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"SPINACH CHLOROTIC SPOT VIRUS: A NEW STRAIN OF BEET YELLOWS VIRUS"

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BY

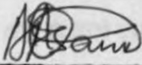
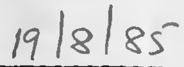
ANNE ADHIAMBO OSANO

A thesis submitted to the University of Nairobi in partial fulfilment of the requirements for the degree of Master of Science in plant pathology

June 1985

DECLARATION

This thesis is my own original work and has not been presented for a degree in any other university.

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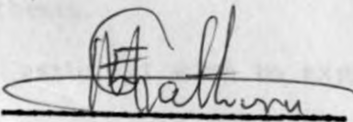
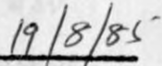

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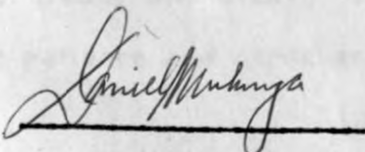
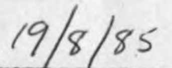

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ACKNOWLEDGEMENT

I wish to express my sincere gratitude to my supervisors, Dr. E. M. Gathuru and Prof. D. M. Mukunya, for their devotion, guidance, advice, criticisms and constant encouragement during the course of this investigation and preparation of this manuscript.

I gratefully acknowledge the technical assistance I received from the technical staff of Crop Science, Food Science, Pathology and Public Health departments of the College of Agriculture and Veterinary Sciences. In particular, I wish to recognise Mr. F. Ngumbu, formerly of Plant Virology Unit, for his special technical assistance and Messrs D. N. Kahara and J. G. Waweru for their help in taking the electron micrographs.

My special appreciation to the Kenya Government, which through the Ministry of Agriculture, provided the funds which enabled me to undertake my post graduate course.

I also wish to thank Mr. J. K. Kiiru for typing this thesis.

Lastly, I wish to express in a more personal way my hearty appreciation with love to: my husband, Peter, for his assistance, encouragement and endurance which I needed very much for the success of this study to my sons, Teddy and Nick, and to my daughter, Mercy, for their patience and perseverance. I wish also to recog-

nise my parents, Mr. and Mrs Jonathan Ngode, my brothers and sisters, especially Mr and Mrs Luke Wasonga, my other relatives and friends for their constant support and encouragement.

TABLE OF CONTENTS

	Page
TITLE	i
DECLARATION	ii
ACKNOWLEDGEMENT	iii
TABLE OF CONTENTS	v
List of tables	vii
List of plates	ix
list of figures	xi
ABSTRACT	xiii
INTRODUCTION	1
LITERATURE REVIEW	5
MATERIALS AND METHODS	19
Source of virus	19
Inoculation procedure	19
Bioassay	19
Source of plants	20
Experimental host range	20
Transmission	21
Insect transmission	22
Tuber transmission	22
Physical properties in crude sap	23
Dilution end point	23
Thermal inactivation point	23
Longevity in vitro	23

Table of contents (contd)

Purification.................................... 24

Mincing of plant materials................ 24

Clarification of the sap.................. 25

Ultraviolet absorption spectrophotometry......... 27

Electron microscopy.............................. 29

Leaf dip preparation..................... 29

Partially purified virus................. 29

Source of antiserum...................... 30

Serological test............................ 31

Particle "Normal length"....................... 31

RESULTS.. 33

Field symptoms in spinach..................... 34

Experimental host range and symptomatology...... 34

Transmission................................... 44

Insect transmission...................... 44

Tuber transmission....................... 44

Physical properties............................ 44

Purification................................... 45

Reducing agents.......................... 45

Clarifying agents........................ 45

Buffers.................................. 46

Ultraviolet absorption spectrum............... 55

Electron microscopy........................... 62

Particle "Normal length"...................... 62

Serology...................................... 62

DISCUSSION AND CONCLUSION..................... 64

Table of contents (contd)

LITERATURE CITED.............................. 78

APPENDICES.................................... 89

Table 7 Comparative study of spinach chlorotic spot virus response with other literature reported viruses in spinach 67

Table 8 Comparative "normal length" of SCSV particles and some viruses reported in spinach and beet root.................. 69

Table 9 Comparative physical properties studies in crude sap of SCSV and other viruses reported in spinach and beet root................................. 74

chlorotic spot virus) showing lateral and end to end aggregation (magnification 45,000x)................ 56

Plate 9 An electron micrography of a partially purified preparation of SCSV treated with urea to break aggregation, (magnification 45,000x)................ 57

Plate 10 An electron micrography of a partially purified preparations of SCSV showing the particle forming a helix, (magnification 45,000x)................ 58

Plate 11 An electron micrography of a leaf dip preparation from spinach leaves showing the presence of slightly flexuous rod shaped virus particles (magnification 45,000x)............................... 59

Plate 12 A histogram showing the distribution of SCSV particle lengths 63

LIST OF FIGURES

Figure 1 Concentration of SCSV in leaves of spinach (*Spinacea oleracea*)......... 60

Figure 2 Ultraviolet absorption spectrum for partially purified preparation of SCSV.. 61

SPINACH CHLOROTIC SPOT VIRUS:

A NEW VIRUS OF BEET YELLOWS VIRUS

ABSTRACT

A Virus causing systemic chlorotic spot symptom in spinach (Spinacea oleracea) was characterized and identified as a strain of beet yellows virus and was referred to as spinach chlorotic spot virus (SCSV). The virus particles were flexuous and approximately 840 nm in length. In glasshouse all the beet varieties (Crimson globe, Eclipe and Detroit) and spinach varieties (lucullus, Foordhook giant and Swiss chard) tested were susceptible.

The virus isolate was limited in host range to the family Chenopodiaceae. In Beta vulgaris, a member of chenopodiaceae family, systemic necrotic lesions were observed about 8-10 days after inoculation. The necrosis was more marked in mature leaves and there was general intensification of red colour. A few leaves showed yellow spots and the plants were also stunted. Necrotic lesions, 2-3 mm in diameter, were also observed in Chenopodium amaranticolor 5 days after inoculation. Systemic tip necrosis was also observed 2-3 weeks after inoculation. Chenopodium quinoa and C. album showed the same type of symptoms already described for C. amaranticolor. White necrotic spots, about 2-3 nm in diameter, with reddish outline, observed in Gomphrena

globosa. Chenopodium amaranticolor was used as an assay host.

SCSV was successfuly transmitted in a semi-persistent manner by the aphid *Myzus persicae*. The virus isolate had a thermal inactivation point (TIP) at 56 ^{o}C and not 54 ^{o}C; dilution end-point (DEP at 10^{-4} and not 10^{-5}; longevity in in vitro (LIV) at one days and not 2 days. SCSV was partially purified from infected spinach leaves by differential centrifugation. The virus was extracted with 0.1M phosphate buffer, pH 7.2, and clarified in 7% ether. In homologous reactions, the virus reacted with its own antiserum to the titre of 1/1024 in microprecipitin tests.

On the basis of these criteria and results obtained, the virus inducing chlorotic spots in spinach was identified as a strain of beet yellows virus.

INTRODUCTION

Spinach (*Spinacea oleracea*) is the sort of vegetable one either loves or loathes. It belongs to the family chenopodiaceae and is rich in vitamin A and iron (Davidson, 1977).

Greenvill (1968) discussed four types of spinach:

i English spinach (*Spinacea oleracea*) which is grown in temperate America and Europe, though it can be grown in the tropics at the higher altitudes;

ii New Zealand spinach (*Tetragonia expansa*) grown in Australia and New Zealand;

iii Indian spinach (*Basella alba* and *B. rubra* which is a succulent vine from Asia, Africa and tropical America;

iv African spinach (*Amaranthus caudatus*, *A. tricolor* and *A. spinsus*).

Spinach is grown mainly as a vegetable and its nutritional value cannot be disputed. Salunkhe (1973), in his survey of nutritional quality of 42 fruits and vegetables had the following report on the nutritional quality of spinach:

Spinach ranks first in iron and magnesium, is second to lima beans in potassium and third to celery and kale in sodium.

Iron is an important constituent of blood and several enzymes. As well as controlling anemia, iron is

also important in the respiratory electron transport. Sodium acts with potassium to provide proper nerve stimulation.

Apart from minerals discussed here, spinach was reported by the same author as containing 3.2% proteins which are needed for the provision of nitrogen for hormones, enzymes and blood. Proteins form the network structure in bones, act as biological buffer system as well as having immunological effects.

Spinach ranks fifth in Vitamin A content which maintains healthy mucous membrance of the eyes, mouth and gastro-intestinal, respiratory and genito-urinary systems. Vitamin A protects against infection, promotes normal skin and tooth growth, stimulates reproduction and lactation, and is essential for light and dark adaptation of vision. Thiamine and Riboflavin were also reported in spinach.

Spinach is grown widely in Nairobi and Kiambu districts and it serves as food to the residents of Nairobi City. The Ministry of Agriculture reports from Coast Province (1977, 1979 and 1981) showed that spinach is also grown at the coast especially in Taita Taveta District, where in 1977 8.9 hectares of land was used to grow spinach and 18.6 tonnes of the vegetable was harvested. This was sold for K£756 outside the district. In 1981, spinach was grown in 16.6 hectares of land and 131.2 tonnes of the vegetable was harvested which was sold for K£6,560 outside the district.

Spinach is attacked by a large number of fungi and viruses. The fungal diseases include downy mildew of spinach caused by Peronospora spinaciae (Leach, Borthwick, 1934);Fusarium wilt by Fusarium oxysporum f. s. spinaciae (Hungerford, 1923); white rust by Albugo occidentalis (Raaba and Pound, 1952); leaf spots by Alternaria spinaciae, Ascochyta spinaciae, Cercospora bertrandii, C. beticola, Cladosporium macrocerpum Heterosporium variable, Phyllosticta chenopodii, Ramularia spinaciae (1960); anthracnose by Colletotrichum spinacicola (Singh, et al., 1951); phoma heart rot by Phoma betae (Herberget et al., 1948) and rust by Puccinia aristidae (Ramsey and Smith, 1952).

Viral diseases reported in spinach include yellows diseases by lettuce mosaic virus (LMV) (Provvidenti, 1972); tobacco rattle virus (TRV) (Kurppa, A. et al., 1981); beet yellows virus (BYV), (Ksiazek and Roland, 1973); blights by broad bean wilt virus (BBWV) (Provvidenti et al.,1970); cucumber mosaic virus (CMV) (Bailiss and Okonkwo, 1979); stunting by spinach yellow dwarf (SYD) (Halliwell, et al., 1973); necrosis by tomato spotted wilt virus (TSWV) (Inouye and Inouye, 1972); mosaic by spinach mosaic virus (SMV) (Naqui and Mahmood, 1975); latent infection by spinach latent virus (SLV) (Bros, et al., 1980). Beet western yellows virus (BWYV) (Duffus, 1960, 1961) and beet mosaic virus (BMV) (Naqui, et al., 1975) were also reported.

In Kenya, the diseases reported are mainly downy mildews by Peronospora offusa (Grew) Tul (Singh,1973).

In Nairobi and Kiambu districts where spinach is widely grown, it was noted that plants exhibited chlorotic spots on the leaves, indicative of viral infection (Gathuru and Bock, 1974). The two reported a virus causing severe systemic chlorotic spots in spinach. All available beet and spinach varieties tested were susceptible. Field incidence was 80%.

In this study, the new virus strain on spinach will be isolated and studied. It will be referred to as spinach chlorotic spot virus (SCSV). The results of symptomatology and host-range through mechanical inoculation, transmission through tuber and the aphid Myzus persicae Sulz, ultraviolet absorption, eletron microscopy will be compiled. This should lead to a possible identification of the virus strain.

LITERATURE REVIEW

In Nairobi and Kiambu districts where spinach is widely grown, it was noted that plants exhibited chlorotic spots on leaves (Gathuru and Bock, 1974). They isolated a virus causing severe systemic chlorotic lesions and stunting on spinach and beet root. The host range appeared to be restricted to chenopodiaceae and solanaceae families. The virus was transmitted by *Myzus persicea* but not *Aphis fabae.*

Susceptible species include *Amaranthus retroflexus*, *Chenopodium amaranticolor*, *C. quinoa*, *Gomphrena globosa*, *Nicotiana clevelandii*, *N. rustica*, *N. tobaccum*, *Rapharus sativum*, *Solanum nigrum*, *Tetragonia expansa*, *Zinnia elegans* and all available varieties of beet root (Crimson, Eclipse, Globe and spinach beet (Lucullus, Swiss Chard, Hollonder Montrous). The dilution end point of the virus was between 10^{-3} and 10^{-4}, thermal inactivation point was at 54 ^{o}C and longevity in vitro between 4 to 5 days. Leaf dips showed rod shaped filamentous particles about 750 nm long. Attempts to purify the virus were not successful.

This research was done to study the above virus further. The virus was called isolate 71-35 by Dr. Gathuru and Bock. Since attempts to purify the virus failed, a lot of work was undertaken to study each purification stage, step by step as published by Noordam

(1973), and hence determine a possible purification method.

