h. The dH_2O was removed and 300ml of prepared sensitizing solution was added and the gel soaked in this for 30min with gentle shaking. The sensitizing

solution was replaced with prepared stain solution and agitated for 10min. The stain solution was removed and the gel rinsed twice for 1min each in dH_2O. Prepared developer was then added and the gel soaked with gentle agitation for 5 – 15min until bands of the desired intensity were found. The reaction was then stopped by the addition of 5ml of the supplied stopper concentrate and the gel agitated vigorously for 5min.

3. Combined Coomassie Blue and Amersham silver staining

After destaining of a gel stained with Coomassie Blue, the gel was stained with the Amersham silver staining kit as described above from the point at which the gel is soaked in dH_2O for 1h.

APPENDIX 10

Electron microscopy

1. Colloidon – carbon coating of grids

Copper, nickel or gold grids were spread shiny side up on a circle of filter paper. 0.15% butvar in chloroform was dropped over the grids with a Pasteur pipette. The grids were allowed to dry. The surface of a bowl of ddH_2O was cleaned with a small drop of 2% colloidon. A fresh drop of colloidon was added to the surface of the water and the

grids floated (shiny side down) on the film. A piece of Whatman 3MM filter paper was placed on top of the grids, allowed to get wet, and then quickly picked up so that the grids remained on the filter paper. The grids were left to dry overnight before being coated with a thin layer of carbon by workers in the EM unit of the University of the Witwatersrand.

2. Lead citrate stain (as practised at Dublin University)

The following three stock solutions were prepared and kept at 4°C.

Solution A: 37.7g trisodium citrate was added to 100ml dH_2O.
Solution B: 33.1g lead nitrate was added to 100ml dH_2O.
Solution C: 1N NaOH was prepared.

The final staining solution was prepared by mixing the solutions in 16ml dH_2O as follows:
3ml solution A added and stirred 2ml of solution B added and stirred until the mixture became milky and precipitated. 4ml of solution C was then added before passing the solution through a millipore filter.

3. Beasley Buffer

1% BSA, 0.1% gelatin and 0.05% Tween 20 were added to 0.05M Tris pH 7.4.

APPENDIX 11

dsDNA extraction

1. Grinding buffer

0.1M NaCl, 0.1M NaEDTA and 1% SDS were added to 0.1M Tris-HCl pH 8.

2. RNase

A stock solution of 10 mg / ml RNase was prepared in dH_2O. This was diluted as required.

3. Proteinase K

A stock solution of 10 mg / ml proteinase K was prepared in dH_2O. This was diluted as required.

4. Phenol / chloroform extraction

An approximately equal volume of phenol / chloroform (8 : 2 v / v - phenol TE saturated) was added to the extract. This was shaken well and then centrifuged at 5000 rpm for 5 min. The upper phase was retained and the procedure repeated once more.

5. TE buffer

0.1M Tris pH 8 containing 0.1M NaEDTA.

APPENDIX 12

Agarose gels

1. TE buffer

0.01M Tris pH 8 containing 0.001M NaEDTA.

2. TBE buffer

A 10X stock solution of TBE buffer was prepared as follows:

108g Tris, 55g boric acid and 9.3g NaEDTA were added to 1l of dH_2O. The solution was autoclaved before use. Dilutions were prepared in sterile dH_2O.

3. TAE buffer

A 5X solution of TAE buffer was prepared.

0.04M Tris, 0.02M sodium acetate and 0.001M NaEDTA were mixed together and the pH adjusted to 8 by adding acetic acid.

4. Preparing gels

0.36g, 0.28g or 0.16g "Seakem" agarose was dissolved by microwaving in 40ml of the appropriate buffer (giving 0.9%, 0.7% and 0.4% agarose gels respectively.

Once the mixture had cooled slightly, 1ug ethidium bromide / 20ml agarose solution was added. (Ethidium bromide was prepared as a stock solution of 10mg / ml in TE buffer). A mini-gel apparatus was prepared and 40ml agarose was poured per apparatus. Gels were left to polymerize at 4°C for at least two hours before loading.

Running buffer was the same as was used to make the gel (ie. TE, TBE or TAE).

5. Tracking dye

2% bromophenol blue was dissolved in 40% RNase - free sucrose prepared in sterile TE buffer.

6. Molecular weights of markers

a) Lambda cut Hind III MW markers (Boehringer Mannheim MW markers II)

Fragment lengths (base pairs): 23130, 9416, 6557, 4361, 2322, 2027, 564, 125.

b) Lambda cut EcoRI + Hind III MW markers (Boehringer Mannheim MW markers III)

Fragment lengths (base pairs): 21226, 5148, 4973, 4277, 3530, 2027, 1904, 1584, 1330, 983, 831, 564, 125.

APPENDIX 13

Cleaning up DNA

TE was added to the restriction digest to a volume of 25ul, then 7.5ul TE-saturated phenol was added and the mixture vortexed well. 25ul chloroform / isoamyl alcohol (25 : 1 v/v) was added, the mixture vortexed well and then centrifuged for 2 min in an Eppendorf centrifuge. The supernatant was retained and a 1/10th v of 3m sodium acetate and 1 volume isopropanol added. This was mixed and

centrifuged for 10 - 15 min in an Eppendorf centrifuge. The supernatant was withdrawn from the tube and the pellet was air-dried in an open tube on the bench for 5 - 10 min before resuspension with vortexing in 10ul TE.

