VIRUS IDENTIFICATION AND ELIMINATION BY MERISTEM-TIP CULTURE IN ZAMBIAN CASSAVA (*Manihot esculenta Cranz*) LANDRACES

By

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DECLARATION

I VINCENT MKUYAMBA, do hereby declare that this dissertation represents my own work and that, to the best of my knowledge, it has not been previously submitted for the award of a degree at this or any other University.

Signed:----------

Date:12/06/95

APPROVAL PAGE

This dissertation of Vincent Mkuyamba is approved as fulfilling part of the requirements for the award of the degree of Master of Science in Agronomy by the University of Zambia.

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ABSTRACT

The production of cassava in Zambia is concentrated in the Western, North Western, Northern and Luapula provinces. Pests and diseases have been listed among the major constraints to increased production of the crop. The present study was done to identify the viruses attacking cassava in Zambia and to evaluate the efficacy of heat treatment of meristem donor plants in regenerating virus-free cassava plants through meristem-tip culture. Cassava leaf samples bearing virus disease symptoms were collected from different locations and tested by triple antibody sandwich enzyme linked immunosorbent assay (TAS-ELISA) and immunosorbent electron microscopy (ISEM). Meristem-tips obtained from heat treated mother plants (37°C for 4 weeks) were cultured on a modified Murashige and Skoog (MS) medium. Indirect (TAS)-ELISA confirmed the presence of a geminivirus, African cassava mosaic virus (ACMV) in all varieties tested. Additionally, in the variety "Kapumba" a second and only recently discovered virus called cassava Q virus (CQV), was detected by ISEM. Based on the number of shooting meristems, results revealed 40% higher shoot induction on meristem-tips derived from heat treated mother plants compared to meristem- tips obtained from untreated donor plants. On the other hand basal callus formation was lower for meristem-tips arising from heat treated donor plants in comparison to the meristems arising from untreated mother plants. The study shows that heat treatment of meristem donor plants does enhance shoot regeneration of cassava plantlets as well as improve virus elimination. Lower basal callus formation allowed for better shoot growth and plantlet regeneration.

DEDICATION

To my dear brother Edward and to my departed friend Shalo Matandiko.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACMV	African Cassava Mosaic Virus
ANOVA	Analysis of Variance
BAP.....	Benzyl amino purine
CEC.....	Cation Exchange Capacity
CMD.....	Cassava Mosaic Disease
COSCA.....	Collaborative Study On Cassava in Africa
CQV.....	Cassava Q Virus
DMRT.....	Duncan's Multiple Range Test
EACMV.....	East African Cassava Mosaic Virus
ELISA.....	Enzyme Linked Immuno-Sorbent Assay
GA3.....	Giberellic Acid
ICMV.....	Indian Cassava Mosaic Virus
IITA.....	International Institute of Tropical Agriculture
ISEM.....	Immuno Sorbent Electron Microscopy
LRRS.....	Luapula Regional Research Station
LSD.....	Least Significant Difference
mAb.....	Monoclonal Antibody
meq.....	Milli-equivalents
MS.....	Murashige and Skoog medium (1962)
NAA.....	Naphthalene acetic acid
PBS.....	Phosphate Buffered Saline
ppm.....	Parts per million

PVP.....	Polyvinyl Pyrrolidone
PVS.....	Potato Virus S
PVX.....	Potato Virus X
RTIP.....	Root and Tuber Improvement Programme
SCCI.....	Seed Control and Certification Institute
SCRI.....	Scottish Crops Research Institute

1.0 INTRODUCTION

1.1 Cassava Production

Cassava is a vital crop for many developing countries primarily as a food source and has more recently begun to assume greater importance as an export product in some countries (Nestel, 1973). On the African continent, cassava has continued to play a major role in alleviating famine conditions by providing a sustained food supply where other crops have failed, largely the result of its ability to adapt to diverse environmental conditions and farming systems (Chitundu and Soenarjo, 1993). Cassava requires few production skills and can be grown with only a limited amount of inputs making it an ideal crop for resource poor small scale farmers (Hahn et al. 1979).

A collaborative study on cassava in Africa (COSCA), was started in 1988 as an initiative of the International Institute of Tropical Agriculture (IITA) with the objective of collecting authoritative information on African cassava production systems, processing methods, consumption patterns and market prospects. Findings of COSCA in Zambia indicate that cassava production in the country is increasing by land area as well as in the quantities being produced (Anon, 1993a). In Zambia, the Root and Tuber Improvement Program (RTIP) located at the Luapula Regional Research Station (LRRS) in Mansa, has the mandate to carry out research and development of root crops in the country. Among its various activities the annual provision of free cassava and sweet potato cuttings to intending growers has had a very stimulating effect on the production of the crops (Anon, 1993a).

Over the past five years the demand for cassava and sweet potato planting material by farmers from the RTIP has increased considerably. In the 1993/94 season, RTIP supplied a total of 320,000 cuttings to cassava growers compared to 62,000 cuttings supplied in the 1988/89 season. Annual cassava production in Zambia is estimated to be in excess of 240,000 mt (Chitundu and Soenarjo, 1993). The main cassava growing areas in Zambia are found in the Western, North Western, Luapula and Northern provinces where production is done primarily by small scale farmers (Simwambana, 1982).

1.2 Justification

Pests and diseases are ranked high among the major factors contributing to low cassava yields and have placed a handicap on efforts to further expand production of the crop in the country. Mosaic disease symptoms probably caused by virus infections are fairly widespread on most of the cassava cultivars presently grown in Zambia. Results from the observations and field screening of over 700 cassava clones grown at various sites in the country showed that up to 80% of the local cultivars were susceptible to cassava mosaic disease (Anon,1993b). Other data from various variety trials conducted in the country by RTIP revealed yield losses in the range of 33-37% when susceptible clones were planted (Anon, 1993b). However, no study has been done to clearly identify the viruses infecting cassava in various parts of Zambia.

While there has been reports making reference to the presence African cassava mosaic virus (ACMV) in Zambia estimates of losses attributed to ACMV in the country are still unknown. The ACMV, a geminivirus transmitted by the whitefly (*Bemisia tabaci*) is reported to cause losses in yield in the range 20-90% in other countries (Hahn et al. 1979).