Other viruses reported in spinach can be grouped into eight groups, potyviruses which include parsnip mosaic virus (PMV), beet mosaic virus (BMV), lettuce mosaic virus (LMV) and turnip mosaic virus (TMV); a tabravirus, that is tobacco rattle virus (TRV); a comovirus, broad bean wilt virus (BBWV); a cucumovirus, cucumber mosaic virus (CMV); tomato spotted wilt virus (TSWV), a type member of tomato spotted wilt virus group, a cleosterovirus, beet yellows virus (BYV); a luteovirus, beet western yellows virus (BWYV) and a tobamovirus, beet necrotic yellow vein virus (BNYVV).

Potyviruses

Potyviruses have rod shaped flexuous particles, 680-900 nm long and 11 nm wide with helical symmetry. The particles are transmitted by seed, sap and aphids in a non-persistent manner.

Parsnip mosaiv viruc (PMV) was reported to infect 8 Chenopodium species including spinanch (Murant, et al., 1970). It has flexuous particles, about 730-760 x 14 nm, transmitted in the non-persistent manner by the aphids such as Myzus persicae. There is no information on the seed or dodder transmission. Chenopodium quinoa, C. amaranticolor are good local lesion hosts. C. quinoa was also found to be assay host in whose leaf extracts the virus lost infectivity after dilution from

10^{-3} to 10^{-4} or storage for ten minutes at 55-58 ^{o}C or 7-10 days at 8 ^{o}C (Murant et al., 1970).

Beet mosaic virus (BMV) another potyvirus, with flexuous filamentous particles measuring 700 nm long and 13 nm wide, causes mosaic disease in sugar beet (Beta vulgaris) and spinach (Spinacea oleracea (Naqui et al., (1975). The virus is transmitted by more than 28 aphid species (Kennedy, et al.,1962) but notably by Myzus persicae and Aphis fabae.

In beet sap, the thermal inactivation point is between 55 and 60 ^{o}C: dilution end point is up to 1/4000 and infectivity retained for 24-48 hours at 20 ^{o}C in beet leaves (Yamuguchi, 1964). Symptoms in spinach may take up to 3 weeks to appear and start as sudden backward arching of the young foliage together with the development of bright golden flecks. The flecks are circular or irregular in outline, possibly with a dark central point. They frequently coalesce to produce large chlorotic areas. As the growth ceases and stunting begins, a yellow discoloration spreads over the foliage. The mature leaves may become necrotic with leaf death advancing from the tip and spreading to the inner foliage and eventually the plant is reduced to a whorl of small mottled and puckered leaves. Young leaves in Beta vulgaris show a pronounced light and dark green mottling and are often puckered. Infected plants are at times stunted.

Lettuce mosaic virus (LMV) has long flexuous, filamentous rod shaped particles, averaging 750 nm. It is sap-transmissible, and is seed-borne in lettuce. It is transmitted by several aphid species in a non-persistent manner (Kennedy, et al.,1962), mainly by <u>Myzus persicae</u>. Symptoms caused by LMV in spinach include numerous light yellow circular spots on developing leaves (Provvidenti and Schroeder, 1972). Subsequent leaves are small, mottled and often distorted. Such plants are usually stunted, the extent of stunting depending on age of infection. Older leaves wither, turn yellow and die. In lettuce (<u>Lactuca sativa</u>), the symptoms vary but usually consist of vein clearing and yellow mottling with veinal necrosis. Plants fail to 'heart' and inner leaves remain dwarfed and rosetted (Costa and Duffus, 1958). <u>Chenopodium quinoa</u>, <u>C. amaranticolor</u> and <u>Gomphrena globosa</u> are good local lesion hosts. In lettuce sap, the thermal inactivation point is 55-60 ^{o}C, dilution point is 10^{-1} to 10^{-2} while infectivity of LMV is retained at 20 ^{o}C for 1-2 days (Jaggar, 1921).

Bauliss and Okonkwo (1979) isolated turnip mosaic virus (TMV) from field grown spinach. TMV, a member of potyvirus group, has flexuous filamentous particles approximately 680 nm (Shepherd and Pound, 1960). It is not transmitted by sap but by 40-50 species of aphids, notably <u>Myzus persicae</u> and <u>Brevicoryne brassicae</u>

(Kennedy, et al., 1962). Infective sap kept at $2^{o}C$ retains infectivity for several months while at $20^{o}C$ it is retained for 3-4 days only. Thermal inactivation point is generally below $62^{o}C$ while dilution end point is between 10^{-3} and 10^{-4}.

Tobravirus group

Tobacco rattle virus (TRV), a tobravirus, was reported by Komuro and his group to infect spinach in 1970. Members of this group have rod shaped particles of 180-215 nm (L) and 46-114 nm (S) lengths, and are transmitted mainly by nematodes, *Paratrichodorus* and *Trichodorus* spp. TRV, an RNA-containing virus, has straight tubular particles of two predominant lengths, 170-180 nm and 70-80 nm (Komuro, et al., 1970).

Some isolates are readily sap transmissible; others are not. At least 11 *Trichodorus* spp (Stubby root nematodes) are natural vectors of the virus in Europe and the U.S.A. (Taylor and Cadaman, 1969). *T. pachydermus* and *T. primitivus* seem to be the most important vectors in Europe. Komuro et al., (1970) reported the virus to be transmitted by *Trichodorus minor*. Seed transmission is between 1 and 6% (Murant and Lister, 1967). Thermal inactivation point is 80-85 ^{o}C, dilution end point about 10^{-6} and infectivity is retained at $20^{o}C$ for more than 6 weeks. TRV causes yellow mottle in spinach and is called spinach yellow mottle (SYMV) virus (Kurppa et al., 1981). They

studied the properties of SYMV in the U.K. plants and concluded that it is a strain of TRV and they called it spinach yellow spot strain of TRV. Diagnosis hosts include *Chenopodium amaranticolor*, *Cucumis sativus* (cucumber), *Nicotiana clevelandii* and *Phaseolus vulgaris* which are all local lesion hosts. *N. clevelandii* is also a systemic host. Yellow blotch is formed on sugar beets.

Comovirus group

A destructive blight of Spinacea oleracea is incited by a strain of broad bean wilt virus (BBWV) (Provvidenti and Schroeder, 1970). BBWV belongs to comovirus group, the members of which are sap transmissible, and some are transmitted by seed. They have beetle as vectors in which they persist for several days. The virus particles are good immunogens. Serological relationships exist between members. They have a fairly narrow host range. The symptoms caused by BBMV in spinach include mottling followed by severe chlorosis and finally chlorosis of the growing point (Provvidenti and Schroeder, 1970). In broad bean (*Vicia faba*) infection is followed by systemic vein-clearing, necrosis of terminal leaves, general wilting and often death. Concentric necrotic ring-shaped local lesions which coalesce are formed in *Datura stramonium.* Inoculated leaves may absciss. Chlorotic or necrotic ring spots and line or oak-leaf patterns are formed on new growth. In *Chenopodium quinoa*, chlorotic local

lesions form on inoculated leaves after 4-5 days. This is followed by systemic chlorosis of the whole plant or of apical leaves (Stubbs, 1960). Myzus persicae is the most efficient vector of the disease (Stubbs, 1960). Transmission through seed or dodder has not been reported. In broad bean sap, the thermal inactivation point is 58 ^{o}C, dilution end point is 10^{-4} to 10^{-5} and infectivity is retained at 21 ^{o}C for 2-3 days (Stubbs, 1947). The particles are isometric, about 25 nm in diameter. In the U.S.A., Schroeder and Provvidenti, (1970) and in Japan, Komuro, (1970) reported it to cause a severe disease of spinach which may be confused with that caused by cucumber mosaic virus. Bailiss et al.,(1975) reported a natural occurrence of BBWV in spinach in Britain.

Cucumovirus group

Cucumber mosaic virus (CMV) causes spinach blight and is therefore also called spinach blight virus (Doolittle and Jagger, 1916).

CMV is the type member of cucumovirus group. The members have poyhedral particles approximately 28 nm in diameter, with a wide host range. Transmission is by mechanical inoculation, seeds and aphids in a non-persistent manner. Green or yellow/green systemic mosaic are produced on cucumber (cucumis sativus) (Doolittle and Jagger, 1916). Chlorotic or necrotic local lesions, is not systemic and are formed on

Chenopodium amaranticolor and *C. quinoa*. Sixty species of aphids can transmit CMV but it is mostly transmitted by *Aphis gossypii* and *Myzus persicae* (Kennedy, Day and Eastop, 1962). Seed transmission is not common while ten species of Cuscuta (dodder) can transmit the virus (Anerson, 1957). In tobacco sap, the thermal inactivation point is about 70 ^{o}C, dilution end point about 10^{-4} and infectivity is retained at 20 ^{o}C for 3-6 days. The particles are isometric, about 30 nm in diameter. The virus is poorly immunogenic.

Tomato spotted wilt virus group

Tomato spotted wilt virus (TSMV) causes severe necrotic symptoms and death of inoculated leaves followed by occasional systemic necrosis in spinach (Inouye and Inouye, 1972). TSWV, a type member of tomato spotted wilt virus group, has isometric particles, 70-90 nm in diameter. Members of this group have spherical particles approximately 85 nm in diameter, which are relatively poor immunogens. The virus, TSWV, has a wide host range and is transmitted by thrips in a persistent manner, acquired only by larvae (Inouye and Inouye, 1972). The virus is readily transmitted experimentally by sap inoculation. Local necrotic lesions are formed 2 to 4 days after inoculation in *Petunia hybrida* cvs. Pink Beauty and Minstrel. *Nicotiana tabacum* cv. Samsun NN, *N. clevilandii* and *N. glutiuosa* show local necrotic lesions followed by systemic necrotic patterns and leaf

deformation. Ninety six per cent seed transmission was reported in Cineraria and tomato (Jones, 1944), while there have been no reports on dodder transmission.

Tomato spotted wilt virus is one of the most unstable plant viruses. Thermal inactivation point is 40-46 ^{o}C, longevity in vitro at room temperature is 2-4 hours and dilution end point is between 10^{-2} and 10^{-3}.

Geminivirus group

The geminivirus group, of which beet curly top virus is a member, has isometric particles, 18-20 nm in diameter, occurring in pairs. Virus particles are efficient immunogens. Some members are transmitted by leafhoppers and others by white flies in a persistent manner. Some members are transmitted experimentally by mechanical inoculation. Beet curly top virus (BCTV) has isometric particles, found in pairs ('geminate'). It has a wide host range and causes debilitating often lethal yellows type diseases, usually accompanied by leaf-curling and distortion in beet, spinach and other hosts (Giddings, 1949). In *Beta vulgaris* BCTV causes vein clearing in young leaves by upward and inward leaf rolling. A conspicuous vein-swelling and galling produces a roughened lower leaf surface, rasp-like to the touch. Droplets of phloem necrosis may be observed as dark concentric rings in transverse sections of roots. In *Nicotiana tabaccum* (tobacco) the virus causes young leaves to be dwarfed with swollen distorted

veins and downward rolled margins, forming a rosette at the growing point. Normal growth resumes after a few weeks. Leaf hopper, Curculifer tenellus is the only natural vector known to occur in North America (Stahl and Carsner, 1923). At least three species of Cuscuta can transmit the virus but it is not seed transmitted (Bennet and Esau, 1936). The thermal inactivation point is 80 C and dilution end point is about 1/4000. Infectivity is retained for up to 8 days at room temperature and more than eleven months at -18 C (Bennet, 1935).

Cleosterovirus group

Beet yellow virus (BYV) is a type member of cleosterovirus group. Particles of this group are unstable in high concentrations of NaCl and CsCl but stable in Cs So . Particles are very flexuous rods, 600-2000 nm long, and 10 nm wide, with helical symmetry and pitch 3.7 nm. Particles may be joined in pairs by up to 10 cross-bridges originating at a morphologically distinct end of the particles. Serological relationship exists between some members. The particles often aggregate in cross-banded masses in phloem cells. Members are transmitted experimentally by mechanical inoculation with difficulty. Some members are transmitted by aphids in semi-persistent manner. BYV causes a yellows disease in Beta vulgaris; young leaves of glasshouse plants often show vein clearing and 'vein-etch'. Older leaves of the field and glasshouse beet

become yellow, thickened and brittle and usually have numerous small red or brown necrotic spots (Roland, 1936; Watson, 1942 and Hull, 1950). It is worth noting, however, that the symptoms of the virus vary depending on the virulence of the strain involved, variety attacked, light condition and possibly other factors (Leach, 1969). Some strains rarely or never produce vein clearing and those that do, do not do so under all conditions. Intensity of yellowing of affected plants also varies depending on the strains of the viruses. A few selections have shown marked necrosis in mature leaves and plants with red pigments show an intensification of red colour.