APPENDIX 14

Southern blotting

20X SSC

3M NaCl in solution with 0.3M sodium citrate and the pH adjusted to 7.

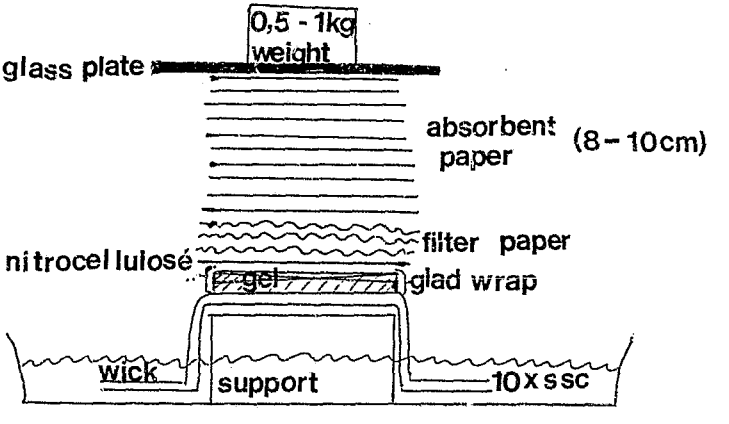
The solution was di. ıted in dH 0 as required.
2

Preparing the blot

After electrophoresis, the gel was photographed in ultraviolet light. The gel was then transferred to a container with 3 v of 1.5M NaCl containing 0.5M NaOH for 1h at room temperature with agitation. This solution was replaced with 1M Tris-HCl pH 8 containing 1.5M NaCl and 0.001M NaEDTA. The gel was shaken in this for 1h at room temperature.

A piece of nitrocellulose paper cut to the same size as the gel was soaked in 2X SSC for 10 min. Three pieces of Whatman 3MM filter paper cut to the same size as the gel were also soaked in 2X SSC for 10 min.

Transfer of DNA was carried out overnight as demonstrated in the diagram below.



Next day the gel was removed and checked under UV light to see that transfer of the DNA had occurred.

The blot was then baked for 2h at 80 °C under vacuum between two sheets of Whatman 3MM filter paper.

APPENDIX 15

Labelling and hybridization according to Boehringer Mannheim's nonradioactive labelling and detection kit

1. Labelling - solutions

0.2M NaEDTA

4M LiCl

70% (v/v) ethanol

10% (w/v) SDS

20X SSC pH 7 (see Appendix 14)

Labelling procedure (solutions labelled * supplied in kit)

DNA was denatured by heating for 10 min at 95 °C and chilling quickly on ice and methanol. 1 ug of freshly denatured DNA, 2 ul hexanucleotide mixture * and 2 ul dNTP labelling mixture * were made up to 19 ul with sterile dH_2O in a sterile Eppendorf tube on ice. 1 ul Klenow enzyme * was then added. The mixture was incubated for 1 - 3 h at 37 °C. The reaction was stopped by adding 2 ul 0.2M NaEDTA. The labelled DNA was precipitated with 2 ul 4M LiCl and 60 ul

prechilled (−20 °C) ethanol. The mixture was left at −70 °C for 1 h, then centrifuged for 15 min in an Eppendorf centrifuge. The pellet was dried and resuspended in TE buffer (see Appendix 14).

2. Hybridization - solutions

20X SSC stock (see appendix 14)

Prehybridization mix: 5X SSC containing 0.5% (w/v) BSA (supplied in kit) and 0.02% (w/v) SDS.

Hybridization solution: Prehybridization solution containing freshly denatured probe

Washing solutions: A. 2X SSC containing 0.1% SDS

B. 0.5X SSC containing 0.1% SDS

Procedure (solutions labelled * supplied in kit)

Nitrocellulose membranes (prepared dot blots and Southern blots) were placed in a box containing at least 20 ml prehybridization solution per 100 cm^2. This was shaken gently at 65 °C for 2 h. The filters were then placed in plastic bags, hybridization solution containing at least 7 ul probe per filter was added and the bags were sealed. The hybridization reaction was carried out, with shaking, overnight at 65 °C. The filters were then returned to the box and washed twice for 10 min at room temperature in washing solution A and then twice for 20 min each at 65 °C in washing solution B.

In the first experiment, filters were air-dried and stored before detection of hybridized DNA. In the second experiment, detection was carried out immediately.

3. Immunological detection - solutions (solutions marked * are supplied in the kit)

Buffer 1: 0.1M Tris-HCl pH 7.5 containing 0.15M NaCl

Buffer 2: 0.5% blocking reagent * (BSA) in buffer 1

Buffer 3: 0.1M Tris-HCl pH 9.5 containing 0.1M NaCl and 0.05M $MgCl_2$

Buffer 4: 0.01M Tris-HCl pH 8 containing 0.001M NaEDTA

Colour solution (freshly prepared): 45 ul NBT-solution * and 35 ul X-phosphate solution * added to 10 ml buffer 3 and centrifuged at 5000 rpm for 10 min before use

Procedure

Filters were briefly washed in buffer 1 before incubating with buffer 2 for 30 min. The filters were again washed briefly in buffer 1. Antibody-conjugate * was diluted to 1:%000 in buffer 1 and filters were incubated in this solution for 30 min. Unbound antibody-conjugate was removed by washing twice for 15 min each in buffer 1. The membranes were then equilibrated for 2 min in buffer 3 before incubation in colour solution for about 24 h. The reaction was stopped by washing the membrane for 5 min in buffer 4. The results were documented by photographing the wet filter.

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