CHAPTER ONE

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a perennial semi-woody shrub with edible starchy roots that is widely grown in the tropical regions of the world. Cassava roots give a carbohydrate production per hectare of 40% higher than that of rice and 25% more than maize (Tonukari, 2004), making it the cheapest source of calories for both human nutrition and animal feeding. The fresh leaves provide a valuable source of protein, vitamins and minerals in most cassava producing areas (Latham, 1979).

Global production of cassava is estimated at 152 million tonnes per year (CIAT, 2001). Half (50%) of the 16 million hectares of cultivated cassava worldwide is grown by small-scale farmers in Africa, 30 percent in Asia and 20 percent in Latin America (CIAT, 2001). Small-scale farming is characterized by cultivation using traditional methods with little or no inputs and largely in intercropping practices. Cassava's role as a traditional human food is changing to an efficient industrial crop in some parts of Africa, for instance in Nigeria (Nweke, 2004).

Ghana produces 7.2 million tonnes annually and is the third largest producer in Africa after Nigeria and the Congo (Alyanak, 1997). In Ghana, cassava is the most favoured among all the root crops and indeed all food crops by consumers. This is reflected by the per capita consumption (PCC) index. The PCC for cassava is high (148kg/year) followed by that of plantain (83kg/year: Annor-Frempong, 1991). It is widely

consumed in various forms in many parts of Ghana, therefore playing a role as the leading food security base.

Under optimal conditions, annual harvest up to 80 tonnes per hectare of cassava can be reached (CIAT, 1979), but currently the annual yields rarely exceed 10 tonnes per hectare (FAO, 1995). Cassava is expected to become even more important as human populations and pressure on the available land continue to increase and soil fertility declines (Cockcroft, 2004). However, the productivity of cassava in sub-Saharan Africa is generally low, in part due to the deleterious effects of pest and diseases.

Cassava mosaic disease (CMD) is considered the most important biotic constraint and it greatly reduces yields (Calvert and Thresh, 2002). As a vegetatively propagated crop, CMD is easily spread from one field to another in most cassava growing areas as farmers continue to use infected stem cuttings as planting materials. Although quantitative data on the losses due to CMD are scanty, a number of estimates have been made. Thresh *et. al.* (1997) reported an overall loss of 15-24% in Africa, equivalent to 12-23 million tonnes of cassava, while Zhang *et al.* (2005) estimated losses due to cassava mosaic disease throughout Africa to be 19.6-27.8 % of the actual production.

The disease is transmitted by the whitefly, *Bemisia tabaci* Genn., which is further disseminated through the stem cuttings used routinely for propagation (Pita *et al.*, 2001). The coastal areas of Nigeria, Sierra Leone, and Ghana recorded the disease in 1929 (Fauquet and Fargette, 1990). The symptoms of cassava mosaic virus (CMV) disease are usually conspicuous to diagnose and much of the evidence and spread is based on visual observation on the leaves. The most visible symptom of the disease is

the expression of the characteristic leaf chlorotic blotches, distortion and a reduction of the leaf area, thereby adversely affecting photosynthetic efficiency and the overall root yield and quality of leaves as vegetables (Almazan and Theberge, 1989).

Resistance of cassava genotypes to ACMV has not yet been reported (Jennings, 1994). However, in genotypes that show resistance, the virus seems to occur mainly towards the base of shoots so that uninfected cuttings for use as planting material could be obtained from the shoot tips (Cours-Darnes, 1968; Jennings, 1994). According to Thresh *et al.* (1994), spread of the disease within and between plants of resistant varieties is relatively slow. In addition, some cuttings propagated from infected plants may produce healthy progeny giving rise to clean plants from a diseased mother plant, a phenomenon termed 'reversion'. Reversion may be linked to varietal characteristics, since it is mostly marked in highly resistant cultivars (Fauquet *et al.*, 1987). It is also possibly dependent on environmental conditions, as preliminary experiments suggested that reversion was greater at high temperatures than during relatively cool periods (Fauquet and Fargette, 1990). Hence, the high chances of obtaining disease-free stocks as planting materials from some infected cassava cultivars when cuttings are taken from the upper section of shoots.

Unlike bacterial and fungal diseases, viral diseases have no effective chemical control on infected plants, thus, causing heavy yield losses in most vegetatively propagated plants. The supply of virus-free planting materials is therefore pivotal to sustainable crop production. Efficient methods developed for production of healthy vegetatively propagated crops has been established (Roca, 1984). Three methods currently in use are thermotherapy, tissue culture and chemotherapy (Kassianof, 1992).

In vitro tissue culture has contributed significantly to crop improvement by overcoming certain limitations associated with conventional techniques. For instance, meristem culture has been widely used to remove yield-limiting pathogens from plant cuttings (Delgado and Rojas, 1992), as well as other systemic diseases in vegetative materials (Ng, 1989; Kassianof, 1992). Elimination of specific viruses by meristem-tip culture has been reported to lead to a dramatic yield increase and rejuvenation of plant varieties that are propagated by vegetative means (Murashige, 1980).

The technique of meristem tip culture uses the meristematic dome and sub-adjacent leaf primordium (Hollings, 1965), suggesting a size of a meristem and the subtending leaves ranging from 0.1-0.5 mm. However, the meristematic dome itself measures from 0.1-0.25 mm (Hollings, 1965). The meristem tip must therefore be small enough to eradicate viruses and other pathogens, yet large enough to develop into a shoot. If larger meristems are used for cultures, it is likely that the virus will be retained in the progeny.

It was found that CMV particles could invade meristems (Walkey and Web, 1968) and that a gradient of increasing virus concentration from the dome to the successive leaf primordia exists. This means that the possibility of obtaining virus-free plants is inversely related to the size of the meristem excised. Therefore, to improve virus elimination using meristem culture, the use of either chemotherapy or thermotherapy as antiviral treatment of donor explants is necessary. This heat therapy is most often applied with meristem tip culture to produce plants that are "virus free" (Li *et al.,* 2002). According to Dodds *et al*., 1989; Griffiths *et al*.,

1990 heat treatment complement well in those plants in which viruses cannot be eradicated just by meristem tip culture alone.

Conventionally, viruses are eliminated by thermotherapy of whole plants in which plants are exposed to temperatures between 35-40°C for a few minutes to several weeks depending on the 'host-virus combination (Nyland and Goheen, 1969). It is based on the fact that most viruses are destroyed at temperatures much below those which kill their host plants. In many cases where the viruses' in the plants are not destroyed (ten-Houten *et. al.*, 1967), the treatment is reported to inactivate or inhibit virus development in infected plant tissues so that a newly developing tissue may be obtained which is free of virus.