Symptoms caused by beet yellows virus on spinach are more variable in severity but chlorosis of mature leaves is frequent. Vein clearing and curling may develop together with necrosis of the growing apex and death of the host. Many minor variants have been isolated from lesions, producing symptoms in sugar beet, ranging from very mild yellowing to very severe vein-etch and leaf necrosis (Leach, 1969). All are apparently serologically related and there is usually complete cross protection between them (Russell, 1964). More than 22 species of aphids can transmit the virus but Myzus persicae and Aphis fabae are the principal vectors in the field (Kennedy, Day Eastop, 1962). Transmission is of the semi-persistent type (Watson, 1946). Thermal

inactivation point in sap is about 55 ^{o}C, and dilution end point is up to 10^{-4}. Infectivity is retained in frozen sap for more than a year but rarely for more than one day at 20 ^{o}C (Watson, et al,.). The virus is very unsatable and therefore presents some problems in purification. Partial purification from clarified sap has been achieved by differential centrifugation. The particles are flexuous filamentous, about 1250 nm long, and 10 nm in diameter, with sub-units arranged around a hollow core in helix of pitch between 3.0 and 3.4 nm. The particles are associated with degeneration of phloem tissues. Arrays of the virus particles have been found in chloroplasts.

Luteovirus group

Beet western yellows virus (BWYV), a member of luteovirus group, causes stunting and chlorosis of a wide range of hosts including sugar beet, spinach, radish, turnip, etc. Particles of this group are isometric, about 25 nm in diameter. They are strongly immunogenic, most members being serologically related. Aphids are the virus vectors, which transmit the particles in a persistent manner, while members are not transmitted mechanically. BWYV has isometric particles, 26 nm in diameter (Esau and Hoefert, 1972a; Ruppel, 1968). The virus is transmissible by eight species of aphids, the most important being *Myzus persicae*. The transmission by seed or dodder is not reported. The

thermal inactivation point is about 65 ^{o}C; Dilution end point of unconcentrated sap is 1/8 while extracts are infectious after 16 days at 24 ^{o}C (Duffus, 1960).

Tobamovirus group

Beet necrotic yellow vein virus (BNYVV), a member of tobamovirus group, is associated with "rhizomania" disease in sugar beet (*Beta vulgaris*) (Kanzawa and Ui, 1972). This disease is characterized by abnormal proliferation of the rootlets. The virus also causes yellow mottle and stunting in spinach. Members of tobamovirus group have very stable elongated and rigid particles, about 18 nm in diameter and 300 nm long, with helical symmetry and pitch about 2.3 nm. They are readily mechanically transmissible while some are also seed transmitted. The particles of BNYVV are straight rods helically constructed, about 20 nm wide and 390 nm, 270 nm and 65-105 nm long (Tamada, et al., 1975). The virus is transmitted by the *plasmodiophromycetes* fungus, *Polymyxa betae*. Transmission by seed or dodder is not yet tested (Tamada, et al., 1975). Thermal inactivation point, in sugar beet sap, is 65 to 70 ^{o}C and dilution end point is about 10^{-4} while infectivity is retained for 5 days at 20 ^{o}C and for 8 days at 4 ^{o}C (Tamada and Baba, 1973). Local lesions are formed on *Beta vulgaris*, *Chenopodium amaranticolor* and *C. quinoa*. Systemic infection, observed in *Beta vulgaris* includes chlorotic or yellow spotting, yellow vein-banding, vein

necrosis, leaf distortion, wilting and stunting (Tamada, et al., 1970).

This project therefore aims at establishing whether the new virus reported in spinach (Spinach chlorotic spot virus, SCSV) is any one of the viruses reviewed here, or a strain of anyone of them or a completely new virus.

MATERIALS AND METHODS

a. Source of virus

The unknown virus was isolated from plants growing in experimental plots at the field station of the College of Agriculture and Veterinary Science of the University of Nairobi.

b. Inoculation procedure

All inoculations were done using carborandam forefinger method. The test plants, at appropriate leaf stages, were dusted with 500-mesh carborandum powder (Rawlins and Thompkins, 1936), inoculated and rinsed with tap water and placed in the green house. Inoculum was prepared by grinding freshly picked spinach leaves in sterile mortar and pestle with 0.01M phosphate buffer, pH 7.2 at the rate of 1 g/ml of buffer.

c. Bioassay

Plants of *Chenopodium amaranticolor* L., *C. quinoa* responded to SCSV with the development of necrotic local lesions about 2-3 mm in diameter, 58 days after inoculation. Generally the leaves of such plants also exhibited systemic tip necrosis after 14-21 days of infection. Consequently plants of the species *Chenopodium amaranticolor* L. were used for all recovery

inoculations and bioassay tests.

d. Sources of Plants

Plants used for host range and symptomatology studies were grown in the greenhouse from seeds obtained from virology germ plasm laboratory of the Faculty of Agriculture, Kabete, and from the East African Quarantine Station, Muguga, Kenya.

Seeds of spinach and beet roots were purchased from Simlaws Seeds, Simpson and Whitelaw Ltd, Nairobi. Other vegetables seeds used were purchased from the same company.

e. Experimental host range

Ten plants of each plant species or cultivar were mechanically inoculated with inoculum prepared from SCSV infected spinach. Three plants of each species or cultivar were left as controls in each inoculation. *Chenopodium amaranticolor* L. and *C. quinoa* were inoculated when the plants had formed six mature leaves and about two months old. *Beta vulgaris* plants were inoculated when plants had five leaves and were five weeks old, while *Spinacea oleracea* L. plants were inoculated when they were four weeks old and had eight leaves. The reactions were observed through three to four weeks after which recovery inoculations were made.

f. Transmission

Besides mechanical sap inoculations, the following methods of transmission were also studied:

1. Insect transmission

Gathuru and Bock (1974) found that SCSV was transmitted by green peach aphid, Myzus persicae L. Sulz. Therefore the aphid was used in this study. They were collected from Kale plants and freed of any virus by rearing them at 20-24 C on healthy Chinese cabbages (Brassicae pekinensis Rupr) which were grown in an aphid cage. Spinach plants were used as the virus source and test plants.

The aphids, starved for one hour, were allowed acquisition feeding periods of fifteen to twenty minutes for the first group, and twenty-four hours in the second and third group. The first group had twenty aphids, while the second ground and the third had forty aphids each. The first group was allowed an inoculation feeding time of one hour while the second group was allowed inoculation feeding time of a maximum of ninety-two hours.

The last group was allowed four weeks inoculation feeding periods. The second and the third groups of aphids were used for several successive inoculations of healthy plants, allowing twenty four hours in each stage

of inoculations.

2. Tuber transmission

The tubers formed from mechanically infected beet plants were crushed and the juice so formed inoculated on C. amaranticolor L.

g. Physical properties in crude sap

For the determination of the relative stability and concentration of SCSV in crude sap, physical properties: dilution end point (DEP) thermal inactivation point (TIP) and longevity in vitro (LIV) were conducted. Concentration of the virus in the production host (Spinacea oleracea L.) was investigated by inoculation of Chenopodium amaranticolor L. leaves with inoculum prepared from freshly picked spinach leaves. Spinach plants were inoculated with the virus and allowed incubation period of ten days. The leaves were then picked at two days interval, for fifty four days. They were ground with sterile mortar and pestle with 0.01M phosphate buffer pH 7.2 at the rate of 1 g/ml of buffer. The inoculum so formed was inoculated onto twelve leaves on three C. amaranticolor L. plants. The number of lesions formed on the leaves were counted and these were equivalent to the virus concentration on the production host (Fig. 1).

Systemically infected spinach leaves were then harvested twenty-five to thirty days (Fig. 1) after

inoculation, macerated with a sterile pestle and mortar and the sap expressed through two layers of cheese cloth. The crude sap was given the following treatments.

1. <u>Dilution end point</u>

A two-fold dilution series up to 10 of the crude sap was made using distilled water as the diluent, 2 ml of undiluted crude sap with 2 ml of each dilution was inoculated separately on ten plants of *C. amaranticolor* L. for each dilution.

2. <u>Thermal inactivation point</u>

Glass tubes 1.5 x 10 cm used in the test were first heated in a water-bath. The crude sap was exposed for 10 minutes between 50 ^{o}C and 60 ^{o}C at 2 degrees intervals. After heating for 10 minutes the contents of the test tubes were immediately cooled under running tap water and then bioassayed for infectivity by mechanical inoculation of five plants of *C. amaranticolor* L. Five plants of the assay host were also inoculated with unheated crude sap as controls.

3. <u>Longevity in vitro</u>

Crude sap was pippetted into test tubes (2 x 14.5 cm) covered with aluminium foil and left at room temperature between 20-25 ^{o}C. Samples of 1.5 ml of the

crude sap were removed after agitation at 1, 2, 3, 4, 5, and 6 days. The crude sap samples were each bioassayed for infectivity by inoculating five plants of _C. amaranticolor_ L.

h. Purification

The unknown virus presented some difficulties in earlier attempts to purify it (Gathuru and Bock, 1974). Therefore a detailed study was undertaken to test the usefulness of the steps of purification as reported by Noordam (1973).

1. Mincing of plant material

Crude sap of most plants contain poly-phenoloxidase and other oxidative agents, which destroys virus particles. To avoid this, reducing agents, such as Na_2SO_3, thioglycollic acid and certain buffers were compared.

0.2% sodium sulphite (Na_2SO_3)

Sodium sulphite (0.2%) was added onto spinach leaves showing SCSV which were macerated and the crude sap inoculated onto ten _Chenopodium amaranticolor_ L. leaves.

0.2% thioglycollic acid

To stop virus aggregation 0.1% thioglycollic acid was added to phosphate buffer, 0.01M sodium phosphate, pH 7.2. The mixture was added onto spinach leaves infected with the virus, macerated and the crude sap inoculated onto five _Chenopodium amaranticolor_ L. leaves.

Buffers

Three buffers, sodium phosphate, borate and sodium citrate were studied to find out which one and at which molarity and pH could best be used to isolate the virus. Phosphate buffer was tested at pHs 5.8, 6.2, 6.6, 7.0 7.2, 7.4, 7.6, 7.8 and 0.02, 0.05, 0.08, 0.1, 0.3 and 0.5 molarities. Borate buffer was tested at pHs 7.6, 8.0, 8.4, 8.8 and similar molarities as the phosphate buffer. Sodium citrate buffer was tested at pH 3.4, 4.0, 4.6, 5.4 and 6.2 and similar molarities as above. Each buffer at each molarity and each pH was added to leaves being macerated and the crude sap inoculated onto five _Chenopodium amaranticolor_ L. plants. As control, crude sap from leaves macerated in distilled water was inoculated onto five _C. amaranticolor_ plants.

2. Clarification of the sap

Clarification aims at removing as much plant material as possible from the sap containing the virus. The effects of 8% butanol, 7% ether, 50% chloroform and

thirty-three and a third per cent carbontetrachloride were tested by adding the clarifying agents to the crude sap formed from macerated infected leaves and inoculating the mixture onto ten Chenopodium amaranticolor L. plants.

Using the results of the above tests, a method published by Wetter (1960) and modified by Noordam (1973) was used to purify the virus. Systemically infected leaves of spinach plants were harvested twenty-five to thirty days after inoculation (Fig. 1). Concentration tests had been done to determine the age which gives highest concentration of the virus. The leaves were cut with scissors, 0.2% Na_2SO_3 crystals added and ground in a commercial waring blendor. The crude sap extracted was expressed through a double layer of cheese cloth and 7% ethyl ether which had been previously shaken with 0.2% Na_2SO_3 to remove peroxides added. After mixising with a magnetic stirrer, the contents were left for about one hour and 10% ether was added once more and then stirred for five minutes.

The homogenate was clarified by centrifuging at 5000 rpm for 5 minutes in MSE-Superminor centrifuge. The fluid was poured with a pad of wadding in the stem of the funnel, first wetting the pad to stop the ether from being absorbed. 10% ether was added again, stirred for 5 minutes and then clarified by centrifuging at 5000 rpm for 5 minutes in MSe-Superminor centrifuge. The supernatant was filtered through a wet

wadding in a funnel, the pH adjusted to 7.5 with immidazole 0.2M (containing 7% ether and 0.2% Na_2SO_3) and centrifuged at 12,000 rpm for 10 minutes in MSE-superminor centrifuge.

For differential centrifugation, the supernatant was centrifuged at 30,000 rpm for 90 minutes in Beckman's L5-50 ultracentrifuge. The pellets obtained were resuspended in 3 ml of 0.01M phosphate buffer each pH 7.0, and left overnight. The suspension was clarified again by centrifuging at 10,000 rpm for 10 minutes in MSE-Superminor centrifuge and the supernatant given a high speed run as before. The pellets obtained were resuspended in phosphate buffer as before to constitute the partially purified virus preparation (Table 1).

i. Ultra-violet absorption spectrophotometry

The ultraviolet absorption spectrum of partially purified preparations of the unknown virus were determined in Beckman's M25 spectrophotometer with a slit of 0.05 mm, using 1 cm quartz cubette. The partially purified virus was diluted in 0.01M phosphate buffer, pH 7.2, and placed in one cuvette. Optical density readings were obtained at wave lengths from 230 to 300 nm intervals, using 0.01M phosphate buffer pH 7.2 as the reference standard.