The perpetuation of cassava mosaic disease (CMD) from one season to the other in the country is largely through the use of infected planting material. Cassava growers in Zambia have been particularly vulnerable to the disease owing to their continued reliance on cuttings obtained from their old and in most cases infected fields for planting in subsequent seasons (Anon, 1993a). While efforts to introduce varieties resistant to cassava mosaic disease from IITA have continued little has been done in Zambia to produce disease-free cassava clonal stocks using tissue culture techniques as a way of supplementing the breeding efforts.

1.3 Rationale

Simwambana (1982) states that even under traditional farming methods the current low yields obtained by Zambian farmers could be increased significantly by using disease-free planting material. Meristem-tip culture has been used successfully to obtain `specific pathogen-free' plants in many crop plants (Bhojwan and Razdan, 1983). The success of meristem-tip culture in eliminating viruses in crop plants is based on the observation that viruses may not be present in the cells of the meristem or may only be present in very low titre (Vasil, 1990). Physical elimination of the virus by excision and culture of the shoot meristem with only one or two leaf primordia was confirmed by Bhojwan and Razdan (1983). The same authors stated that through micro-propagation techniques virus-free meristems can be rapidly multiplied in culture to regenerate whole plants which can be kept healthy through the provision of an insect free environment.

The objectives of the present study were as follows:

1. To identify virus isolates responsible for cassava mosaic disease in various parts of Zambia.
2. To compare virus elimination by meristem-tip culture with the heat treatment method in local cassava landraces.

2.0 LITERATURE REVIEW

2.1 Importance and constraints in cassava production

Cassava ranks fourth as the most important source of food in the tropics after rice, wheat and maize (Cock, 1982). On the African continent in 39 countries the crop is grown exclusively as a staple food crop. Nigeria and Zaire stand among five of the world's largest cassava-producing countries. Sub-Saharan Africa currently plants more than one-half of the world's cassava area, but because of low yields it produces less than 40% of the worlds output (Hahn et al. 1979). Some of the cited constraints to increased cassava production in Africa are insect pests, diseases, weeds, soils, cultural and socio-economic factors. In addition the transportation of harvested tuberous roots and of planting material is difficult because of bulkiness. The tuberous roots and processed products are low in protein and cannot be easily stored for a long time (Cock,1982).

Annual cassava production in Zambia is estimated to be in excess of 240,000 metric tonnes (Chitundu and Soenarjo, 1993). The main cassava producing areas in Zambia are Western, North western, Luapula and Northern provinces where production is done primarily by small scale farmers (Simwambana ,1982). Pests and diseases are among the main factors leading to low cassava yields and have placed a handicap on efforts to further expand the production of the crop in the country (Anon, 1993a). Mosaic disease symptoms probably caused by virus infections are fairly widespread on most of the cassava cultivars presently grown in Zambia. The same author further states that cassava growers in Zambia are particularly vulnerable to Cassava Mosaic Disease (CMD) owing to their continued reliance on cuttings obtained from their old and in most cases infected fields for planting in subsequent seasons.

2.2 Virus identification

Many of the world's economically important virus diseases of crops in tropical and subtropical countries are caused by geminiviruses transmitted by either whitefly (*Bemisia tabaci*) or leafhopper *(Cicadulina spp.*) vectors. Cassava mosaic disease (CMD) is the most widespread economic disease of cassava in Africa owing to the magnitude of losses in the yield of infected cassava plants (Hahn et al., 1979). In Africa alone, the value of crop loss resulting from CMD is estimated to exceed two hundred million British pounds annually (Harrison et al., 1990). While Cassava Brown Streak (CBS) has been reported in East Africa, it is still not considered a major threat to cassava cultivation in this region. Recent studies on cassava mosaic disease in Africa and the Indian sub-continent have shown that the disease is caused by three distinct geminiviruses, ACMV, EACMV and ICMV, which all have different geographical distributions but all transmissible by the whitefly *Bemisia tabaci* (Harrison et al., 1990). These viruses can be detected and distinguished by serological tests.

Storey and Nichols (1938) reported wide variations in the symptoms of CMD and attributed these variations to differences in the strains of the pathogen. The symptoms of CMD are characteristic of a mosaic disease, showing primarily the chlorosis of discrete areas of the leaf lamina and an overall distorted, twisted and small sized leaf with bright yellow spots. Transmission of a cassava mosaic virus by a species of whitefly was first reported by Ghesqueiere (1932) in the Belgium Congo as cited by Hahn et al. (1979).

Both the vector and the disease are prevalent in all parts of East, West, Central and Southern Africa. This is probably why the spread of the disease and seasonal infection pressure are

closely related to vector behaviour, its migration and population dynamics. Chant (1958) observed that non-virus carrying whiteflies had to feed for at least 4 hours on the young leaves of mosaic infected cassava plants to become viruliferous and thus be able to transmit the disease following a minimum feeding period of 15 minutes. Under field conditions spread by the vector is done when the population is high.

2.2.1 Enzyme Linked Immuno Sorbent Assay (ELISA)

Serological methods for the detection of pathogens, particularly Plant viruses have been available for many years. ELISA provides high sensitivity, ease of use and speed compared to other detection methods (Bhojwan and Razdan, 1983). The binding of antigen and specific antibody is made visible through the use of a "tag" enzyme which acts upon its substrate to generate a coloured product. Antibodies are the most critical components of this immunoassay and therefore standardised antibody reagents with the appropriate specificity and sensitivity are required for consistent, interpretable results. ELISA techniques have been used successfully for the detection of plant viruses (Ng, 1992). Sequeira and Harrison (1982) in their serological studies of cassava mosaic geminiviruses applied ELISA techniques to detect the viruses.

2.2.2 Immuno Sorbent Electron Microscopy (ISEM)

Immuno sorbent electron microscopy techniques combine the techniques of electron microscopy and serology involving the detection of complexes of antigen (virus) and specific antibody. In this procedure, support films are precoated with specific antibody to which homologous virus particles in the tissue extract become attached and concentrated. The

method is usually used to detect viruses preset in host plant's sap in concentrations too low to permit detection in conventional electron microscopy. Sequeira and Harrison (1982) state that this technique gives an effective concentration of virus particles on the microscope grid and may sometimes be used to trap more than one virus at once. The author was also able to use the method to effectively compare serological relationships between different virus strains.