Heat treatment (with hot water or hot air) resulting in inactivation or an inhibition of the replication of the virus has been successful in several cases including fruit trees (Manganaris *et al.*, 2003). This technique allows quick propagation of plant material, producing healthy plants from a single individual in a short period, regardless of location or season of the year. In cassava production, positive yield responses after thermotherapy to obtain clean planting materials have been shown in Cuba (Garcia *et al.*, 1993), Cameroon (Zok *et al.*, 1992), and Peru (Delgado and Rojas, 1992).

Application of meristem culture combined with thermotherapy is reported to increase the survival rate of *in vitro* explants (Manganaris *et al.*, 2003), since larger tips can be obtained from heat-treated plants while ensuring virus-free plant production. Propagative materials by this method can serve as guideline for the safe movement of cassava planting materials as outlined by Frison (1994), since the production of virus-

free plants is prerequisite for the international exchange of clonal material to avoid risks of introducing diseases to uninfected areas.

It is against this background that this study was initiated to find out the effect of size of initial meristem tip explant on regeneration rate and eradication of cassava mosaic virus disease in four (4) local cassava cultivars using thermotherapy-meristem culture. This is to ascertain effectiveness of thermotherapy and *in vitro* culture of cassava meristem tips to generate large numbers of virus-free plants.

Objective of Study

The objective of the current investigation was to use the simple box system with incandescent lamps as treatment for virus elimination of four cassava cultivars.

Specific Objectives:

1. To eliminate African Cassava Mosaic Virus disease and its symptoms on infected cultivars based on absence of symptoms and confirmed by a molecular test
2. Assess effect of heat therapy on the survival and growth of the four cultivars.
3. To determine the appropriate explant size that could regenerate whole plant *in vitro.*

CHAPTER TWO

LITERATURE REVIEW

2.1 Aetiology of the African Cassava Mosaic Virus (ACMV)

Pioneering work on cassava mosaic disease revealed that the disease is caused by a geminivirus (Bock *et al*., 1978) transmitted by a vector agent, the whitefly, *Bemisia tabaci* Genn:(Homoptera: Aleyrodidae) and distributed in vegetative propagules, making it the most prevalent and economically important disease of cassava in Africa (Hahn *et al*., 1980).

Three species, the *African cassava mosaic virus* (ACMV), the *East African cassava mosaic virus* (EACMV) and the *South African cassava mosaic virus* (SACMV) occur in Africa. SACMV is closely related to the EACMV but probably resembles more a virus from tomato since a recombination likely to be from a tomato virus was detected in its AC1, Rep-gene (Berrie *et al*., 2001).

In sub-Saharan Africa, mainly African cassava mosaic virus (ACMV) and East African cassava mosaic virus (EACMV) cause the disease (Bock and Woods, 1983; Hong *et al*., 1993). These two viruses belong to the genus *Begomovirus*, family *Geminiviridae* (Murphy *et al*., 1995; Mayo and Pringle, 1998). For several years, when ACMV was confirmed as the causal agent of CMD (Bock and Woods, 1983), the virus remained the only known causal agent of the disease in some countries in the western coast including Nigeria and Ghana.

Later studies (Gibson, 1996; Ogbe *et al.*, 1996; 1997; Legg *et al.*, 1999; Fondong *et al.*, 2000; Offei *et al.*, 1999) showed that EACMV occurs over a much wider area including Western Kenya, Western Tanzania, Zambia, Nigeria, Togo, Guinea, Ivory Coast, Cameroon and Ghana. Similarly, serological techniques initially developed to detect ACMV (*Geminiviridae: Begomovirus*) were subsequently used to demonstrate the occurrence and distribution of distinct serotypes (Swanson and Harrison, 1994). Three cassava mosaic geminivirus (CMG) species were described based on DNA sequence comparisons (Hong *et al.*, 1993), two occurring in Africa, namely African cassava mosaic virus (ACMV), East African Cassava Mosaic Virus (EACMV), and one restricted to India, designated Indian cassava mosaic virus (ICMV).

Geminivirus replication mostly occurs in the nuclei of the infected cells, where virus particles and virus induced doughnut structures can be seen in electron microscopy (Fontes *et al.*, 1994) and result in the mosaic symptoms on the affected plant leaves.

2.2 Genome structure of the Cassava Mosaic Geminivirus

Cassava geminiviruses have a bipartite genome, composed of two circular single-stranded DNA (ssDNA) components termed DNA-A and DNA-B that are packaged into separate particles (Chellappan *et al.*, 2005). These components have protein-coding sequences (ORFs or open reading frames) in the virus and complementary strands respectively.

Particles are germinate (c. 30 x 20 nm), with obvious "waist" at the mid-point (CRA and CRB, respectively) of the long axis (Figure 1). Each half has apparently pentagonal profile, with the faces in contact being longer than the others. In both

genome parts, open reading frames occur both in the virus particle strand and in the complementary strand, implying that transcription is bidirectional.

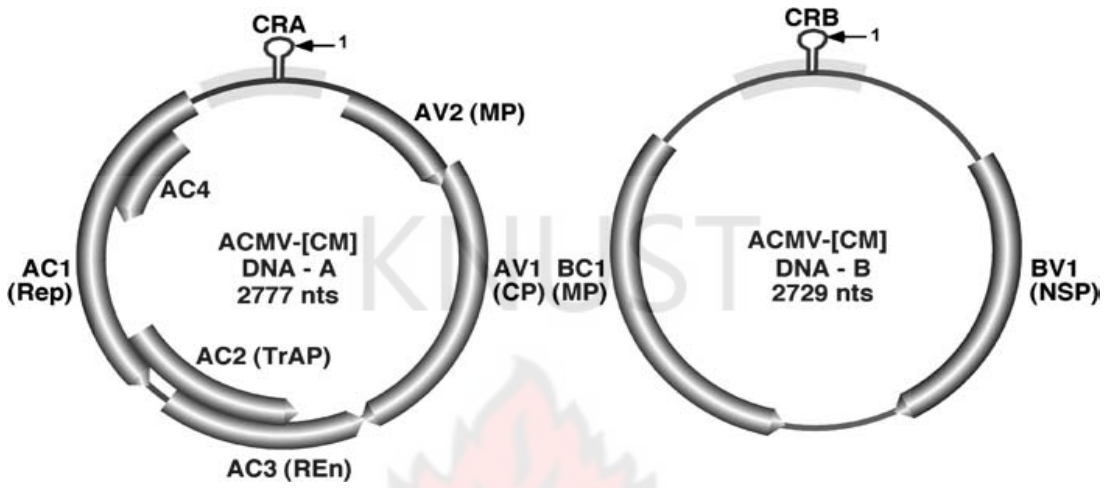


Figure 1. Genome organisation of a typical begomovirus. Arrows show arrangements of ORFs in the genomic DNA-A (left) and DNA-B (right) of ACMV. ORFs (AV1, AC1, AC2, AC3, AC4, BC1 and BV1) indicated occur in all cassava begomoviruses and the CRs contain shared sequences in both components. [Source: Chellappan *et al.*, 2005.].