Table 1: Flow chart for partial purification of spinach chlorotic spot virus (SCSV)

Step and Fraction treated	Treatment	Fraction discarded
1. Systemically infected leaves of *Spinacea oleracea* 25-30 days after inoculation	Add 0.2% Na_2SO_3 srystal and macerate in a vitamix blender; expresss through layers of cheese cloth	Pulp
2. Expressed extract	Add 7% ethyl ether containing 0.2% Na_2SO_3; stir for 5 minutes and leave to stand for (30-60) minutes. Add ether (10% of the sap volume) and stir for 5 minutes. Clarify the homogenate at 5000 rpm for 5 minutes.	Pellets
3. Supernatant	Pour the supernatant in a funnel through a wetted pad. Add more ether (10% of the sap volume) and stir for 5 minutes. Clarify once more by low speed centrifugation at 5000 rpm for 5 minutes.	Pellets
4. Supernatant	Pour the supernatant once more in a funnel through a wetted pad, adjust the sap pH to 7.6 with 0.2 M imidazole containing 7% ether and 0.2% Na_2SO_3. Centrifuge one more at 10,000 rpm for 10 minutes	Pellets
5. Supernatant	Filter the supernatant as in point 3 and 4 above. Centrifuge at 30,000 rpm in a Beckman L5-50 ultracentrifuge set at 4-8°C.	Supernatant
6. Pellets	Resuspend pellets in 0.01 M phosphate buffer pH 7.2 containing 0.5 M urea. Clarify at low speed (10,000 rpm for 10 minutes)	Pellets
7. Supernatant	Give one more cycle of differential centrifugation	
8.	Partially purified virus	

j. Electron microscopy

1. Leaf dip preparation

This was made using the technique described by Brandes (1964). A drop of 2% potassium phosphotungstate, pH 6.5, was placed on the air-dried grid using 25 gauge hypodermic needle. A fresh cut edge of infected triangular leaf-piece was held on the drop for 3 seconds and the grids left to air dry without draining the excesss stain. The supporting substrate was made by placing one drop of formovar on the surface of water in a glass dish. On the formed film, pairs of 3 mm, 200 mesh copper grids were slightly pressed against the film with a glass rod to ensure proper contact with the substrate. The grids were picked with 2.6 x 7.6 cm glass microscope slide and the films were air-dried for 2-3 hours before use.

The grids, with the virus particles were examined in Carl Zeis EM 9A model electron microscope. Particles were compared to polysterene particles (0.109 microns in diameter) as internal standards.

2. Partially purified virus

A drop of a partially purified preparation was placed on a clear surface of 2.6 x 7.6 cm glass slide. The specimen grids were then floated on the drop of 35 minutes after which they were removed and air dried. Then a drop of 2% potassium phosphotungstate, pH 6.5,

was placed on the grid. Excess liquid was removed and the grid examined in the electron microscope.

k. Source of Antiserum

The SCSV antiserum was prepared by injecting partially purified preparations of the virus intravenously and intramuscularly into a New Zealand white rabbit obtained from veterinary section of the Kenya Agricultural Research Institute, Muguga. Nine days prior to virus injections, blood for normal serum was collected by dripping, after making a cut on the marginal vein of the ear.

Initially, two injections were given, one intramuscular, consisting of 1 ml of the virus mixed with an equal amount of Freud's incomplete adjuvant and another, intravenous, consisting of 0.5 ml of the virus mixed with an equal amount of physiological saline (0.85% NaCl). Three similar intravenous injections were given at 5 days intervals.

Ten days after the last injection, the rabbit was bled on the marginal ear vein by dripping and the blood allowed to stand for 2 hours at room temperature, the clot rung and then left overnight at 4 ^{o}C. The antiserum was decanted, centrifuged at 6,000 rpm for 15 minutes in Superminor MSE centrifuge and 0.01% sodium azide added to the supernatant as a preservative. The antiserum was then kept in a deep freezer.

l. Serological test

This test was used to determine the titre of the SCSV. Two fold dilution series of the partially purified virus (antigen) and antiserum were made with sterile 0.85% NaCl in 0.01M Tris oxymethyl aminomethane buffer pH 7.0. The microprecipitin tests were done in petri dishes (10-13 cm diameter) previously coated with formvar by pouring 1% solution of polyvinyl formaldelyde in the dish and the solution immediately poured onto the next dish and the dishes left to dry for about eight hours.

Charts showing all the dilution were prepared on paper and placed below the petri dishes. Controls consisted of normal serum, crude sap from healthy spinach leaves clarified by low speed centrifugation, physiological saline and crude sap from leaves of infected spinach leaves. Combinations and controls were placed in their respective positions on the petri dishes as shown by charts. They were then flooded with paraffin oil, inocubated at 37 ^{o}C for 2 hours. The petri dishes were put at 4 ^{o}C, left overnight and examined the following day. Examination was done using the dark field illumination stereomicroscope at magnification 40 and 60x.

m. Particle "Normal length"

To determine the normal length of SCSV, the virus

particles were measured with a ruler from projected images of the photomicrograph negatives of both leaf dips and partially purified virus preparations.

RESULTS

a. Field symptoms in spinach (Spinacea oleraceae)

Field symptoms included systemic chlorotic spots in spinach. The spinach plants were mottled and severely stunted (plate 1)

Plate 1: A photograph showing mottled and stunted spinach plants on a field infected with spinach chlorotic spot virus

b. Experimental host range and symptomatology

Fifty-six cultivars in 23 species distributed among 15 genera in six families of test plants were tested for their response to SCSV following mechanical inoculation (Table 2).

Most of the plant species tested were not susceptible to infection by SCSV and the virus was not recovered from them.

Spinaceae oleracea L.

Systemic vein clearing was observed 5-7 days after inoculations. This was followed by formation of chlorotic spots which at times coalesced forming yellow patches between greenish parts of the leaf, although proper mosaic pattern was not formed. The plants became stunted, the extent of stunting depending on the age of inoculation (Plate 2 and 3).

Beta vulgaris L.

Systemic necrotic lesions were observed about eight to ten days after inoculation. The necrosis was more marked in mature leaves. At first, numerous necrotic spots developed with a brown colour but later turned red. In some plants, the whole leaf or even the whole plant turned red. Generally, there was intensification of red colour (Plate 4&5) A few leaves showed yellow spots and the plants were also stunted.

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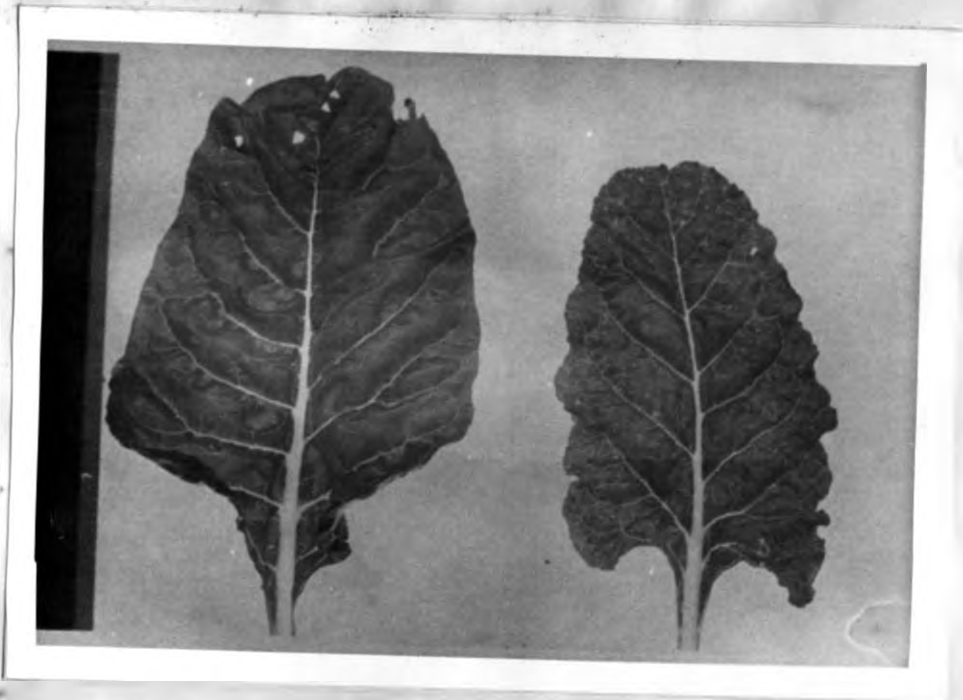

Plate 2: A photograph of a systemically infected leaf *Spinacea oleracea* (right) exhibiting tiny chlorotic spots 30 days after inoculation, compared with a healthy leaf (left)

Plate 3: A photograph of a systemically infected plant of Spinacea oleracea L. (right) showing stunting compared with a healthy plant, 60 days after inoculation (left)

Plate 4: A photograph of a systemically infected leaf of <u>Beta vulgaris</u> L. (right) showing numerous necrotic spots 30 days after inoculation compared with a healthy leaf (left)

Plate 5: A photograph of a systemically infected plant of Beta vulgaris L. (left) showing stunting 60 days after inoculation compared with a healthy plant (right)

Table 2: Response of experimental hoststo spinach chlorotic spot virus (SCSV) following mechanical inoculation

Species and cultivars	Response		incubation period in days
	Local	Systemic	
AMARANTHACEAE			
Gomphrena globosa L	NL	0	7
CHENOPODIACEAE			
Spinacea oleracea L.			
'Lucullus'	0	CS, M, ST	10
'Foordhook giant'	0	CS, M, ST	10
'Swiss chord'	0	CS, M, ST	10
Beta vulgaris L.			
'Crimson globe'	0	NL, ST	10
'Eclipse'	0	NL, ST	10
'Detroit'	0	NL, ST	10
Chenopodium amaranticolor Coste and Reyn			
C. quinoa willd	NL	TN	5
C. morale	NL	TN	5
C. album	NL	TN	5
COMPOSITAE			
Zinnia elegans jacq.			
'Dark Jewels'	0	0	
CRUCIFERAE			
Brassica oleracea L			
'Capitata'	0	0	-
Brassica rapa L.	0	0	-
CUCURBITACEAE			
Cucumis sativus L.	0	0	-
'London long green'	0	0	-
'Cool and Crisp'	0	0	-

Table 2 continued

Species and cultivars	Response Local	Response Systemic	Incubation period in days
Cucumis melo L			
'Honey Dew'	0	0	-
'Chilean Black'	0	0	-
'Sugar Baby'	0	0	-
LEGUMINOSAE			
Cassia occidentalis L.	0	0	-
Phaseolus vulgaris L.	0	0	-
'Canadian Wonder'	0	0	-
'Mexican White'	0	0	-
'Mwezi Moja'	0	0	-
'Rosecoco Small'	0	0	-
'Rosecoco Large'	0	0	-
'Rosecoco Spherical'	0	0	-
'Golden Oblong'	0	0	-
'Cream Oblong'	0	0	-
'Red Harricot'	0	0	-
'White Harricot'	0	0	-
'Light Brown'	0	0	-
'Zebra'	0	0	-
'GLP[2]'	0	0	-
'Black Harricot'	0	0	-
Pisum sativum L.			
'Green Feast'	0	0	-
SOLANACEAE			
Capsicum annum L.			
'Yolowonder'	0	0	-
Datura metal L.	0	0	-
Datura stramonium L.	0	0	-
Lycopersicon esculentum Mill			
'Beeuaty'	0	0	-
'Supermarmande'	0	0	-

Chenopodium amaranticolor

Necrotic spots which started as tiny reddish spots were formed about 5 days after inoculation. The centre became necrotic which then enlarged upto 2-3 mm in diameter.

In about 2-3 weeks systemic infection was observed. This started with younger leaves at the top, becoming chlorotic at the tips and then necrotic and the leaves withering from the leaf tips towards the base. Soon the whole tip of the plant became necrotic (Plate 6 and 7). *C. quinoa* Willd and *C. album* showed the same type of symptoms already described for *C. amaranticolor*.

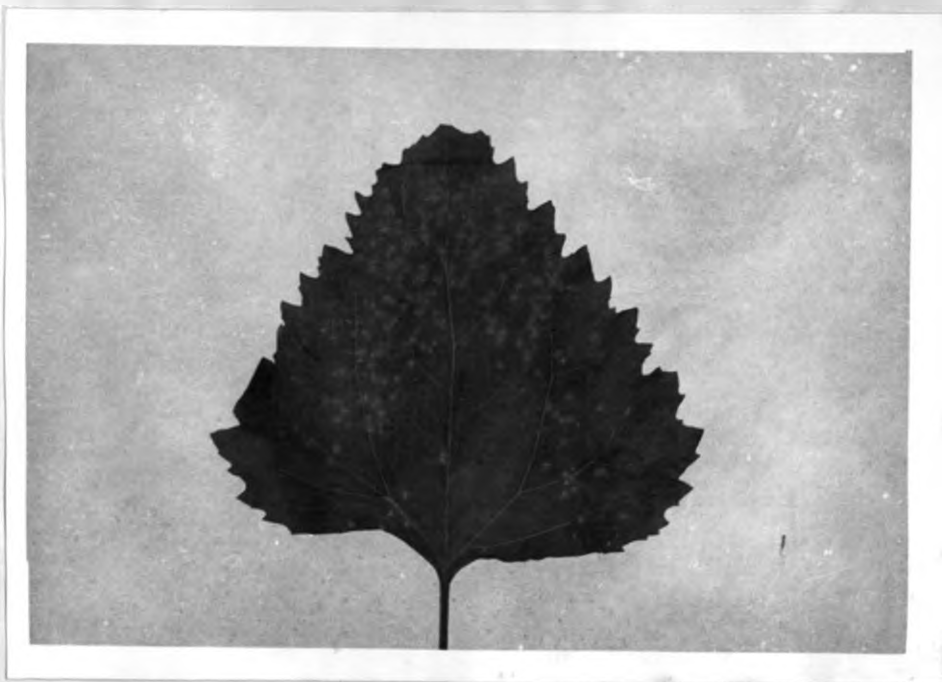

Plate 6: A photograph of an inoculated leaf of *Chenopodium amaranticolor* showing necrotic lesions, 14 days after inoculation

Plate 7: A photograph of systemically infected plant (right) of Chenopodium amaranticolor showing tip necrosis, 21 days after inoculation, compared to a healthy plant (left)

Comphrena globosa L.