2.3 Production of virus free planting material

2.3.1 General considerations

Plant tissue culture as a field of plant biotechnology has continued to contribute steadily to world wide crop improvement efforts. A study by Murashige (1990) estimated that there are about 1000 plant species that can be propagated by tissue culture, with about 300 commercial laboratories world wide already engaged in micropropagation. An increasing number of Universities, Government institutions, research venture companies and botanical gardens are actively involved in the application of tissue culture technologies. The most widely used and commercially successful application of plant biotechnology is in the rapid and large scale clonal multiplication of plants by meristem-tip culture (Vasil, 1990). Other important applications of meristem-tip culture have been in virus elimination and in the in vitro conservation of germplasm (Ng, 1987). Virus-free plants can be useful experimental materials for studying the effects of specific viruses on host plants. More importantly virus-free plants have been shown to contribute to vigour, quality and the yield of host plants. The relative ease with which viruses are eliminated depends on the type of virus and the host-virus combination (Thottapilly et al.,1992). While control of plant bacterial and fungal diseases can

be achieved with the use of chemicals, the control of virus diseases through the use of chemicals has not been successful. Chemicals affecting virus multiplication usually exert high phytotoxicity on the host plant. Additionally these are expensive and once treatment is stopped the virus concentration may rapidly rise again (Bhojwan and Razdan, 1983).

Cassava virus diseases in the field show various levels of mosaics, leaf distortions and other deformities depending on the interactions between virus strains and cassava genotypes (Agrios, 1988). Symptoms caused by viruses in themselves are not adequate in providing a reliable diagnosis because they sometimes resemble those caused by mutations, nutrient deficiencies or toxicities (Agrios, 1988). Detection and identification require the use of modern diagnostic technology based on serology and electron microscopy. It is therefore imperative to know what kind of virus(es) present in the crop concerned in order to formulate sound disease management practices.

2.3.2 Methods of producing virus-free planting material

2.3.2.1 Meristem-tip culture

Most of the tissue culture work on cassava has been directed at producing virus-free cassava clonal stocks. Meristem-tip culture has enabled the regeneration of a wide range of virus-free clones in a wide range of economically important crops (Quak, 1977). Bhojwan and Razdan (1983) cited other workers as being the first to obtain virus -free dahlias and potatoes by meristem-tip culture. White (1934) observed that Tobacco Mosaic Virus (TMV) was unequally distributed throughout the different zones of the tobacco root with the root tip it self being virus-free. Quak and Hakkaart (1966) stated that there was competition in the

meristem between the production of virus particles and cell production. In the meristematic tissue, the capacity for nucleic acid synthesis is utilised for cell production to the detriment of virus multiplication. In cells below the meristem which are increasing in size rather than dividing, virus multiplication occurs unhindered. The absence of vascular elements and plasmodesmata in the meristem retards the transport of virus particles. Bhojwan and Razdan (1983) have accepted this theory as the most plausible explanation to the absence of virus particles in the meristematic tissue.

Quak (1977) alluded to the fact that the possible occurrence of high concentrations of cytokinins and auxins in the dividing cells (meristematic cells) hindered the penetration of virus particles or that the virus particles would be inactivated by them. Mellor and Stace-Smith (1969) speculated that some enzymes which are needed for virus multiplication were absent in meristematic tissue. They also expressed the view that the presence of naturally occurring inhibitors was responsible for low virus concentrations in the meristem.

Ng (1992) stated that one of the most important features of using meristem-tip culture is that the regenerated plants usually retain the genetic integrity of the parents. This is thought to be probably due to the more diploid nature of the meristem (Murashige, 1974). A sound understanding of the etiology of the disease coupled with the use of reliable virus indexing methods is needed in order to obtain virus-free regenerated plants from meristem-tip culture particularly given that some viruses may be latent. The success of virus eradication by meristem-tip culture is affected by a number of factors such as culture medium, explant size, culture storage and physiological condition of the explant (Bhojwan and Razdan, 1983). The

physiological state of the explant may sometimes have a very strong influence on the success of virus eradication by meristem-tip culture. Bhojwan and Razdan (1983) found it preferable to obtain meristem-tips from actively growing buds. They also found that the efficiency of meristem-tip culture depends on, in addition to the survival of the explants and shoot development, the rootability of the shoots and their freedom from viruses.

Nutritional requirements for optimal growth of the tissue in vitro may vary with the species and even for the same plant it may depend on the source of the tissue (Murashige and Skoog, 1962). The survival of the meristem-tips under optimal culture conditions is related to the size of the explant (Bhojwan and Razdan, 1983). A large explant enhances the chances of survival. Light incubation has been studied and found to have a bearing on the ability of meristem-tip cultures to regenerate plants while the effect of temperature is still unclear.

2.3.2.2 Heat treatment

Heat treatment or thermotherapy has been effectively used for a long time to obtain virus-free plants from infected individuals of many crop species. The basic principle by which viruses are eradicated using heat is that at temperatures higher than normal many viruses in plant tissues are partially or completely inactivated with little or no damage to the host tissues (Baker,1962). Although the apical meristems are quite often free of viruses, it cannot be regarded as a phenomenon of universal occurrence. Langhans et al. (1977) provided evidence showing that some viruses actually invaded the meristematic regions of the growing tips.

Heat treatment is usually given through hot water or hot air depending on the circumstances. Whereas, hot water treatment has proved better for dormant buds, hot air treatment has in general given better elimination of viruses in actively growing shoots and better survival of the host (Hollings 1965). Heat treatment can be given to the mother plants before excising the meristem-tips or alternatively the meristem-tip cultures are exposed to high temperatures. For hot air treatment which is comparatively easier to apply, actively growing plants are usually transferred to a thermotherapy chamber in which they are exposed to temperatures of 35-40° C for a period of time. For successful heat treatment an adequate supply of humidity should be maintained during the process (Wang and Hu, 1980).