DNA-A contains two genes (AV1 and AV2) in the virus strand and four genes (AC1, AC2, AC3 and AC4) in the complementary strand and encodes replication-associated protein (AC1 or Rep), transcriptional activator protein (AC2 or TrAP), replication enhancer protein (AC3 or REn), and coat protein (AV1 or CP). The virus (BV1) and complementary (BC1) strands of DNA-B contains one gene each and encodes nuclear-shuttle protein (BV1 or NSP) and movement protein (BC1 or MP) involved in cell-to-cell and systemic movement of the virus throughout the plant (Ingham *et al.*, 1995).

The nucleotide sequences (nts) of DNA-A and DNA-B are different except for a region of approximately 200 nts that share more than 90 percentage nucleotide sequence identity (Timmermans *et al.*, 1994), defined as the common region. The common

region carries regulatory sequences essential for viral DNA replication and transcription (Timmermans *et al.*, 1994). Among all the gene products, Rep is indispensable for viral DNA replication (Argüello-Astorga *et al.*, 1994; Chatterji *et al.*, 1999; Eagle *et al.*, 1994; Fontes *et al.*, 1994; Laufs *et al.*, 1995; Zhan *et al.*, 1991)

2.3 Virus replication and movement in plant

The ssDNA genomes of the virus replicate in the nucleus of infected cells via a rolling circle mechanism using a dsDNA intermediate (Saunders *et al.*, 1991; Stenger *et al.*, 1991). Sequence comparisons have shown that the viral replication-associated proteins are DNA binding proteins and are related to proteins involved in the initiation of replication of some ssDNA plasmids (Koonin and IIyina, 1992).

The origin of replication includes a conserved 30-nucleotide putative stem-loop element that is present in all geminiviruses (Revington *et al.*, 1989). The synthesis of ssDNA is regulated by the synergistic activity of TrAp and REn proteins that act as activator of transcription and enhancer of replication respectively (Sunter and Bisaro, 1991).

According to Pascal *et al.* (1994) and Sanderfoot *et al.* (1996), the genome complexes of BV1 that serves as the nuclear shuttle protein are directed to the cell periphery through interactions with the BC1 products, and the establishment of the virus infection depends upon the spread of the virus through the plant host.

The movement of the virus occurs at two different levels, thus short distance cell-to-cell movement and long distance movement that involve delivery of the virus to distal parts of the plant by the vascular system (Lazarowitz, 1992).

Gibson and Otim-Nape (1997) reported that the virus moves downwards to infect basal parts of stems even in resistant plant genotypes as the virus was first introduced to the young upper leaves. This occurs along with photosynthates in phloem sap moving towards tuberous storage roots. However, the growing shoots also attract the upward flow of the virus in phloem to supply the young shoots (Jennings, 1960; Anon, 1976).

2.4 Cellular and tissue relation

African cassava mosaic virus in cassava is able to induce symptoms within approximately five-days' post-inoculation (Chellapan *et al.*, 2004a), causing micro-structural changes in the nucleus of the host cells. In some crop species like tomatoes, the geminivirus induces fibrillar bodies and virus-like particles in the nuclei of phloem-associated parenchyma cells and sieve elements (Kim *et al.*, 1986). Similar abnormalities occur in cassava but are less common (Horvat and Verhoyen, 1981). However, chlorotic pattern over the entire leaf, narrowing and distortion of leaflets, severe mosaic and reduction in leaf size are observed (Njock, 1994), thereby affecting storage root yield of cassava.

The virus particles antigen accumulates in the nuclei of many phloem parenchyma or companion cells of in other tissues, such as cortex, mesophyll and epidermis (Sequeira and Harrison, 1982). These have the capacity to trigger post-transcriptional gene silencing (PTGS) with the production of virus-induced short-interfering RNAs (siRNAs) in infected host plants (Lucioli *et al.*, 2003; Chellapan *et al.*, 2004) to suppress disease symptom expression. As a counter defence, viruses have evolved to encode a

protein(s) that suppresses the host PTGS to establish infection in plants (Vance and Vaucheret, 2001). It is possible for ACMV and EACMV to co-infect plants, presumably due to the combined action of PTGS suppressors AC4 and AC2, from ACMV and EACMV respectively (Vanitharani *et al.*, 2004). A co-infected cassava plant produces increased mosaic symptoms, a phenomenon called synergistic interaction.

2.5 Transmission and spread of the cassava mosaic virus

Bemisia tabaci Gennadius is the natural vector of cassava begomoviruses in sub-Saharan Africa (Storey and Nichols 1938; Otim-Nape *et al.*, 1998). This vector allows rapid and efficient spread of the virus because it is an indiscriminate feeder. However, Bock *et al.*, (1978) suggested that humans are the principal vectors of ACMV because of the inadvertent use of infected cuttings as planting materials.

The adult whitefly must feed for about three and half hours to acquire the virus and needs a latent period of at least eight hours, and about ten minutes of inoculation feeding to transmit the virus (Mound and Halsey, 1978; Bethke *et al.*, 1991). The whitefly remains viruliferous for more than a week and does not lose the virus during molting (Dubern, 1979). The virus is not passed through the egg of the insect to the next progeny (Dubern, 1979).

Vector distribution, virus concentration, and susceptibility to virus inoculations are all related to leaf age (Fauquet and Fargette, 1990). Ninety-five percent of whiteflies found on cassava are concentrated on the abaxial surface of the five youngest leaves of the shoot. The percentage of individual whiteflies that becomes viruliferous when given access to infected cassava ranges from 0.15% to 1.7% (Dubern, 1979).

2.6 Spread of *Bemisia* whitefly population

The major peak of whitefly population is between March and June in the humid forest and derived/coastal Savannah (Leuschner, 1977). This period coincides with the beginning of the rains and a new cropping season, with young plants that are suitable for whitefly development (Fauquet and Fargette, 1990; Fishpool and Burban, 1994; Palaniswami *et al.*, 1996). A minor peak in the population of the insect occurs between September and November after the usual break of rains in the month of August in the two agroecologies (Leuschner, 1977).

The disease follows the pattern of whitefly population, and the spread is rapid in the humid forest. The severity of the viral disease is associated with large population densities of the whitefly vector *Bemisia tabaci* (Otim-Nape *et al.*, 2000).The life span of *B. tabaci* depends on temperature but on average is 21 days (Dubern, 1979).