White necrotic spots, about 2-3 mm in diameter were observed with reddish outline.

Zinnia elegans, *Brassica oleracea*, *B. rapa*, *Cucumis sativus*, *Cucumis melo*, *Cassia occidentalis*, *Phaseolus vulgars*, *Pisum sativum*, *Capsicum annum*, *Datura metal*, *D. stramonium*, *Lycopersicon esculentum*, *Nicotiana glutinosa*, *N. tabaccum*, *N. rustica*, *N. debney*, all did not respond to infection. Recovery inoculations done on *Chenopodium amaranticolor* revealed no virus in the plants.

c. transmission

1. Insect transmission

Chlorotic spots were observed in 10-15 days after inoculation feeding periods. Three out of four spinach plants given inoculation feedings periods of one hour and ninety-two hours both showed the chlorotic spots.

Tuber transmission

C. amaranticolor plants inoculated with juice from crushed tubers all showed systemic necrotic lesions in six days.

d. Physical properties

Crude sap preparations of SCSV were infectious at a dilution of 10^{-4} but not at 10^{-5}. The preparations

were inactivated after 10 minutes exposure between 54° and 56°C and were infectious only for one day when left at room temperature (25-29° C), but not for 2 days.

e. Purification

Reducing agents

The usefulness of various reducing agents, such as sodium sulphite and sodium thiogylcollate to prevent the virus from losing infectivity through oxidation was investigated. Sodium sulphite gave more number of local lesions (576) as compared to thioglycollic acid, although the statistical analysis gave non-significant difference between the use of the different reducing agents (Table 3).

Clarifying agents

Leaves clarified with 7% ether, inoculated on C. amaranticolor, gave the highest number of local lesions as compared to 50% chloroform, 80% butanol and ether: Carbon tetrachloride: sap, 1:1:1 ratio combination. A statistical analysis between the ether 7% and chloroform 50% gave a significant difference between the two (Table 5). Since 8% butanol and ether: carbon tetrachloride: sap combination gave no local lesions, the two were not included in the analysis. Seven per cent ether therefore poved effective as a clarifying agent for the removal of normal host contaminants.

Buffers

Buffer tests on infected leaves gave significant statistical difference between the three buffers tested: phosphate, borate and sodium citrate (Table 4). Phosphate gave the highest number of lesions compared to the three and so proved more effective as an extraction medium of the SCSV.

Table 3: Analysis of variance of observed effect on reducing agents on the extraction of spinach chlorotic virus (SCSV) from infected leaves

	Number of local lesions on *Chenopodium amaranticolor* leaves								
	Replicates								
Reducing sugars	1	2	3	4	5	6	7	8	Total
Sodium sulphate	78	102	12	180	54	75	46	29	576
0.1 Thioglycolic Acid	185	25	60	107	46	68	68	4	532
Distilled water	179	13	23	3	47	96	14	40	415
Buffer -0.01M PO4, pH 7.5	284	118	61	0	48	69	42	59	681

_0

Anova Table (Table 3)

SOURCE	DF	SS	MSS	F
Total	32	275134		
Level	1	154004.5		
"Total"	31	12333.5		
Treatment	3	4547.70		0.41
Error	28	104248.25		

Fcal: 0.41 F^{5}_{35} (0.05) :2.56

There is non significant difference between the treatments

Table 4: Analysis of variance of observed effect of sap infected with SCSV treated with various clarifying agents

	Number of local lesions			
	Agents			
Replicates	Ether 7%	Chloro- form 50%	Butanol 8%	Ether: sap: CCL_4 (Carbon tetra chloride) 1:1:1
1	1	10	0	0
2	30	13	0	0
3	13	5	0	0
4	27	23	0	0
5	37	23	0	0
6	10	12	0	0
7	4	15	0	0
8	24	12	0	0
9	12	4	0	0
10	32	5	0	0
11	13	24	0	0
12	20	21	0	0
13	7	10	0	0
14	41	10	0	0
15	52	27	0	0
16	13	7	0	0
17	2	2	0	0
18	40	7	0	0
19	30	23	0	0
20	29	18	0	0
21	10	5	0	0
22	63	1	0	0
23	17	5	0	0
24	27	19	0	0

Anova Table

Source	DF	SS	MSS	F
Total	48	23851.00		
Level	1	15229.69		
"Total"	47	8621.31		
Treatment	1	1333.52	1333.52	8.42
Error	46	7287.79	158.43	

Fcal : 8.42 F^{1}_{46} (0.05) : 4.07

There is significant difference between the treatments.

Table 5: Analysis of variance of observed effect of sap infected with SCVS treated with three different buffers at different molarities and pHs

	No. of local Lesions				
	Replicates				
Treatment No.*	1	2	3	4	Total
1	92	112	0	0	252
2	0	16	66	303	385
3	28	39	9	13	89
4	0	1	51	7	59
5	391	19	56	3	469
6	270	28	4	173	475
7	1	0	24	19	44
8	40	1	36	5	82
9	10	49	77	7	143
10	31	13	1	3	48
11	8	16	60	16	100
12	11	27	54	63	155
13	27	0	0	3	30
14	8	2	8	0	18
15	8	20	20	2	50
16	161	16	180	255	582
17	1	106	266	2	375
18	46	183	153	127	507
19	140	49	17	5	211
20	25	0	17	10	52
21	6	0	0	188	194
22	19	0	36	15	70
23	5	28	4	0	37
24	12	412	8	3	435
25	42	194	166	22	624
26	74	12	17	25	128
27	59	133	15	13	220
28	76	18	103	174	271

Table 5 continued

	No. of Local Lesions				
	Replicates				
Treatment No.	1	2	3	4	Total
29	22	21	0	20	63
30	112	185	3	103	413
31	10	89	0	6	105
32	12	29	0	0	41
33	10	17	57	7	91
34	6	19	5	4	34
35	35	19	44	3	91
36	46	50	311	31	438
37	0	9	143	12	164
38	9	2	18	25	54
39	15	12	78	18	123
40	1	3	41	0	45
41	66	9	0	4	79
42	234	0	159	36	429
43	184	35	9	22	250
44	0	73	0	28	101
45	0	5	37	33	75
46	19	31	6	9	65
47	58	17	144	44	263
48	22	261	15	18	316
49	62	10	37	9	118
50	261	12	23	13	309
51	313	8	5	62	388
52	70	8	31	36	145
53	146	4	37	38	215
54	158	179	225	64	576
55	39	220	27	45	331
56	267	3	46	0	316
57	83	41	9	44	179

Table 5 continued

Treatment No.	No. of Local Lesions Replicates 1	2	3	4	Total
58	120	57	13	22	212
59	43	17	12	0	72
60	28	30	61	5	1
61	38	1	120	3	177
62	42	38	0	37	117
63	22	27	6	35	90
64	20	10	7	15	52
65	19	83	24	9	135
66	25	3	57	6	91
67	23	38	8	14	83
68	40	22	0	27	89
69	0	166	4	55	226
70	8	25	10	20	53
71	0	9	6	19	34
72	116	78	61	85	340
73	30	107	56	64	257
74	44	64	32	118	258
75	91	166	67	108	438
76	57	38	100	35	230
77	96	22	8	34	166
78	71	33	14	17	135
79	53	27	70	66	216
80	0	0	138	69	207
81	0	16	98	0	114
82	59	77	78	0	214
83	81	217	121	104	423
84	52	27	22	48	149
85	0	22	14	60	96
86	6	46	105	64	221

Table 5 continued

Treatment No.	No. of Local Lesions Replicates 1	2	3	4	Total
87	46	2	63	11	122
88	0	98	0	49	147
89	5	144	11	0	160
90	93	81	70	58	170
91	80	49	30	12	302
92	95	32	63	63	171
93	7	81	49	19	253
94	66	70	33	49	156
95	138	81	0	73	218
96	95	90	120	75	292
97	84	46	10	26	380
98	115	45	40	85	166
99	19	58	125	50	285
100	133	28	61	57	252
101	0	20	128	55	279
102	80	146	100	56	390

* Treatment numbering explained in appendix I

Anova Table

Source	DF	SS	MSS	F
Total	365	2685508.00		
Level	1	154292.00		
"Total"	364	1531215.74		
Treatments	2	928477.21	464238.61	278.82
Error	362	602738.53	1665.01	

Fcal: 278.82 F^{2}_{362} (0.05): 3.00

There is significant difference between the treatments.

Table 6: Analysis of variance of observed effect of sap extracted from plants infected with SCSV treated with phosphate buffer at different pHs and molarities

Treatment* No.	No. of local lesions Replicates 1	2	3	4	Total
1	152	0	40	85	277
2	103	0	0	4	107
3	36	36	0	0	72
4	93	4	99	86	282
5	13	108	43	130	294
6	88	51	113	31	283
7	160	112	28	26	326
8	126	22	68	59	275
9	2	21	20	109	152
10	21	90	35	2	148
11	120	100	13	237	470
12	56	76	126	107	367
13	86	130	153	132	501
14	93	149	69	83	394
15	158	68	10	10	255
16	154	171	232	194	751
17	50	0	19	0	69
18	10	205	67	182	464
19	0	59	105	0	164
20	0	2	0	43	45
21	24	0	11	0	35
22	37	3	14	0	54
23	7	15	49	30	101
24	0	48	2	44	94

*Treatment numbering explained in appendix II

Anova Table

Source	DF	SS	MSS	F
Total	81	720576.00		
Level	1	441486.42		
"Total"	80	279089.58		
Treatment	23	136164.00	5917.57	2.36
Error	57	142985.58	1508.52	

Fcall: 2.36 F^{23}_{57} (0.05) : 1.84

Conclusion: There is a significant difference between the treatments

Anova Table

Source	DF	SS	MSS	F
Total	81	720576.00		
Level	1	441486.42		
"Total"	80	279089.58		
Treatment	23	136164.00	5917.57	2.36
Error	57	142985.58	1508.52	

Fcal: 2.36 F^{23}_{57} (0.05) : 1.84

Conclusion: There is a significant difference between the treatments

The difference between the three treatments (phosphate, borate and sodium citrate buffers) is strongly significant. Highest number of local lesions was observed when phosphate buffer was used and so phosphate is better than both borate and sodium citrate buffers. Therefore the concentration of the virus from the clarified preparation was accomplished by high speed centrifugation. A partially purified preparation of SCVS was obtained by differential centrifugation (30,000 rpm) and low speed (6,000 rpm and 12,000 rpm). The pellets were resuspended in 0.01M phosphate buffer (pH 7.2).

Although the method gave an acceptable purified preparation, the virus particles were aggregated both side by side and end by end (plate 8). When urea was added to the preparation, considerable reduction of virus particle aggregation was achieved (Plate 9).

Concentration tests results (Fig. 1) showed that the highest concentration of the virus in the leaf occurred 25 to 35 days after inoculation.

f. Ultraviolet absorption spectrum

Virus being nucleoproteins absorb ultraviolet radiation in a characeristic manner and show maximum absorption near 260 nm and a minimum at 240 [Donbrow (1967) and Hiskey (1955)]. Concentration of a purified virus and its purity can be determined. Partially purified preparation of SCSV showed a maximum

ultraviolet absorption at 256 nm with 0.01M phosphate buffer pH 7.0 used as reference standard (figure 2).

g. Electron microscopy

Electron microscopy of leaf-dip preparations from infected spinach leaf tissue showed the presence of flexible rod virus like particles, approximately 840 nm in length (Plate 11). Flexous rod virus particles forming a helix about 840 nm long were observed in the electron microscope, when partially purified preparations of SCSV, negatively stained with 2% potassium phosphotungstate pH 6.5 were examined (Plate 10). The shape and form of these rod virus particles were associated with closterovirus group.