Some viruses cannot be readily eliminated by thermotherapy or meristem culture alone (Ng, 1987). One of the major limitations of thermotherapy for virus eradication has been that not all viruses are sensitive to the treatment. Walkey (1978) states that a prolonged heat treatment may lead to the inactivation of the resistance factor(s) in the plant tissues and thereby increasing the incidence of infectivity of the cured plants compared to the control. The size of the meristem-tips is critical for virus eradication when they are excised from plants that had been heat-treated for only a short period or not at all.

A combination of chemotherapy, thermotherapy, and meristem-tip culture could increase the efficacy of virus elimination. At IITA the percentage of cassava plants regenerated from meristem-tip culture and free of ACMV was increased from 25 to 100 % when the parent plants were heat-treated at 37° C for one month (IITA 1979; 1980). Potato Virus S (PVS) and Potato Virus X (PVX) have been eliminated successfully by a combination of thermotherapy

and meristem-tip culture while Mild Yellow Edge, a disease of strawberry, was eliminated by meristem-tip culture without a heat treatment (Bhojwan and Razdan, 1983). It was observed by Quak (1977) that heat treatment was most effective against isometric viruses and against diseases resulting from mycoplasmas. This has been particularly established in such crops as sugar cane, cassava and some fruit trees (Quak, 1977; Kartha and Gamborg,1975).

2.3.2.3 Visual selection

In India, Uganda and Kenya, mosaic-free cassava planting material has been obtained through selection of symptomless plants from infected fields followed by careful rouging (Cock, 1985). In areas with low incidence of whiteflies, the use of mosaic-free planting stocks could probably greatly reduce the incidence of the disease or even eliminate it. Varietal resistance in combination with preventive measures such as the use of clean planting materials can reduce the losses. International exchange of vegetative material previously hampered by the fear of transferring virus infected material is no longer a problem when it is done by tissue culture (Bhojwan and Razdan, 1983). Ng (1987) reports that the International Institute of Tropical Agriculture (IITA) has produced more than 22 such cassava varieties by tissue culture for distribution to national breeding programmes of interested countries.

3.0 MATERIALS AND METHOD

3.1 Virus identification

In this study particular emphasis was placed on the detection of the virus isolates responsible for cassava mosaic disease. Viruses were detected in the leaf samples by a combination of serological tests and by electron microscopy.

3.1.1 Source of leaf samples

Samples of cassava leaves showing mosaic, vein banding and other deformities were collected from specific locations in six provinces of Zambia during the 1993/4 cropping season (Table 1). Samples were collected at random without any preference given to varieties. Each sample consisted of 3 young symptom bearing leaves picked from the shoot of a diseased cassava plant. Leaf samples were also collected from five of the most popular and widely grown varieties "Kapumba", "Mung'anga, "Nalumino", "Chinyimba and "Bangweulu (Table 2). Each sample was packed in a separate plastic bag labelled to show the date of collection, variety (where possible) and name of district sampled.

Table 1: Specific locations where cassava leaf samples were collected in Zambia in 1993/4 cropping season

Province	District	Location
Lusaka	Lusaka	Mt.Makulu
		UNZA
Luapula	Kawambwa	Shikalaba
		Kapako
		Musambeshi
	Mansa	LRRS[1]
Northern	Mpika	Market centre
	Kasama	Mulaushi
North Western	Solwezi	Market centre
Central	Kabwe	Katondo
	Serenje	Market centre
	Kapiri Mposhi	Central[2]
	Mkushi	Market centre
Copperbelt	Kitwe	Chimwemwe
	Mufulira	Murundu, Mokambo
	Luanshya	Mikomfwa
	Ndola Rural	Mpongwe
	Chingola	Lufwanyama

1) Luapula Regional Research Station
2) Central Agricultural Block

3.1.2 Enzyme Linked Immunosorbent Assay (ELISA)

Collected fresh leaf samples were immediately sent to the laboratories of the University of Zambia (UNZA) and the Scottish Crops Research Institute (SCRI)[1] for testing.

Serology tests involving indirect (TAS)-ELISA with a panel of monoclonal antibodies (mAbs) raised against ACMV and ICMV were carried out at SCRI and UNZA. In the first TAS-ELISA tests conducted at SCRI a battery of 19 different monoclonal antibodies was used. Out of these monoclonal antibodies 15 were raised against ACMV and 4 raised against Indian Cassava Mosaic Virus (ICMV). Some of the monoclonal antibodies reacted with geminiviruses other than the ones used to prepare them . ACMV reacted with all 15 anti-ACMV mAbs and 1 of the anti-ICMV mAbs. ICMV reacted with 2 of the anti-ACMV mAbs and all 4 anti-ICMV mAbs while EACMV reacted with up to 9 of the anti-ACMV mAbs with the exception of not reacting with any of the anti-ICMV mAbs. From the pattern of reactions given by an individual virus isolate it was possible to readily deduce which virus was present in the tested sample. Based on the tests at SCRI two identifying monoclonal antibodies (mAbs) SCR23 and SCR33 were chosen for TAS-ELISA tests at UNZA involving samples collected from different parts of Zambia. The polyclonal coating antibody and the two monoclonal identifying antibodies SCR23 and SCR33 including the ELISA protocol were kindly provided by Professor Brian Harrison (University of Dundee, Scotland).

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The monoclonal SCR23 reacted with all ACMV isolates likely to be found in Zambia while mAb SCR33 reacted similarly with the exception of not reacting with East African cassava mosaic virus (EACMV). Microwell polystyrene microtitre plates by Nuclon were used in the ELISA tests at UNZA. The rabbit antimouse alkaline phosphatase conjugate used in the tests including all chemical reagents for the preparation of buffer solutions were obtained from Sigma Chemical Company (St Louis, USA). The substrate 4-Nitrophenyl phosphate was obtained (from Boeringer Mannheim, Germany). Crude sap was manually extracted from leaf samples by use of a mortar and pestle. Micro plates were coated with 100µl of diluted polyclonal antibody 1:10000 in coating buffer and incubated 3 hours at 37°C (buffer compositions are presented in Appendix C). Crude sap was extracted at a ratio of 1:10 by volume in extraction solution and 100µl of the extract was added to each well of the micro plate followed by incubation overnight at 4°C.