2.7 Reversion and Recovery as natural control of cassava mosaic disease (CMD)

One characteristic of mosaic disease-resistant cassava cultivars is that they often become symptomless, because of incompletely systemic nature of the virus in the plant (Pacumbaba, 1985). Therefore, in infected cassava clones, it is possible to select healthy cuttings that will produce symptomless progenies. This is the result of the biological

phenomenon called "reversion" (Fauquet *et al.*, 1987). Similarly, a mother plant initially infected produce new shoots on the plant that show symptom-free branched leaves (Rossel *et al.*, 1987), a phenomenon known as 'Recovery'. Hot weather is reported to be associated with recovery from other virus diseases in other crops elsewhere in the tropic and semi-tropics (Frazier *et al.*, 1965). Both recovery and reversion are characteristics of MD-resistant genotypes (Fargette *et al.*, 1996), and have been linked to ACMV being incompletely systemic in cassava (Rossel *et al.*, 1987; Rossel *et al.*, 1994; Njock *et al.*, 1994; Fargette *et al.*, 1996).

It is also reported that environmental factors, such as high temperature, greatly influence plant virus interactions (Gibson, 1994), associated with frequently attenuated symptoms (heat masking), and with low virus content (Chellappan *et al.*, 2005). In contrast, low temperature is often associated with rapid spread of virus diseases and the development of severe symptoms (Hine *et al.*, 1970; Gerik *et al.*, 1990). Therefore, this natural interrelation makes thermotherapy the method of choice to free vegetative materials from infected viruses (Manganaris *et al.*, 2003).

For most of the clones, the percentage of reversion and recovery is nonexistent (Gibson and Otim-Nape, 1997), therefore making it easier for the application of thermotherapy technique to disease-free plants in large quantities (Mellor and Stace-Smith, 1970).

2.8 Virus Elimination through Meristem Culture

Meristem culture is reported to be an effective method of eliminating viruses and other systemic diseases from vegetative materials (Ng, 1989; Kassianof, 1992). The term "meristem-tip culture" has been suggested to distinguish the large explants from those

used in conventional propagation (Bhojwani and Razdan, 1983). Hollings (1965) suggested the term "meristem-tip" for explants that were cultured in order to produce virus free plants. Meristem-tip refers to the meristematic dome and the first pair of sub adjacent leaf primordia, ranging in length from about 0.1 to 0.5 mm. The size of the meristem, however, varies from species to species.

Elimination of plant viruses has been successfully achieved owing to their mode of replication and mechanism of movement within the plant. Morel and Martin (1952) concluded that, the success of virus elimination is achieved due to factors such as:

(a) the uneven distribution of virus in plant which is much less in the meristem,

(b) the inability of viruses to travel quickly enough through plasmodesmata to keep

up with the actively growing tip.

Belkengren and Miller (1962), Mullin *et al*., (1974) and Boxus (1976) subsequently added that, the virus could only travel through the plant vascular system, which is absent in the meristem. More so, the high metabolic activity of meristematic cells, usually accompanied by elevated endogenous auxin content in the shoot apices, protects that region from virus replication. Based on these findings, it is clear that meristem culture provides the best explants for eliminating viruses, bacteria and fungi from plants. However, excision and regenerating such small meristems is difficult *in vitro* thus larger 'meristem tips' (1.0 mm) is usually excised, to enhance plant regeneration from explants (Appiano and Pennazio, 1972; Roberts *et al*., 1970). When the infected plantlets were introduced to thermotherapy *in vitro*, it resulted in markedly enhanced virus eradication (Cooper and Walkey, 1978).

2.9 Effects of heat on Virus

The effect of heat on virus is not well understood but it is believed to be effective in inhibiting viral replication and synthesis of movement proteins mainly by blocking transcription in plants (Mink *et al.*, 1998), so that subsequently developed shoots are free from the virus.

Harrison (1956) speculated that the virus content of a plant represents equilibrium between replication and degradation of the virus by the host system and that the activity of the virus-degrading system increases with temperature. At higher temperatures, synthesis does not occur, and inactivation of the virus results from heat (Mink *et al.*, 1998), suggesting that high temperature treatment is an effective therapeutic method for the elimination of virus diseases.

Thermal inactivation of plant viruses *in vitro* follows the course of first-order chemical reaction (Babos and Kassanis, 1963). The most plausible hypothesis for the mechanism of heat therapy is that high temperatures cause the destruction of essential chemical activities in both virus and host but the host is better able to recover from the damage. Geard (1958) has therefore suggested that, the temperature coefficient of thermal inactivation for the host exceeds that of the virus at certain temperatures.

It is also reported that, posttranscriptional gene silencing (PTGs) in plants serves as a natural antiviral defence response (Waterhouse *et al.*, 2001). This phenomenon involves sequence-specific suppression of gene expression in plants. This RNA-silencing is termed RNA interference (Fire *et al.*, 1998) The effect of temperature was

found to be associated with gene silencing, and increasing temperature drastically elevated virus-derived short-interfering RNA (siRNA) accumulation, resulting in less symptom development (Szittya *et al.*, 2003).

A unique feature of RNA silencing is the cleavage of long doubled-stranded RNA (dsRNA) into short interfering (21-24 nts) RNAs (Hamilton and Baulcombe, 1999) by a ribonuclease III-like enzyme termed DICER (Bernstein *et al.*, 2001).

2.10 Virus elimination by the combined effect of thermotherapy and meristem culture

Thermotherapy and meristem tip culture, although, are reported to eliminate plant viruses when each is applied alone, there is evidence that they fail to completely free some species of plants of some viruses (Dodds *et al.*, 1989; Griffiths *et al.*, 1990). According to these authors, Potato Virus S (PVS) and Potato virus X (PVX) are difficult to eliminate by thermotherapy or meristem-tip culture alone. In such cases, thermotherapy can complement meristem-tip culture to produce virus-free plants.

According to Stace-Smith & Mellor (1968) and Slack (1980), meristem culture has resulted in efficient elimination of most systemic and viral diseases in plants when used in combination with other antiviral therapies, with the percentage of virus elimination being greater with the simultaneous adoption of thermotherapy. Hillocks (1997) generated virus-free plants for subsequent multiplication and use by farmers through meristem culture coupled with thermotherapy.