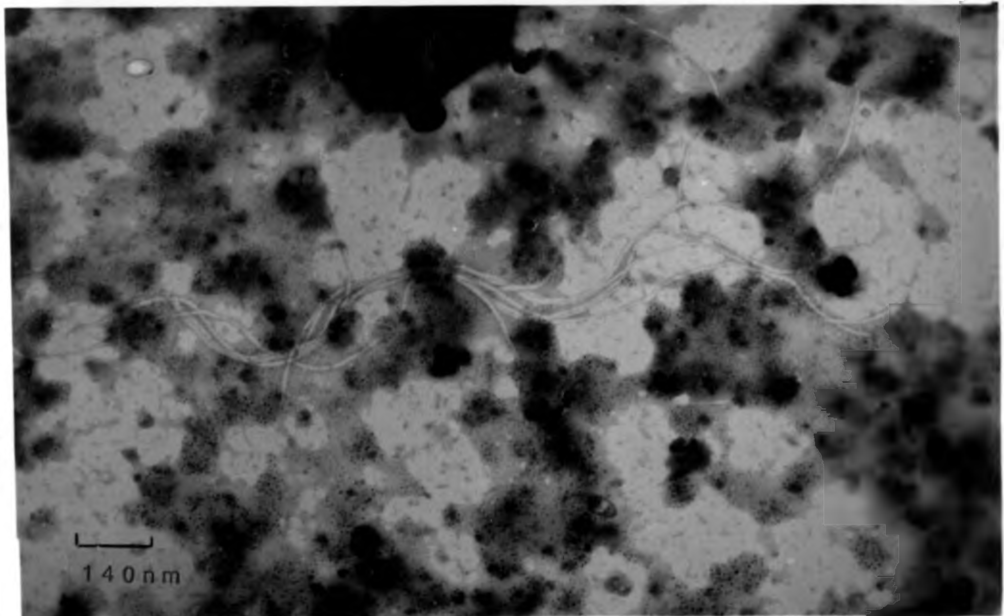

Plate 8: An electron micrograph of a partially purified preparation of SCSV (spinach chlorotic spot virus) showing lateral and end to end aggregation (magnification 45,000x)

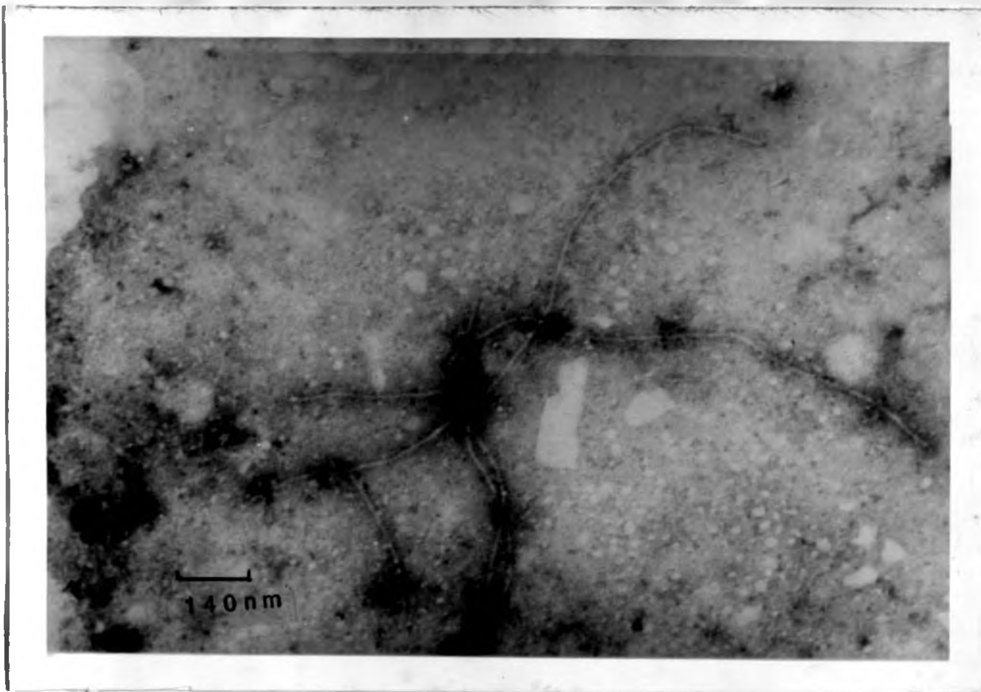

Plate 9: An electron micrograph of a partially purified preparation of SCSV, treated with urea to break aggregation (magnification 45,000x)

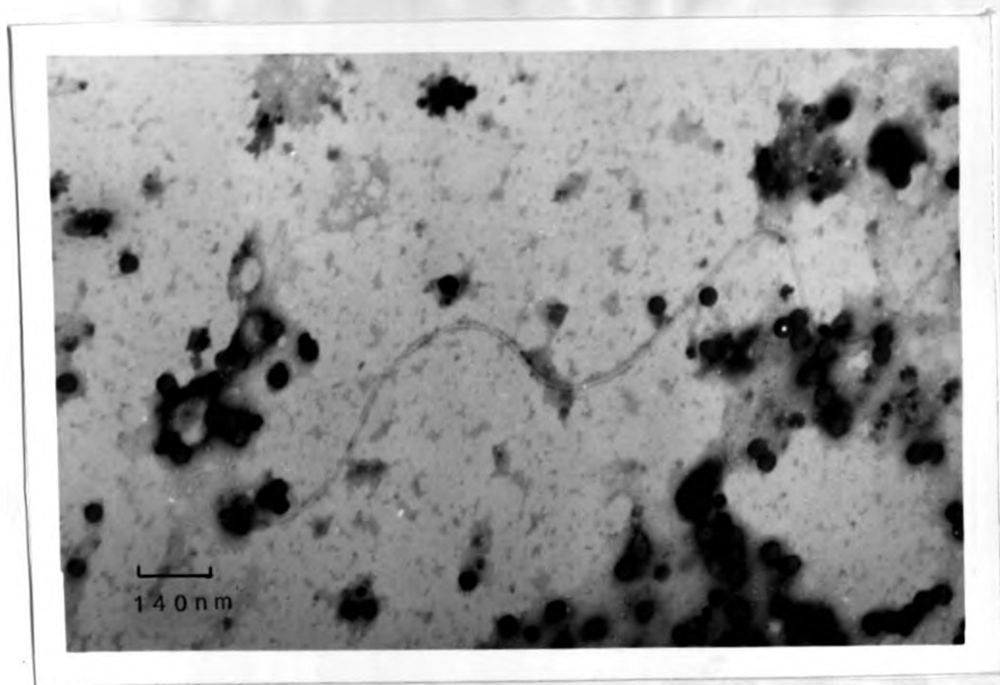

Plate 10: An electron micrograph of a partially purified preparation of SCSV showing the particle forming a helix (magnification 45,000x)

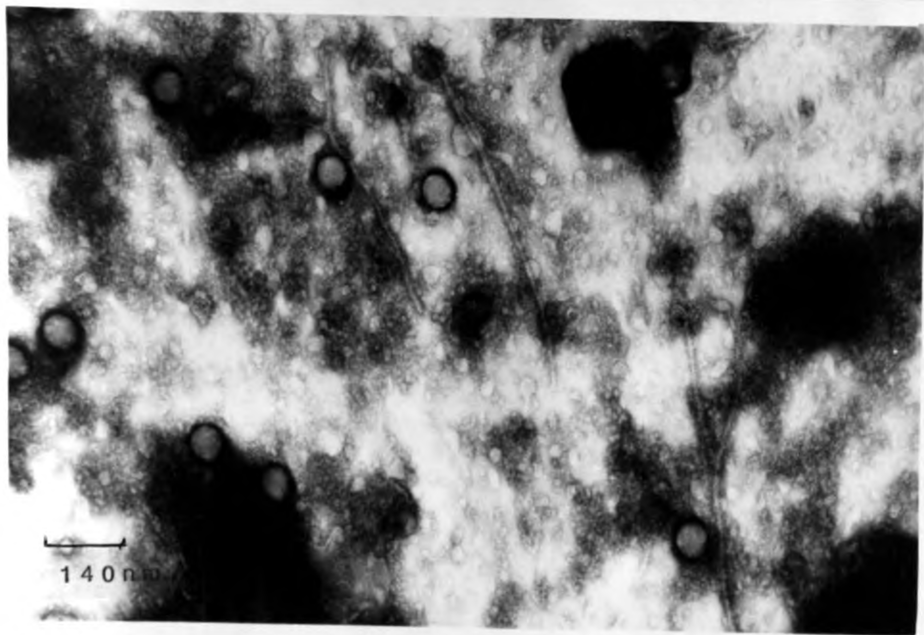

Plate 11: An electron micrograph of a leaf-dip preparation from spinach leaves showing the presence of slightly flexuous rod shaped virus particles (magnification 45,000x)

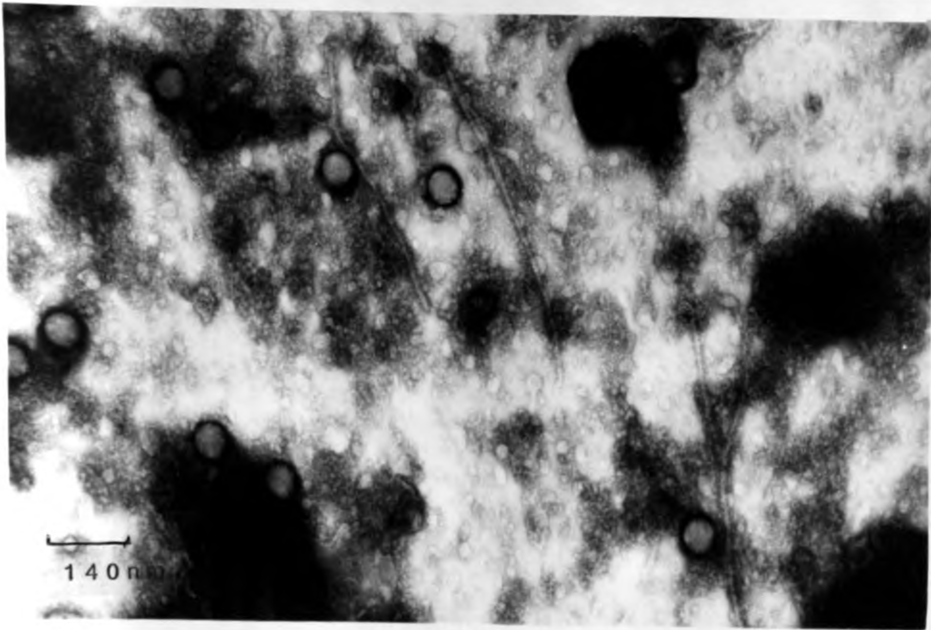

Plate 11: An electron micrograph of a leaf-dip preparation from spinach leaves showing the presence of slightly flexuous rod shaped virus particles (magnification 45,000x)

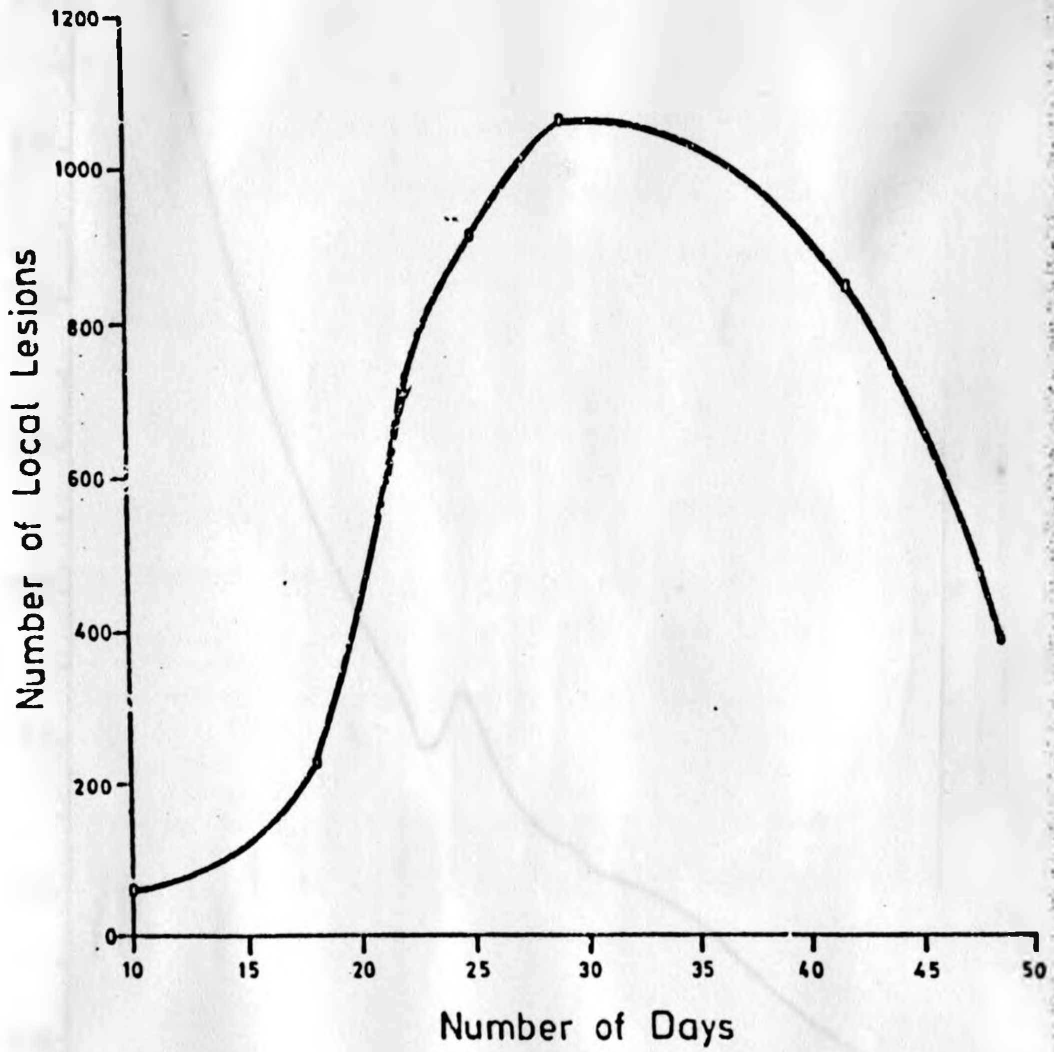

Fig. 1: Concentration of SCSV in leaves of spinach (*Spinacea oleracea*)