Unless stated otherwise, each step was preceded by washing the wells three times using Phosphate Buffered Saline(PBS) mixed with Tween solution for 3 minutes. In the subsequent step 200µl of blocking solution was added per well and the plates incubated for 30 minutes at 37°C before proceeding to add one of the identifying monoclonal antibodies (mAbs) SCR23 or SCR33. Wells were loaded with 100µl of identifying mAbs diluted 1:10 and incubated 3 hours at 37°C. Rabbit anti-mouse-alkaline phosphatase conjugate diluted 1:1000 in conjugate buffer was added to each micro well at 100µl per well. This was followed by adding 150µl of freshly prepared 4-nitrophenyl phosphate per well diluted at 0.6mg/ml in substrate buffer.
The micro plates were incubated at room temperature for 2 hours and results read. Results were read again after incubating overnight at 4°C.

3.1.3 Immunosorbent Electron Microscopy (ISEM)

At SCRI samples tested by TAS-ELISA were further examined by immunosorbent electron microscopy (ISEM) for the presence of other viruses not detected by the ELISA technique. For this test, the carbon filmed electron microscope grids were first floated face-down on Cassava Q Virus (CQV) antiserum diluted 1/1000, and then on virus containing leaf sap. The virus particles were then able to be attached to the antibody molecules already bound to the carbon film. In order to outline the virus particles, the microscope grids were treated with the negative stain uranyl formate. Cassava Q Virus particles were then observed through the microscope and recorded. Virus-like particles were observed on the antiserum coated grids while nothing was observed on the control (uncoated) grids. Since the particles were trapped specifically by CQV antibodies, this was an indication that the attached particles were CQV particles.

3.2 Virus elimination

3.2.1 Donor plant establishment

Five cassava landraces namely; "Munganga", " Nalumino", "Kapumba", "Chinyimba", and "Bangweulu" were chosen as meristem donors. Symptomless as well as diseased mother plants of at least 2 years were selected in the field to provide the planting material for a cassava nursery at UNZA. The nursery was established in the month of November, during the 1993/4 cropping season at the University field station. A spacing of 1m x 1m in between plants and ridges was used. Each variety was planted on a separate ridge at random.

Table 2: Agronomic characteristics of local cassava landraces sampled

	Variety[1]				
Traits	BAN	NAL	KAP	CHI	MUN
Yield (T/Ha)	31.3	28.9	21.7	21.8	22.1
Harvest index %	58.3	52.7	56.7	-	-
CMD reaction[2]	MR	R	MR	MR	VS
Maturity[3]	2-16	16-24	16-24	-	-
Root shape	good	poor	fair	fair	fair

1) BAN = Bangweulu, NAL = Nalumino, KAP=Kapumba, CHI=Chinyimba, MUN = Munganga

2) R = Resistant, MR = Moderate Resistance, S = Susceptible, VS = Very Susceptible

3) Maturity measured as months after planting

Source: Anon. 1993a ,RTIP Annual Report.

Other cuttings of the same landraces were planted on sterilised soil in plastic pots and left to grow in the open around the School of Agriculture at UNZA. No nutrient fertilizers were applied to the donor plants throughout their growth. Weeding was done with a hand hoe at different stages of growth depending on weed pressure. Since the potted plants were left in the open, only supplemental irrigation was done to maintain the soil at field capacity. Soil samples were collected in the field station at the site of the cassava nursery (Appendix A). Weather conditions at UNZA during the growing season are presented in Appendix B.

3.2.2 Heat treatment

The experiment was set up to determine the efficacy of heat treatment on virus elimination by meristem-tip culture in cassava. It is important to recognise that even after taking all the necessary precautions in excising small sized meristem-tips and subjecting them to various treatments favouring virus eradication, only a proportion of the cultures actually yield virus-free plants for several reasons. This therefore, made it necessary to test all the meristem-tip derived plants for specific viruses before they were used as mother plants in the production of virus-free stocks.

In this experiment before any treatment was applied, plants to be used as meristem donors were subjected to an indirect (TAS)-ELISA test to ascertain their disease status. The same test was repeated after transplanting the regenerated plants on to a soil medium. Part of the potted cassava plants testing positive for ACMV were selected to undergo heat treatment at 37°C for a period of 6 weeks in an improvised heat chamber at Seed Control and Certification Institute (SCCI). Plants in the field station testing positive for ACMV by TAS-ELISA were selected as meristem donors not having undergone any heat treatment.

A randomised complete block design (RCBD) in 3 replications was set up to determine the effect of heat treatment on virus elimination. The two treatments in the experiment were: heat therapy (4 weeks at 37°C) followed by meristem-tip culture and meristem-tip culture without carrying out any heat therapy. For each treatment a total of 45 meristem-tips measuring 0.5-1.2mm with at least two leaf primodia were excised from axillary buds obtained from selected 3 months old cassava plants.

The replications in this experiment consisted of the three cassava varieties used as meristem donors: "Munganga","Bangweulu" and "Kapumba."

3.2.3 Cassava meristem-tip culture

An experiment to eliminate ACMV by meristem-tip culture was set up in the tissue culture laboratory at UNZA. Meristem donor plants were raised in the field from cassava stakes collected from the RTIP in Mansa, Luapula Province. Chemical reagents including phytohormones, test tube racks, and culture tubes were all obtained from Sigma Chemical Co. St Louis, USA. For aseptic conditions a Steag laminar flow cabinet was used. Cultures were kept in a growth cabinet and a scientific stereo microscope of 30x magnification fitted with zoom lenses was used during excision of meristems.

Different culture medium compositions have been used successfully to regenerate cassava plantlets by meristem-tip culture. In this experiment a modified MS medium composition (Roca, 1985) was used. Culture medium composition for the initiation stage lasting 2-4 weeks consisted of MS medium with 1.0 μM Thiamine-HCl, 0.6 mM inositol and 0.058 M sucrose. The following growth regulators were used: 0.2μM Naphthalene acetic acid (NAA), 0.1μM Benzyl amino purine (BAP) and 0.1μM Giberellic acid (GA_3). The culture medium was solidified on 0.6 % agar.