The rate of production of healthy vegetative plants using these two methods is affected by the size of explants used. When size of the meristem tip used is very small (0.1 mm), the percentage of growing meristems is not always satisfactory (Hollings, 1965). To increase this percentage, a combination of heat treatment with meristem culture was recommended. Thus, the use of larger meristem tips (1 mm or more) once the virus advance had been slowed, was suggested as it grew better on the nutrient media and was virus-free (ten Houten *et al.*, 1968). Brown *et al.* (1988) obtained 90% eradication of potato virus S (PVS) from more than 20 genotypes of meristems of sizes ranging between 0.2 to 1.0mm of heat-treated plants that were excised and cultured *in vitro*. Similarly, Nyland and Goheen (1969) found that the only obvious significant variable for thermotherapy were temperature, duration of treatment and size of explant used for culture initiation. They observed that, the success of the heat therapy depended on upon removal of a small to large portion (0.2-1.4mm) of the treated plant after the exposure to high temperature. Adejare and Coutts (1981) also reported the absence of mosaic symptoms on the leaves of rooted explants when they subjected diseased donor cassava explants to heat treatment for not less than 30 days at 35°–38°C, and cultured on modified MS (Murashige and Skoog, 1962) medium. This resulted in raising disease-free plants from heat-treated meristems, in comparison with the control (untreated) plants.

In view of the successes reported by the above authors, it is necessary to replicate this technique on some local cassava cultivars. There is therefore a need to investigate the response of some known mosaic-diseased cassava cultivars to the treatment of both heat and meristem-tip culture in the elimination of the virus. Leonhardt *et al.* (1998) have also reported similar effect on potato, suggesting a temperature of 37°C for a

minimum of 30 days as adequate for virus elimination in potato plants cultivated *in vitro* from single node cuttings.

According to Nyland and Goheen (1969), heat need not be applied in sophisticated heat chambers for the successful eradication of plant viruses. Ahloowalia and Prakash (2002) suggested that a simple box, large enough to contain the plants, fitted with heat producing incandescent lamps is adequate to produce virus-free plants. The objective of the current study was therefore to use a simple heat box made with side glass to provide heat therapy combined with meristem tip culture to eradicate cassava mosaic virus from four local cassava accessions.

2.11 Virus indexing

Visual inspection is made relatively easy when symptoms clearly are characteristic of a specific disease. However, many factors such as virus strain, host plant cultivar/variety, time of infection, and the environment can influence the symptoms exhibited (Matthews, 1980). For specificity, reliability and versatility of diagnosis, the use of serological and molecular assays, and indicator hosts in the greenhouse have undergone major breakthrough (Naidu and Hughes, 2003), making virus indexing more accurate.

Indexing for the presence of viruses can be achieved by visual examination, viral symptoms infection tests on indicator plants, serological test, electron microscopy, and direct detection of RNA using molecular techniques (Naidu and Hughes, 2003). Progress in molecular biology, biochemistry and immunology has led to the development of many

new, accurate, rapid and less labour-intensive methods of virus detection (Naidu and Hughes, 2003).

Technologies for the molecular detection of plants pathogens have already undergone two major breakthroughs well over the past three decades. The first was the advent of antibody-based detection, in particular monoclonal antibodies and enzyme-linked immunosorbent

assay (Kohler and Milstein, 1975; Clark and Adams, 1977). More to these, are viral nucleic acid based techniques such as dot blot hybridization/slot blot hybridization, polymerase chain reaction (PCR) with virus-specific primers, nucleic acid hybridization with radio-labelled and nonradio-labelled probes, DNA/RNA probes. (Hampton *et al.*, 1992).

Serological or immunological assays are conducted on a solid surface such as on a micro titre plate or nitrocellulose membrane and the antigen–antibody reaction is visualized by means of a suitable detection system such as an enzyme-labelled antibody (Hampton *et al.*, 1992; Van Regenmortel, 1982; Van Regenmortel and Dubs, 1993). In these methods, Enzyme-Linked Immunosorbent assay (ELISA) is versatile, but is labour intensive in that several steps with different reagents are involved (Gould and Symons, 1983). In addition, ELISA can only be applied for those viruses where specific antisera are commercially available. It is further limited by the uneven patterns of distribution of certain pathogens in the plant (Gould and Symons, 1983), as well as climatic influence, which reduce the titre below the possible level of detection (McLaughlin *et al.*, 1981; Hewings and D'Arcy, 1984). Nucleic acid-based detection methods therefore have the advantage since any region of a viral genome can be targeted to develop the diagnostic test (Gould and Symons 1983).

In molecular laboratory assays, the affinity of one strand of DNA for its complementary sequence is one of the strongest and most specific interactions that have been exploited in developing nucleic acid hybridization assays, which are based on the homology between two strands of nucleic acid (Mullis *et al.*, 1986). In this assay, a single-stranded complementary nucleic acid, which has been "labelled" with a reporter molecule, is used as a probe to form a hybrid with the target nucleic acid. The double-stranded probe-target hybrid molecules are then detected by several methods, depending on the reporter molecule used. The sensitivity of nucleic acid-based detection systems was greatly improved following the development of the polymerase chain reaction (PCR) procedure (Mullis *et al.*, 1986).

PCR is an *in vitro* method for amplifying specific DNA regions producing easily detectable amounts of DNA fragments, using universal primers. These are usually visualized by agarose gel electrophoresis. PCR-based diagnostic technique is 100-1000 folds more sensitive (Henson and French, 1993; Hadidi *et al.*, 1995; Candresse *et al.*, 1998) and therefore, suitable for the detection of infections in an initial stage, even when the pathogen titre in the plant is low. It is a molecular biology method for enzymatically copying target nucleic acid sequence without using a living organism, in which repeated replication of a given sequence forms millions of copies within a few hours (Mullis and Faloona, 1987). The method is a highly specific and versatile method of DNA amplification using thermostable DNA polymerase from *Thermus quaticus* or *Pyrococus furiosus* (Mullis and Faloona, 1987). The procedure starts with extraction of total genomic DNA from plant sample (Aldrich and Cullis 1993).

Since PCR has the power to amplify the target nucleic acid present at an extremely low level and form a complex mixture of heterologous sequences, it has become an attractive technique for the diagnosis of plant virus diseases. A single diagnostic test or assay may provide adequate information on the identity of a virus, but a combination of methods is generally needed for unequivocal diagnosis. While symptoms visually provide vital information on virus diseases, it is necessary that visual inspection for symptoms be done in conjunction with other confirmatory tests such as the polymerase chain reaction to ensure accurate diagnosis of virus infection (Bock, 1980).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Source and establishment of plant materials

Four cassava accessions namely "Amakuma", "Esi-Abaya", "Afisiafi" and "UCC-Bankye" showing various degrees of severe CMD symptoms in cassava fields were collected from the CSIR-Crops Research Institute (CRI) at Fumesua, near Kumasi. Ten (10) dormant woody stem cuttings (5-7 nodes long) taken from diseased mother plants, were randomly selected in two batches of five cuttings from each of the four accessions. Batch-1 cuttings were planted in buckets and subjected to thermotherapy, while batch-2 cuttings were also planted in buckets and placed outside the insectory of the Crop Science Department of the KNUST, to serve as untreated plants. Buckets

were filled with sterile topsoil. The buckets of soil containing the cuttings were watered regularly to maintain adequate water regime for the spouting and growth of plants.