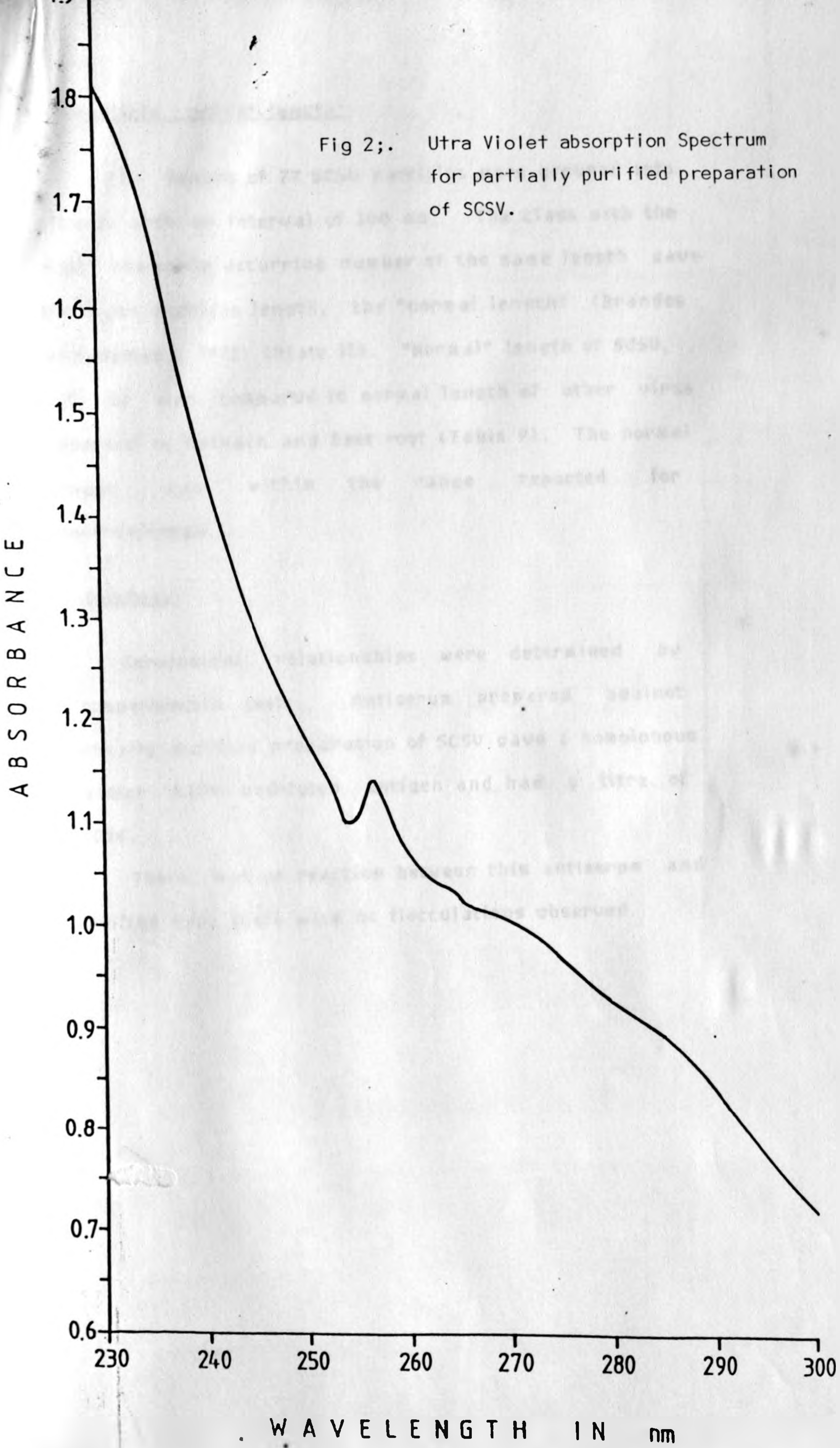

Fig 2;. Utra Violet absorption Spectrum for partially purified preparation of SCSV.

h. Particle 'normal length'

The length of 77 SCSV particles were grouped into classes with an interval of 100 nm. The class with the most commonly occurring number of the same length gave the mean particle length, the "normal length" (Brandes and Wetter, 1972) (Plate 11). "Normal" length of SCSV, 840 nm was compared to normal length of other virus reported in spinach and beet root (Table 9). The normal length was within the range reported for clostroviruses.

i. Serology

Serological relationships were determined by micropercipitin tests. Antiserum prepared against partially purified preparation of SCSV gave a homologous reaction with undiluted antigen and had a titre of 1/1024.

There was no reaction between this antiserum and clarified sap; there were no flocculations observed.

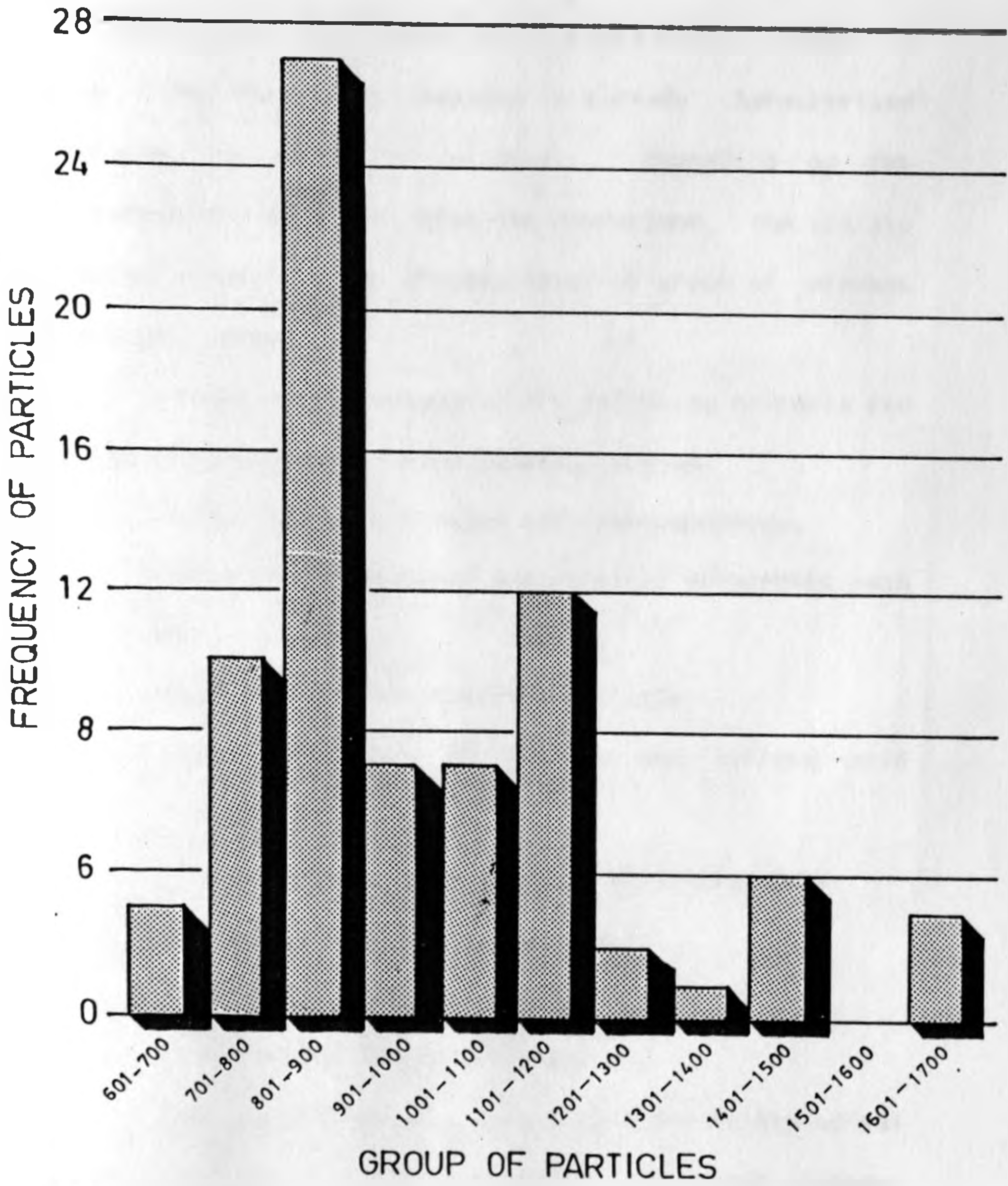

Plate 12:

A histogram showing the distribution of SCSV particle lengths.

DISCUSSION

Characterization and identification of plant viruses depend on a number of criteria (Ross, 1964). A new virus isolate is compared to already characterized viruses or group of viruses. Depending on the information obtained from the comparison, the isolate may be a strain of an already existing group of viruses or a new virus.

Knight (1963) suggested the following criteria for establishing relationships between strains:

(a) Similarity in host range and symptomotology;

(b) similarity in physical and chemical properties such as:

(i) same size and shape of the particle;

(ii) same proportion of protein and necleic acid component;

(iii) sample proportion of necleic acid components;

(c) similarity in method transmission;

(d) positive serological cross reaction;

(e) positive cross protection reaction.

The production of symptoms demonstrate biological activities of the virus particles in plant disease (Mathews, 1970). The activities of a particular protein, coded by the virus RNA are reflected by symptoms. The symptoms are observed on a large number

of hosts confirmed by recovery tests on assay hosts and serve as criteria in virus identification.

Broad bean wilt virus (BBWV) (Provvidenti and Schreoder, 1970) and cucumber mosaic virus (CMV) (Bailiss and Okonkwo, 1979) on spinach are therefore differentiated from spinach chlorotic spot virus (SCSV) on the basis of symptomotology and host range studies. Other viruses, differentiated on the same ground are: tomato spotted wilt virus (TSWV) causing necrosis (Bailiss and Okonkwo, 1979); spinach mosaic virus (SMV) causing a mosaic disease (Naqui and Mahwood, 1975); beet curlytop virus (BCTV) (leach, 1969) and spinach latent virus (SLV) (Bos L. et al., 1980). Yellows diseases are caused by (SCSV) tobacco rattle virus (TRV) (Komuro et al.,1970); lettuce mosaic virus (LMV (Provvidenti and Schroeder, 1972); parsnip mosaic virus (PMV) (Murant et al., 1976); beet yellows virus (BYV) (Watson, 1942 and Hull, 1950); beet western yellows (BWYV) (Duffus, 1960); beet mosaic virus (BMV) (Naqui, et al., 1975); beet necrotic yellow vein (BNYV) (Tamada et al., 1970); spinach yellow mottle virus (SYMV) (Kurppa et al., 1981) and spinach yellow dwarf virus (SYDV) (Halliwell et al.,1973). Beet western yellows virus is differentiated from (SCSV) because it has isometric particles, 26 nm in diameter.

The comparison of SCSV host range, physical properties, and particle size with literature data

reported for the above listed viruses causing yellows disease in spinach (Table 7, 8 and 9) reveal considerable differences.