Culture medium composition for rooting, further growth and hardening was modified to: MS (one third strength), 3.0 M Thiamine-HCl, 0.6 mM inositol and 0.058 M sucrose. The medium was solidified on 0.8 % agar.

For this stage the only growth regulator added was 0.1μM NAA. Routine subculturing was done throughout the culture period in order to maintain growth. A hardening or conditioning treatment in which the cultures were gradually exposed to glass house conditions was done for a period of 1 week. This process affords the cultures a chance to tolerate water stress and enables them to quickly adapt to their new environment once they are potted. Potting was done on a sterilised 2:1 sand/vermiculite mixture in plastic pots.

Temperature conditions for culture initiation and rooting including further growth were maintained at 27-28°C in the Rumed growth cabinet for approximately 6 weeks. Other than day light, illumination not exceeding 2000 lux was provided by Philips TL 20W/29 RS fluorescent tubes for 2 weeks before gradually increasing it to 5000 lux by adjusting the distance between the cultures and the light source. Hardening was done at a light intensity of 8000-10000 lux provided by Philips TLD 58W/84 while the temperature was reduced to 24-25°C. Throughout the culture period the photoperiod was maintained at 16 hours. As a result of space limitations in using the Rumed growth cabinet, an improvised culture room providing similar culture conditions was used to accommodate some of the cultures.

3.3 Data collection

3.3.2 Virus identification

In the TAS-ELISA tests, for virus detection in the samples optical density was recorded by means of a Sigma Diagnostics ELISA plate reader set at a wave length of 405nm. Upon adding the substrate, a visual assessment of the pattern of colour development on micro titre plates was done and recorded using appropriate controls.

The detection of CQV by ISEM involved negative staining techniques with Uranylformate/sodium hydroxide to outline the virus particles which were later observed and recorded through the microscope.

3.3.2 Virus elimination

In the cassava meristem-tip culture experiment, for each treatment the cumulative number of contaminated meristems observed weekly was recorded. Shoot and callus induction of uncontaminated meristems was determined by counting number of meristems showing shoot and callus growth every week. Shoot growth was determined from uncontaminated meristems by randomly measuring the length of at least 10 actively growing shoots once every week and calculating the average length for each treatment. Data for this experiment was collected for a period of 13 weeks before it was decided to discontinue the experiment due to an unexpectedly high rate of contamination.

3.4 Analysis

3.4.1 Virus identification

Optical density data recorded on the Sigma Diagnostics Elisa plate reader were used to analyse samples tested by ELISA. Visual colour differentiation on the micro titre plates was used to assess the samples tested.

Samples tested by ISEM for CQV particles were analysed by observing virus like particles on the antiserum coated microscope grids while nothing was found on the control (uncoated) grids.

3.4.2 Virus elimination

Data recorded in the meristem culture and heat treatment experiment were subjected to analysis of Variance (ANOVA) in order to establish the presence of treatment mean differences. Mean separations were done by the Least Significant Difference Test (LSD) at 5% level of significance. The following computer software packages were used in the analysis; LOTUS 123 R3.1, MSTAT and Hyper Graphics.

4.0 RESULTS

The 1993/94 cropping season was characterised by below normal rainfall in most parts of the country (Anon,1994). The drought adversely affected the yields of most crops including those of the staple food crop maize particularly in the southern half of Zambia. The season could generally not be said to have been conducive to cassava production especially for those farmers who planted new fields. Soil and weather conditions during the season at the site of the cassava nursery used in the study are presented in the appendices.

4.1 Virus identification

At SCRI, TAS-ELISA tests confirmed the presence of ACMV in all the samples collected from Mt Makulu and UNZA in Lusaka district with the only exception of one of the samples from Mt Makulu which did not show any visible mosaic disease symptoms (Table 3). The isolate from the cultivar "Munganga" only differed slightly from the others in also reacting with one of the mAbs raised against ICMV (another geminivirus) while the other four isolates only reacted weakly or not at all. Immunosorbent electron microscopy was only able to detect the presence of Cassava Q Virus (CQV) in the "Kapumba" isolate. All the other isolates tested did not show the presence of CQV.

From a total of 18 samples collected from the main cassava growing areas of Northern, North Western and Luapula provinces, at least 50% of the samples were positive for ACMV when reacted with mAb SCR23 and SCR33 (Table 4). All the samples that tested positive with the mAb SCR23 similarly showed a positive reaction when tested with the mAb SCR33. This confirmed the presence of ACMV in the samples as opposed to EACMV.

Table 3: Reactivity of cassava leaf samples collected in Lusaka district tested at SCRI, 1993/94 season

Isolates	Location	ELISA tests[1]		ISEM[2]
		ACMV[3]	ICMV[4]	CQV[5]
MMZ01	Mt. Makulu	+	-	-
MMZ02	Mt. Makulu	+	-	-
MMZ03	Mt. Makulu	-	-	-
Kapumba	UNZA	+	-	+
Munganga	UNZA	+	(+)	-
Bangweulu	UNZA	+	-	-
Total tested		6	6	6
Total negative		1	5	5
Total positive		5	1	1

1) positive reaction = + ; negative reaction = -

2) ISEM = Immunosorbent electron microscopy

3) ACMV = African cassava mosaic virus

4) ICMV = Indian cassava mosaic virus

5) CQV = Cassava Q virus

Table 4: Reactivity of cassava leaf samples collected from five provinces of Zambia tested at UNZA, 1993/4 season

Province	District	No. Isolates	ELISA tests[1] SCR23 +	SCR23 −	SCR33 +	SCR33 −
Luapula	Kawambwa	5	2	3	2	3
	Mansa	1	1	0	1	0
Northern	Mpika	8	3	5	3	5
	Kasama	2	1	1	1	1
North western	Solwezi	1	1	0	1	0
Central	Kabwe	1	0	1	0	1
	Serenje	1	1	0	1	0
	Kapiri Mposhi	1	1	0	1	0
	MKushi	1	0	1	0	1
Copperbelt	Kitwe	1	0	1	0	1
	Mufulira	4	2	2	2	2
	Luanshya	1	0	1	0	1
	Chingola	2	1	1	1	1
	Ndola Rural	2	1	1	1	1
Total tested		31				
Total negative				17		17
Total positive			14		14	