3.2 Establishment of heat-treated (Thermotherapy) plants.

A batch of five (5) cuttings each selected from the four diseased cassava accessions were subjected to a heat treatment at 37±2°C for four to six weeks. The five cuttings (representing 5 replications) per cultivar were planted in buckets filled with sterile topsoil and placed in a heat chamber to sprout and establish shoots (Figure 2). The heat chamber was a 0.9m^3 wooden box fitted with side glasses (Figure 3), and 100watts incandescent bulbs to generate the heat. Two thermometers were placed in the box to monitor the heat generated. Each bucket was placed 15cm between rows apart in the heat chamber. The nursed cuttings in the buckets were periodically watered to prevent desiccation of the growing plants. The temperature was kept within the range (35-39°C) until the apical buds of the sprouted shoots were excised and aseptically cultured in the tissue culture laboratory.

Figure 2. Five cassava cuttings each of the four (4) cultivars nursed in buckets placed in a heat chamber to sprout. From left: "Afisiafi", "Amakuma", "UCC-Bankye" and "Esi-Abaya"

Figure 3. Sprouted cuttings of four (4) cultivars of cassava growing in the heat chamber. From left- see above legend

3.3 Composition of stock solutions and preparation of culture medium

Murashige and Skoog (1962) basal salts prepared from stock solution was used for culture initiation. The composition of the MS basal salts is shown below:

Stock solution I (macronutrients)

Chemical	*g/l*
NH_4NO_3	33.0 g
KNO_3	38.0 g
$CaCl_2.2H_2O$	8.8 g
$MgSO_4.7H_2O$	7.4 g
KH_2PO_4	3.4 g

Stock solution II (micronutrients)

Chemical	*g/l*
KI	0.16 g
H_3BO_3	1.24 g
$MnSO_4.H_2O$	4.46 g
$ZnSO_4.7H_2O$	1.72 g
$Na_2MO_4.2H_2O$	0.05 g
$CuSO_4.5H_2O$	0.005 g
$CoCl_2.6H_2O$	0.005 g

Stock solution III

Chemical	*g/l*
$FeSO_4.7H_2O$	5.56 g
Na_2. EDTA. $2H_2O$	7.46 g

Vitamin stock solution mixture

Chemical	*g/500ml*
Thiamine HCl	0.01 g
Pyridoxine HCl	0.05 g
Nicotinic acid	0.05 g
Glycine	0.1 g

MS basal medium for stock solution

Stock solution I (macronutrients)	50 ml
Stock solution II (micronutrients)	5 ml
Stock solution III	10 ml
Vitamin stock solution	5 ml
Sucrose	30 g

Myo-inositol	100 mg
Phytagel	3.5 g

The stock solutions of the macro and micronutrients were prepared at one-litre stock of each. This was carried out by filling the one-litre beaker with 300 ml distilled water placed on a magnetic stirrer and the exact amounts of each chemical was weighed and dissolved in the water one after the other. The content of the beaker was then topped-up with distilled water to a one-litre volume.

In the preparation of the stock solution-III, a total solution of 100ml was prepared. The constituent compounds were accurately weighed at 0.556g for $FeSO_4.7H_2O$ and 0.746g for $Na_2.EDTA.2H_2O$ and dissolved separately in 20ml of distilled water. Since the two compounds are not soluble at room temperature, the solutions were thoroughly mixed using a magnetic stirrer hot plate and heated until it turned yellowish.

The vitamin mixture stock solution was prepared by accurately weighing and dissolving thiamine hydrochloride at 0.01g, pyridoxine at 0.05g, nicotinic acid amide at 0.05g and glycine at 0.1g in a beaker containing 200ml distilled water. The mixture was then thoroughly stirred on a magnetic stirrer and topped up with distilled water to a final volume of 500 ml.

3.4 Preparation of hormone-free MS basal medium from stock solutions

A hormone-free Murashige and Skoog (1962) basal medium was prepared from the stock solution described above. It was supplemented with 100 mg (0.1g) L^{-1} inositol, 30g of sucrose and 3.5g of phytagel. The medium was prepared by filling a one-litre

beaker with 300ml distilled water and with a magnetic stirrer in the beaker, placed on a magnetic hot plate. While stirring, 50ml volumes of stock I, 5ml of stock II, 10ml of stock III and 5ml of vitamin mixture were measured and added into the distilled water, respectively. After 10-20 minutes of stirring, 100mg of myo-inositol and 30g of sucrose were weighed and dissolved into the mixture. The solution was then topped-up with distilled water to the 900ml mark of the beaker and the pH of the solution adjusted to 5.8 with drop wise quantities of 0.083M NaOH or 0.083M HCl. With the final volume of 1liter, the solution was transferred into erlymeyer flask, and 3.5g of phytagel, used as a gelling agent, was added to the medium and heated gently until it melted. The content was immediately dispensed into 145 x 25mm Pyrex test tubes at 10 ml per tube, closed with lid, and autoclaved at 121°C for 20 minutes and at a pressure of 1.33bars. The culture tubes containing medium were removed from the autoclave and allowed to cool, solidify, and then transferred to the inoculation room for culture initiation.

3.5 *In vitro* culture of Cassava Meristematic tips

Apical bud explants (1-2cm long) from shoots established in the heat chamber as well as from buckets (control) were excised using scalpel into magenta vessel containing distilled water. Explants were then transferred to the inoculation room in the tissue culture laboratory. The distilled water was discarded and materials taken to laminar flowhood. The shoot-tips were surface sterilized in 5% sodium hypochlorite solution for 20 minutes. The containers were agitated vigorously to ensure complete surface contact of disinfectant on shoot-tips. The sodium hypochlorite was discarded and the buds rinsed in five changes of sterile distilled water at 5 minutes intervals. The shoot-tips were then transferred to sterile Petri dishes lined with moist sterile filter paper.

Meristematic tissues of different sizes (D1+1 measuring 0.2-1.0mm with one pair of leaf primordium, D2+2 measuring 1.0-2.0mm with 2 leaf primordia and D3+3 measuring 2.0-3.0mm with 3 leaf primordia) were excised from the sterilised shoot-tips with a dissecting needle and scalpel blades under the microscope. They were then cultured on a hormone-free MS basal medium supplemented with inositol, sucrose and phytagel contained in the 145x25mm Pyrex tubes (Plate 2). Each explant was embedded with only a small part in the medium, and sealed with parafilm. After inoculation, cultures were transferred to the incubation room, programmed to provide a 16/8 hours photoperiod, a temperature of 24±2°C and 70% relative humidity (RH).