Table 7: Comparative study of spinach chlorotic spot virus response with other literature reported viruses on spinach

Host	Response of Viruses*												
	BMV	BNYV	BWY	BYV	BBWV	CMV	LMV	PMV	PYFV	TRV	SYDV	SMV	SCSV
Beta vulgaris L	VC M P	CLL SCL	ST C	VC VE RNS RNS	NR	NR	NR	NR	NR	NR	NR	NO	VC RNS
Spinacea oleracea L	YF C ST	Y M ST	ST C	Y	S	B	CLL M ST	S	CLL SYF M Y	Y M	Y ST	NO	VC Y ST
Chenopodium quinoa Willd	CLL	CLL NLL	NR	NR	CLL SCL	NLL CLL	CLL ST SYVF	NLL Y	NLL SNF	NLL SN	NR	NR	NLL SCL TN
Chenopodium amaranticolor Coste & Reyn	NR	CLL NLL	NR	NR	NR	NLL CLL	SYVF CLL	NLL CLL	NLL	SN	NR	NR	NLL SCL TN
Gomphrena globosa L.	CLL	NR	NR	NR	NR	NR	NLL	NR	NR	NF D	NR	NR	NLL
Nicotiana clevelandii Gray	NR	NR	NR	NR	NR	NR	CLL Y	NR	CLL M NLL SV N	NR	NR	NR	NT

*Viruses full names appear in appendix V

ABBREVIATIONS FOR SYMPTOM DESCRIPTION

Abbreviation	Description
VC	Vein clearing
M	Mottle
P	Puckering
YF	Yellow Fleck
C	Chlorosis
ST	Stunting
CLL	Chlorotic local lesions
NR	Not reported
SCL	Systemic chlorotic lesions
S	Susceptible
NLL	Necrotic local lesion
Y	Yellowing
VE	Vein etch
RNS	Red necrotic spot
BNS	Brown necrotic spot
B	Blight
SYVF	Systemic yellow veinol fleck
SNF	Systemic necrotic fleck
SVN	Systemic veinol necrosis
SN	Systemic necrosis
D	Distortion
NF	Necrotic flecking
TN	Tip necrosis
NT	Not tested

Table 8: Comparative "normal lengths" of SCSV particles and some viruses reported in spinach and beet root

Virus *	Normal length (nm)	Author
BMV	730	Lind, 1915, Robins, 1921 and Smith, 1957
BMYV	110, 270, 390	Tamada et al., 1970
BWY	Isometric particles	Duffus, 1960
BYV	(800-1300)	Roland, 1983, Watson, 1942, Hull, 1950
BBWV	Isometric particles	Stubbs, 1947, 1947, 1960
CMV	Isometric particles	Doolittle, Jagger, 1916
IMV	750	Jagger, 1921
PMV	730-760	Murant et al., 1970
PYFV	Isometric particles	Murant and Goold, 1968
TRV	190, 45-115	Quanjer, 1943
SYD	250	Thomas et al., 1953
SMV	NR	Naqui and Mahmood, 1975
SCSV	840	(Virus under study)

*Full virus names appear in appendix V

Among the above listed viruses, LMV, PMV and BMV are classified as potyviruses, BYV as cleostrovirus and TRV as tobravirus while BNYV is classified as tobamovirus (Mathews, 1981). Potyviruses, cleostrovirus, tobraviruses and tobamoviruses are differentiated by the modal length of their particles, stability in crude sap and transmission method. Members of tobravirus group have two modal lengths, 180-210 nm length (L) and 46-114 nm (S) length and are transmitted mainly by nematodes (Paratrichodorus and Trichodorus spp). Tobamoriruses are stable particles, 300 nm long and 118 nm wide. Tobamoviruses are readily mechanically transmitted, but they are not transmitted by common vectors such as aphids and whiteflies. Potyviruses have longer particles (680-900 nm) than the tobravirus and tobamoviruses but cleosterovirus have the longest, 600-2000 nm long. Potyviruses are easily mechanically transmitted in a non-persistent manner, while cleostrovirus are transmitted mechanically with difficulty and by aphids in a semi-persistent manner.

The particles of cleostrovirus are very unstable, found joined together in pairs of upto ten cross-bridges and normally aggregated in cross bands in phloem cells.

SCSV, having a normal length between 800-900 nm, belong to cleostrovirus group with 600-2000 nm and not potyvirus group with 680-900 nm long rods and tobravirus group with 180-215 nm and 46-114 nm long rods or tobamovirus (300 nm). Transmission method also groups

SCSV into cleostrovirus group since it is mechanically transmitted with difficulty as members of cleostrovirus group. Potyviruses and tobamoviruses are readily mechanically transmitted. Vector transmission of SCSV is by aphids in a semi-persistent manner as is true with members of cleostrovirus group and not by aphids in a non-persistent manner as in potyviruses. There are no vectors in tobamovirus while tobraviruses are transmitted by nematodes. Therefore it should be concluded that by its modal length, method of transmission and stability, SCSV is a cleostrovirus. The only member of cleostrovirus reported to cause yellows disease in spinach is beet yellows virus (BYV) (Roland, 1966; Watson, 1942 and Hull, 1950). The symptoms caused on spinach by BYV are more prominent. Vein clearing and necrosis may also occur. In sugar beet (*Beta vulgaris*), the symptoms vary depending on the strain of the virus involved, variety attacked, light conditions and possible other factors (Leach, 1969). Some strains rarely or never produce vein clearing and those that do, do not do so under all conditions. Intensity of yellowing of affected plants also varies depending on the strains of virus. A few selections have shown marked necrosis in mature leaves and plants with red pigment show an intensification of red colour. Roland (1936), Watson (1942) and Hull (1950), reporting diagnostic hosts of the virus said that young leaves of

Beta vulgaris, of glasshouse plants often showed vein clearing and vein-etch; older leaves of field and glasshouse became yellow, thickened and brittle and usually had numerous small red brown necrotic spots. SCSV produced systemic vein clearing in 5-7 days after inoculation. This was followed by formation of chlorotic spots which at times coalesced, forming yellow patches. The plants became stunted, the extent of stunting depending on the age at the time of inoculation. In *Beta vulgaris*, systemic necrotic lesions were observed about eight to ten days after inoculation. the necrosis being more marked in mature leaves. There was an intensification of red colour; the necrotic spots were numerous and mostly brown in colour at first but later turned red. A few leaves showed yellow spots and some were stunted. Thus on the basis of symptomatology, SCSV appeared to be related to BYV. Preliminary mechanical inoculation showed SCSV as having host range restricted to chenopodiacea, that is *Chenopodium quinoa*, *C. amaranticolor*, *Spinacea oleracea* and *Beta vulgaris*. BYV also has a narrow host range mainly restricted to chenopodiaceas (Roland, 1936).

The method of transmission is usually similar for related viruses. SCSV is transmitted mechanically by aphids and through tubers, while BYV is also sap and aphid transmitted (Roland, 1936). There was, however, no report on tuber transmission. In 1927, Johnson recognised that characterization of plant viruses by

means of host range and symptomatology presented some difficulties. Viruses of the same group or closely related viruses or strains have similar properties with regard to virus stability and concentration (Ross, 1964). Johnson (1927), therefore, suggested the use of physical properties, that is, dilution end point (DEP), longevity in Vitro (LIV) and thermal inactivation point (TIP) in the identification and characterization of mechanically transmitted plant viruses. The results obtained from such properties should be considered as relative as the properties depend on a number of factors (Ross, 1964), such as:

1. Sensitivity of assay host;
2. environmental conditions prevailing;
3. concentration of virus in the source host;
4. presence or absence of inhibitors in the inoculum; and
5. methods of inoculation.

Dilution end point indicates the concentration of the virus in the crude sap. Thermal inactivation point indicates the stability of the virus during ten minute exposure to a specific temperature while longevity in vitro refers to the stability of the virus to aging at room temperature.

Crude sap preparations of SCSV were infectious at a dilution of 10^{-4} but not at dilution of 10^{-5}. The preparations were inactivated after ten minutes exposure

between $54^{o}C$ to $56^{o}C$ and were infectious only for one day when left at room temperature ($25-29^{o}C$) but not for two days (Table 9). The crude sap kept in deep freeze was infective for all the 60 days during which the study was conducted.

These results correspond closely with values of physical particles, DEP, TIP and LIV reported from BYV, that is DEP is 10^{-4} for SCSV, the same is for BYV; TIP for BYV is $55^{o}C$, while SCSV is between 54° and $56^{o}C$. LIV for BYV is one day and for SCSV is also one day.

SCSV presented some difficulty in early attempts to purify it. Therefore a detailed study of each step in purification was undertaken. Sodium sulphite proved to be effective as a reducing agent, giving more local lesions in the test than thioglycollic acid. Reducing agents aid in preservation of viruses that readily lose infectivity through oxidation (Mathews, 1970).

Although statistics gave non-significant differences between the treatments, sodium sulphite, which is used in a method described by Wetter (1960) and adopted for purification of the virus, was an acceptable alternative.

Table 9: Comparative physical properties studies in crude sap of SCSV and other viruses reported in spinach and beet root

Virus *	Dilution end point	Thermal inactivation point (°C)	Longivity in vitro (days)	Author
BMV	10^{-4}	55-60	1-2	Lind, 1915, Robbins, 1921 and Smith, 1957
BNYV	10^{-4}	65-70	5-8	Tamada *et al.*, 1970
BWY	10^{-8}	65	16	Duffus, 1960
BYV	$10^{-4}-10^{-5}$	55	1	Roland, 1936; Watson, 1942; Bull, 1950
BBWV	$10^{-4}-10^{-5}$	58	2-3	Stubbs, 1947, 1960
CMV	10^{-4}	70	3-6	Doolittle, Jagger, 1916
LMV	$10^{-1}-10^{-2}$	55-60	1-2	Jagger, 1921
PMV	$10^{-3}-10^{-4}$	55-58	7-10	Murant *et al.*, 1970
PYFV	$10^{-3}-10^{-4}$	57.5-65	4-7	Murant and Goold, 1968
TRV	10^{-6}	80-85	6 weeks	Quanjer, 1943
SYD	10^{-1}	50	1	Claudel, et al., 1953
SMV	$10^{-4}-10^{-5}$	70-75	2	Naqui and Mahmood, 1975
SCSV	10^{-4}	54-56	1	(Virus under study)

*Full virus names appear in Appendix V

A study of the use of buffers in the purification procedure gave significant differences between the three buffers tested, namely, phosphate, borate and sodium citrate. Phosphate proved more effective with highest number of local lesions in the test. Phosphate buffer, was, however, only used in resuspension of the virus particles after high speed centrifugation since Wetter's method (1960) adapted for the purification did not use a buffer in early stages of purification. This presented no doubts since tests done involving leaf maceration when reducing agents and buffers are added as extraction media gave non-significant differences.

Ether proved to be the best clarifying agent compared to butanol, chloroform and carbon tetrachloride. A significant difference was observed when the above agents were tested. Therefore considering the above results, the method described by Wetter (1960) and modified by Noordam (1973) gave satisfactory purification results.

Electron microscopy of partially purified preparations of SCSV showed that the virus particles associated with infectious entity were very flexuous and filamentous in shape and had normal length of 840 nm which is in agreement with viruses of cleostrovirus group. Leaf dip preparations gave similar results.

SCSV is a weak immunogen, having a titre of 11024. BYV was also reported to be weakly immunogenic.

CONCLUSION

SCVS, a possible strain of BYV, was first noticed during 1974 in spinach growing areas of Nairobi District and adjacent areas. The virus was isolated from Spinacea oleracea on which it induced yellow spots.

Study and characterization of the virus was carried out through symptomatology, host range, transmission, ultraviolet absorption, electron microscopy and serological reactions.

When the results were compared to other viruses causing diseases in spinach, the following conclusions were reached:

. SCSV has flexious filamentous, rod shaped particles and different from BWYV because it has isometric particles;

. By its modal length of 840 nm, its transmissibility by Myzus persiea in a semi-persistent manner and mechanically with difficulty, is a member of cleostrovirus group, of which only BYV has been reported to cause yellows disease in spinach;

. SCSV showed a narrow host range, being limited to the family chenopodiaceae. It induced vein clearing, yellow spots and stunting in Spinacea oleracea, systemic necrotic red and brown lesions in Beta vulgaris, necrotic local lesions and tip

necrosis in *Chenopodium amaranticolor* and *C. quinoa* and induced local lesions in *Gomphrena globosa*.

Therefore, on the basis of host range, symptomatology, physical properties studies and electron microscopy, SCSV is related to BYV and is probably a strain of the virus.

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APPENDICES

Appendix I: Tables explaining the treatment numbering in Table 5

Phosphate buffer

	pH							
Molarity	5.8	6.2	6.6	7	7.2	7.4	7.6	7.8
0.02	1	7	13	19	25	31	37	43
0.05	2	8	14	20	26	32	38	44
0.08	3	9	15	21	27	33	39	45
0.1	4	10	16	22	28	34	40	46
0.3	5	11	17	23	29	35	41	47
0.5	6	12	18	24	30	36	42	48

Appendix II: Table explaining the treatment numbers in Table 5

Borate buffer

	pH			
Molarity	7.6	8.0	8.4	8.8
0.02	49	55	61	67
0.05	50	56	62	68
0.08	51	57	63	69
0.1	52	58	64	70
0.3	53	59	65	71
0.5	54	60	66	72

Appendix III: Table explaining the treatment numbers in Table 5

Sodium Citrate Buffer

	pH				
Molarity	3.4	4	4.6	5.4	6.2
0.02	73	79	85	91	97
0.05	74	80	86	92	98
0.08	75	81	87	93	99
0.1	76	82	88	94	100
0.3	77	83	89	95	101
0.5	78	84	90	96	102

Appendix IV: Table explaining the treatment numbering in Table 6

	pH							
Molarity	5.8	6.2	6.6	7	7.2	7.4	7.6	7.8
0.3	1	2	3	4	5	6	7	8
0.5	9	10	11	12	13	14	15	16
1	17	18	19	20	21	22	23	24

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Appendix V: Virus abbreviations written in full

BMV	Beet mosaic virus (Lind, 1915, Robbins, 1921 and Smith 1957)
BNYV	Beet necrotic yellow vein (Tamada *et al.*, 1970)
BWY	Beet western yellows (Duffus, 1960)
BBWV	Broad bean wilt virus (Stubbs, 1947, 1960)
CMV	Cucumber mosaic virus (Doolittle, Jaggar, 1916)
LMV	Lettuce mosaic virus (Murant Munthe, Goold, 1968)
PYFV	Parsnip yellow fleck virus (Murant and Goold, 1968)
TRV	Tobacco rattle virus (Quanjer, 1943)
SYD	Spinach yellow dwarf virus (Thomas, C. E., *et al.*, 1953)
SMV	Spinach mosaic virus (Naqui, Q. R. and Mahmood, K., 1975)
SCSV	Spinach chlorotic spot virus (Virus under study)