1) positive reaction = + ; negative reaction = -

The isolate from "Nalumino" which is the only variety that consistently shows field resistance produced only negative results when tested with mAb SCR23 and SCR33 while isolates from "Munganga", "Chinyimba", " Kapumba" and "Bangweulu" were able to test positive for ACMV (Table 3). Field observations and interviews conducted with Agricultural extension workers in these areas confirmed the wide presence of CMD in the majority of cassava fields. In the Northern and Luapula province it is quite common to find large areas of CMD infested cassava fields. It was observed that most farmers usually plant more than one cultivar in the same field and once a field was infested with CMD, in most cases more than half the plants in the field developed typical mosaic disease symptoms.

4.2 Effect of heat treatment on shoot induction

There were significant differences observed in shoot induction of meristem-tips obtained from heat treated donor plants (37°C for 4 weeks) and those from donor plants not having been exposed to any heat therapy. Shoot induction of meristem-tips derived from heat treated donor plants was 40% higher compared to that of meristems arising from untreated donor plants after 13 weeks of culture (see Table 5). However, the shoot induction in either case was still less than 50% in the same culture period. The high rate of contamination of cultures had an effect on the blocking factors used in the experiment as can be seen from the fairly high coefficient of variation. However this is not unusual in experiments of this nature.

Table 5: Effect of heat treatment on shoot induction after 13 weeks of culturing.

Treatments	% Shoot Induction
Heat therapy	38.8
No heat therapy	27.8
Grand mean	33.3
LSD (0.05)	5.56
CV%	51.6

4.3 Effect of heat treatment on shoot size

The was no significant difference in the size of the shoot between cultured meristem-tips derived from heat treated donor plants (37°C for 4 weeks) and those from untreated donor plants (see Table 6). There was no appreciable amount of rooting observed throughout the culture period.

Table 6: Effect of heat treatment on shoot growth after 13 weeks of culture.

Treatment	Shoot Size (mm)
Heat therapy	2.8
No heat therapy	3.1
Grand mean	2.9
LSD (0.05)	0.54 ns
C.V.%	56.7

4.4 Effect of heat treatment on callus induction

There were no significant mean differences observed in callus induction of cultured meristem-tips obtained from heat treated donor plants (37°C for 4 weeks) and those from untreated donor plants (Table 7). Meristem-tips derived from heat treated mother plants had a lower frequency to form callus compared to those obtained from untreated mother plants (see Figure 3). The number of meristems with a basal callus showing shoot regrowth was negligible for either source of meristems cultured. The proliferating callus appeared to have had a repressing effect on shoot growth and plantlet regeneration in both cases.

Table 7: Effect of heat treatment on callus induction after 13 weeks of culturing.

Treatments	% callusing
Heat therapy	55.8
No heat therapy	56.8
Grand mean	51.3
LSD(0.05)	5.6
C.V. (%)	33.5

4.5 Effect of heat treatment on culture contamination

Contaminated cultures of meristem-tips taken from heat treated and untreated donor plants were not statistically different after 13 weeks of culture (Table 8). It was observed that the rate of contamination was very high in this experiment. The experiment was discontinued after 13 weeks of culture when more than 40% of the cultures were observed to be contaminated.

Table 8: Effect of heat treatment on culture contamination after 13 weeks of culturing.

Treatments	% Contamination
Heat therapy	41.8
No heat therapy	43.1
Grand mean	42.2
LSD(0.05)	3.1 ns
C.V. (%)	22.2

5.0 DISCUSSION

5.1 Virus identification

The results from the samples collected in Lusaka district give an indication of the prevalence of ACMV on most of the cultivars grown by Zambian farmers since the majority of the cultivars tested reacted positive for ACMV. This observation ties with the results of the field screening conducted in Zambia by the RTIP which showed that up to 80% of the local cultivars were susceptible to CMD (Anon,1993b). Other than the cultivar "Nalumino" released officially as an improved clone in December, 1993 there has not been many truly resistant varieties released in Zambia from the national research programme and therefore farmers have continued to plant the local landraces that are in most cases susceptible to CMD.

The only cultivar which consistently tested negative for ACMV in the TAS-ELISA tests at UNZA was "Nalumino". The fact that the "Munganga" isolate was able to react with only one of the antibodies raised against ICMV could mean that the isolate detected was merely a minor variant within ACMV. TAS- ELISA tests for detecting ACMV in the samples of the cultivar "Munganga" produced strong positive reactions all the time, which serves as a possible indication of the susceptibility of this cultivar. Soenarjo et al. (1988) made similar observations in a study to evaluate the susceptibility of local cultivars to CMD. The cultivar "Munganga" was found to be very susceptible to CMD while "Nalumino" was classified as resistant. In all the samples tested EACMV reported previously in many countries of East Africa was not detected in the present study. This is rather surprising going by the geographic location of Zambia.

The CQV isolate detected in the sample of the cultivar Kapumba belongs to a recently discovered group of plant viruses associated with mild leaf flecking in cassava. This virus does not appear to be economically important in this country at present. However, efforts should be made at monitoring the spread of the virus by gathering more information on it such as population dynamics, host range and vector behaviour.

The isolate "MMZ03" did not show any typical symptoms of a virus infection and this could possibly be the reason it tested negatively in all tests conducted. The results of this study have showed that the ACMV, which is a whitefly transmitted geminivirus previously reported to the west and south of the Rift valley is prevalent in most of the cassava growing areas of Zambia. In all the TAS-ELISA tests conducted at UNZA, similar positive results were obtained when samples were tested with mAb SCR23 and mAb SCR33 since the two antibodies react in a similar manner for ACMV.

It must, however, be borne in mind that while host range, symptom expression and physical properties have some use occasionally in identification, they are in general a most imprecise guide and their comparative value in most cases is minimal for such purposes. It is evident that the control of ACMV on typically small farms where plantings are grown in an overlapping sequence and in close proximity will mostly be dependent on the introduction of highly resistant varieties of the type now being developed at IITA.