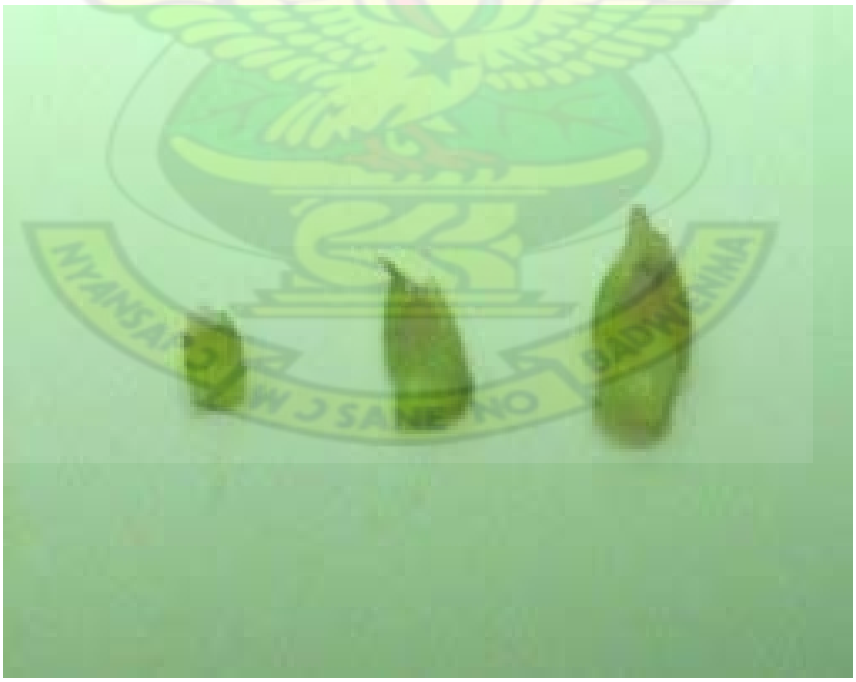


Plate 1. Different sizes of meristem tips excised under aseptic condition. From left: D1+1 (0.2-1.0mm with 1pair of leaf primordium), D2+2 (1.0-2.0mm with 2 pairs of leaf primordia) and D3+3 (2.0-3.0mm with 3pairs of leaf primordia)

Plate 2: Meristematic explants cultured on MS medium supplemented with inositol, sucrose, phytagel and vitamins.

3.6. Virus indexing

3.6.1. Virus detection by visual inspection

Pre and Post-cultured shoots derived from cuttings of the four diseased cultivars with differential disease symptoms of African cassava mosaic virus (ACMV) were evaluated for the presence and severity of symptoms by physical observation of plant leaves.

A month after planting the cuttings, initial disease severity symptoms of the four accessions were scored by visual inspection of sprouted shoots of the heat treated and untreated nursed cuttings. After plantlets were established from *in vitro* explants, disease score was again assessed to determine the status of derived plantlets. Scored data was subjected the square root transformation and analysed. The severity of disease symptoms was scored using a range of 1-5 where

1= no symptoms

2= a mild chlorotic pattern over the entire leaf while the latter appears green and healthy

3= a moderate mosaic pattern throughout the leaf narrowing and distortion in the lower

one-third of the leaflets

4= severe mosaic distortion in two-third of the leaflets and general reduction in leaf size

5= severe mosaic and distortion in the entire leaf.

Index of severity of symptoms based on all plants (ISS_{AP}), and index of severity of symptoms based only on diseased plants (ISS_{DP}) were used. The data was subjected to angular transformation (Njock *et al.*, 1994) and analysed:

$$\text{Incidence (\%)} = (X/Y)\ 100$$

$$ISS_{AP} = (a+2b+3c+4d+5e)/\ (a+b+c+d+e)$$

$$ISS_{DP} = (2b+3c+4d+5e)/\ (b+c+d+e)$$

where X is the number of diseased plants and Y the total number of plants scored; a, b, c, d, and e are the number of plants scored under the respective severity classes 1, 2, 3, 4 and 5.

Shoots derived from cuttings of the four diseased cultivars with differential disease symptoms of African cassava mosaic virus (ACMV) were assessed for index of severity of symptoms (ISS) to determine their status by scoring young resultant plants in a

potted experiment. Data was analysed after square root transformation and are in parenthesis (Table 5).

3.6.2. Extraction of genomic DNA

Genomic cassava DNA was extracted from leaf samples taken from tissue-cultured plantlets of the four cassava cultivars, submitted to heat treatment, and leaf samples from pre-cultured sprouted shoots of these cultivars showing various degrees of severity symptoms. The DNA samples were extracted using a modified CTAB method by Aldrich and Cullis (1993) as follows:

Twenty milligrams (20mg) of fresh sample each were ground in 500µl of 2% CTAB and 0.1% (0.5µl) of mercaptoethanol with intermittent vortexing in 1.5ml microfuge tube. Three hundred (300µl) micro litres of 2% CTAB and 0.5µl (0.1 %) of β-mercaptoethanol was added to the samples and then incubated in a sand bath at 65ºC for 30 minutes. The samples were allowed to cool at room temperature and an equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed by several inversions of the tube to wash DNA from debris. Samples were then centrifuged at 14000rpm for 15 minutes and the supernatant phase of the sample was transferred into a clean 1.5 ml tube. The chloroform: isoamyl alcohol cleaning step was repeated. Two-thirds ($^2/_3$) volume (200µl) of ice cold isopropanol was added to the supernatant to precipitate the DNA, and kept on ice for 30 minutes and centrifuged at 10,000rpm for 5 minutes. The isopropanol was decanted and the DNA pellet was washed with 350µl washing buffer (76% ethanol plus 10mM ammonium acetate) on a rocking surface for 15 minutes and again centrifuged at 6000rpm for 4 minutes. The washing buffer was decanted and washed again in 350µl volume of 70% ethanol, and then centrifuged at 6000 rpm for 4 minutes. The ethanol was decanted and DNA dried in vacuum for 10 minutes, until the smell of ethanol was no longer detectable.

The DNA was then suspended in 100µl TE buffer (1mM Tris HCI, pH 8, 0.1mM EDTA, pH 8). A quantity of 10µl DNA sample + 5µl sample buffer (Bromophenol blue) each was taken and run alongside **λ** Hind III molecular marker on 0.8% Agarose gel containing Ethidium bromide, and photographed under UV light-transluminator for band observation to check the quality and quantity of DNA extracted.