While a range of cultivars with good agronomic characteristics are already available, further information is needed to assess re-infection rates of different local cultivars. The cultivar, Nalumino widely cultivated in the Western province has so far shown a reasonably high level of resistance to ACMV.

The IITA cassava improvement programme has been providing ACMV resistant genotypes to many national breeding programmes in Africa. The RTIP based in the Luapula province of Zambia is currently producing healthy stocks of local cultivars and evaluating these along side those imported from IITA. However, there still remains the aspect of suiting these genotypes to local preferences in terms of tuber-sweetness, leaf palatability and branching habit. In Zambia there appears to be a stronger preference for varieties combining sweet tubers and a less branching growth habit (Soernajo, pers. comm.). In most parts of the country cassava leaves are consumed as a vegetable served with the main meal.

Since there is no immediate prospect of using insecticides, biological control, mild strain protection or truly tolerant varieties the basic approach to control ACMV in many regions has involved an integration of measures such as virus- resistant varieties, crop sanitation and crop deployment (IITA, 1980). In Zambia this would entail a programme of education, sustained support to advisers, agricultural extension workers and farmers. It should be emphasised that sanitation alone will not be effective in areas of high inoculum pressure such as we have in the Luapula and Northern provinces of this country. Resistant varieties are nearly a must under such conditions.

5.2 Virus elimination

Exposure of ACMV- infected plants to temperatures of 35°C and above have been shown to suppress symptom expression within a few weeks and meristem-tips cultured from such plants in vitro have successfully regenerated in to virus-free plants (Ng, 1987). Results from the present study show that shoot initiation was higher for meristems derived from heat treated donor plants than that of meristems arising from untreated donor plants. This meant that heat treatment of meristem donor plants at 37°C for a period of 4 weeks was able to stimulate shoot induction.

However, throughout the course of the experiment it was observed that shoot growth beyond the initiation stage was slow. This accounted for the small size of shoots obtained at the end of the experiment. This situation could be attributed to the below optimum culture conditions of light intensity and temperature 2 weeks after inoculation of the meristems caused by a breakdown of the only available growth cabinet. Shoot induction for heat treated donor plants was 40% higher than that of untreated donor plants.

The proliferating callus formation on the basal part of the shoots could have had a negative effect on subsequent shoot development after shoot initiation as there was no meristem with a callus that showed any shoot regrowth. It was interesting to note that the meristems arising from heat treated donor plants had a lower callusing percent compared to those from donor plants not heat treated.

The high rate of contaminated cultures observed in the experiment could be the result of inadequate surface disinfection of the explants for the varieties used. The use of bleach solution for surface disinfection of the explant is not always successful and may be influenced by the degree of pubescence of the cultivars (Zamora and Gruezo, 1992). Another possible source of contamination in the experiment may have been the frequent rate of subculturing (every 2 weeks) which may have increased the chances of contamination through regular exposure of explants. High contamination rates and callus formation around the base of the isolated meristems is a frequent cause of failure to regenerate plantlets (Zamora and Gruenzo, 1993). This situation suggests a need to further refine the present protocol for regenerating cassava plantlets in vitro under our local conditions.

6.0 CONCLUSION

There is sufficient information to indicate that cassava mosaic disease (CMD) is a major constraint to the production of cassava not only in Zambia but the sub-region as a whole. The two viruses attacking cassava identified in the study are African cassava mosaic virus (ACMV) and cassava Q virus (CQV). ACMV is which appears to be the most prevalent virus attacking cassava in Zambia is at the moment the most serious disease of cassava on the African continent in terms of its geographic distribution and economic losses. More information is needed on the epidemiology of ACMV in Zambia. A broader understanding of the incidence and the rate of field spread of ACMV should be used as the basis for designing control strategies.

Virus elimination by a combination of heat treatment and meristem-tip culture could play a supplementary role in the production of virus-free clonal stocks of cassava for onward distribution to farmers. The study has shown that the heat treatment of meristem donor plants at 37°C for a period of 4 weeks was able to enhance shoot induction and reduce basal callusing of the growing shoots. More research needs to be undertaken to refine the protocol for regenerating cassava plantlets in vitro under our local conditions and also to verify the present observations.

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8.0 APPENDICES

Appendix A. Soil characteristics of the cassava nursery at UNZA field station 1993/4 season

P^H ($CaCl_2$)	Nitrogen %	Phosporous ppm	Potassium meq	Calcium meq	C.E.C M.E %
7.5	0.05	24	0.23	5.7	6.79

Appendix B: Weather data from 13th of December, 1993 to 20th of March, 1994

Dekade	Rainfall (mm)	Mean air temp (C)	Mean RH
1	2.4	24.36	70.64
2	33.6	25.81	29.43
3	39.2	24.16	82.65
4	52.6	25.15	78.50
5	21.5	27.78	76.13
6	21.6	25.24	75.10
7	66.3	25.85	76.75
8	3.2	23.88	78.73
9	0	26.35	82.75
10	0	25.80	76.00
	254.2	25.44	72.67

Dekade = 10 day period: Temp = Temperature

Appendix C. Buffer composition

The composition of the buffers used for ELISA is as follows (Harrison, pers. comm.): Coating buffer (pH 9.6) is 1.59g Na_2CO_3 + 2.93g $NaHCO_3$ in a litre of distilled water. PBS (pH 7.4) is 8.0g NaCl + 0.2g K_2HPO_4 + 2.9g Na_2HPO_4 + 0.2g KCl in a litre of distilled water. Extraction buffer (pH 8.0) is 0.05M Tris-HCl + 0.005M EDTA + 2% PVP + 0.05% Tween-20 in a litre. PBS-Tween is PBS + 0.05% Tween-20 per litre. Blocking solution is 5% dried milk powder + PBS-Tween- Polyvinyl pyrrolidone (PVP). Conjugate buffer is PBS-Tween + 2% PVP + 0.2% ovalbumin per litre. Substrate buffer (pH 9.8) is 10% diethanolamine in a litre.