3.6.3 Preparation of Agarose gel and DNA loading into wells

Three (3.0g) grams of agarose (Sigma company) was dissolved in 100ml of TAE (242g Tris base, 57.1ml glacial acetic, 100ml 0.5 M EDTA) buffer to make 1.5% agarose. The agarose was melted in a microwave oven and allowed to cool to 65°C with constant stirring. After cooling, 4µl of ethidium bromide was added to the 100ml agar and poured into gel plate with comb already set in place to allow for loading of sample. The agarose gel was allowed to solidify for approximately 30 minutes before the comb was removed and the gel immersed in the electrophoresis tank containing 1xTAE buffer.

Two microlitres (2µl) of loading buffer was added to 10µl of each DNA sample and loaded into already prepared wells. The first well was loaded with 10µl of 100 bp ladder. The gel was run at 100 volts for 1.5 hours. After the electrophoresis, the gel was removed and visualised on a UV light-transilluminator and photographed.

3.6.4 Virus Indexing by Polymerase Chain Reaction (PCR) method

Genomic DNA extract from leaves of cassava accessions from the field, thermotherapy and *in vitro* regenerated (Table 2) were used as template for PCR amplifications to detect the presence or absence of cassava mosaic disease (CMD). The amplification was done using specific primers designed previously to detect ACMV (Fondong *et al.*, 2000). The primer sequences are shown in Table 1.

Table 1. Primers used in the virus diagnostics

Primer Sequence	Name	Direction	Virus
5′ TTC AGT TAT CAG CGC TCG TAA 3'	F_1	Forward	ACMV
3′ GAG TGC AAG TTG ACT CAT GA 5′	R_1	Reverse	ACMV
5′ GTG AGA AAG ACA TTC TTG GC 3'	F_2	Forward	ACMV
3' CCT GCA ATT ATA TAG TGG CC 5′	R_2	Reverse	ACMV
5′ GCG GAA TCC CTA ACA TTA TC 3'	AL1/F	Forward	ACMV
3' GCT CGT ATG TAT CCT CTA AGG G 5′	ARO/R	Reverse	ACMV
5′ CTC AAC TGG AGA CAC ACT TG 3'	1	Forward	ACMV
3' CCG GCA ACA TAC TTA CGC TT 5′	2	Reverse	ACMV
5′ TAC ACA TGC CTC RAA TCC TG 3'	AL3/F	Forward	UV
3' CTC CGC CAC AAA CTT ACG TT 5′	$AL1/R_2$	Reverse	UV
5′ TGT CTT CTG GGA CTT GTG TG 3'	$AL1/F_1$	Forward	UV
3' TGC CTC CTG ATG ATT ATA TGT C 5′	CP/R_3	Reverse	
ACMV			

Primer sources: Fondong *et al.*, 2000

Table 2. List of Positive controls used

Well no.	Description
1	"UCC-Bankye" leaf DNA extracts taken after heat treatment only
2	"Esi-Abaya" leaf DNA extracts taken after heat treatment only
3	"Afisiafi" leaf DNA extracts taken after heat treatment only
4	"UCC-Bankye" leaf DNA extracts taken from the field
5	"Esi-Abaya" leaf DNA extracts taken from the field
6	"Amakuma" leaf DNA extracts from meristem-tip culture only.
7	"UCC-Bankye" leaf DNA extracts from meristem-tip culture only
8	"Esi-Abaya" leaf DNA extracts from meristem-tip culture only
9	"Afisiafi" leaf DNA extracts from meristem-tip culture only

Samples of the DNA extract for each treatment were dissolved in TE buffer to a concentration of 10ng/ml for the PCR amplification. Two (2µl) micro litres each of the 10ng/ml DNA was added to one tube of a lyophilized Accupower HotStar PCR premix kit (1 unit of Hot Start Taq, DNA polymerase, 1x PCR Buffer containing 1.5mM $MgCl_2$ and 250 µM of each dNTP; Bioneer Company, Japan), together with 1µl each of both forward and reverse primers (Table 1), and 16µl of distilled water (dH_2O) was added to make a total volume of 20µl. The reaction mixture was amplified using ABI 2720 PCR thermocycler, programmed to run 35 cycles. Amplification cycle consists of denaturation at a temperature of 94°C for 5 minutes, annealing temperature at 52°C for 1 minute and elongation at 72°C for 2 minutes. This initial amplification cycle was followed by 35 cycles of 1 minute at 94°C, 1 minute at 52°C and 2 minutes at 72°C. At the end of the reaction, a final elongation step was achieved at 72°C for 10 minutes. PCR products were separated by electrophoresis in a 1.5% agarose gel containing ethidium bromide under a constant current of 120 mA for 2 hours and 30 minutes. The amplified bands were visualized under UV-light-transluminator.

CHAPTER FOUR

RESULTS

4.1. Virus disease Status in the Cassava varieties studied

The disease scores on the shoots of the four accessions with differential disease symptoms of African cassava mosaic virus (ACMV) are shown in Table 3. There were significant differences ($p \leq 0.05$) between cassava cultivars prior to thermotherapy, with "Amakuma" and "Esi-Abaya" showing 100% disease incidence in the pre-cultured shoots, while "Afisiafi" and "UCC-Bankye" showed 85 and 80% incidence, respectively.

Table 3. Scores of African Cassava Mosaic Virus disease status on shoots before thermotherapy on the four (4) cassava accessions

Cultivar	ISS_{AP}	ISS_{DP}	Incidence (%)
"Afisiafi" (AFC)	2.15 (1.62)	2.30 (1.67)	85 (9.11)
"Amakuma" (AMC)	2.80 (1.81)	2.80 (1.81)	100 (10.02)
"UCC-Bankye" (UCCC)	2.00 (1.58)	2.08 (1.60)	80 (8.94)
"Esi-Abaya" (EAC)	3.30 (1.95)	3.30 (1.95)	100 (10.02)
Grand mean	(1.74)	(1.76)	(81.1)
Lsd	(0.18)	(0.16)	(21.68)
Cv (%)	(6.5)	(5.8)	(9.5)

ISS_{AP} = Index of severity of symptoms on all plants
ISS_{DP} = Index of severity of symptoms on diseased plants only
**Parenthesis = Transformed data used for analysis*

The highest severity index (3.30) of symptoms scores on all plants (ISS_{AP}) and (3.30) on diseased plants only (ISS_{DP}) were observed in pre-cultured "Esi-Abaya". Statistically, the severity scores of "Amakuma" for severity of symptoms on all plants and on diseased plants only (2.80 and 2.80) respectively, were not significantly different from those of "Esi-Abaya". The lowest score for ISS_{AP} and ISS_{DP} was observed in "UCC-Bankye", with 2.0 and 2.08 symptom scores, respectively. However, the scores for ISS_{AP} and ISS_{DP} by "UCC-Bankye" and "Afisiafi" were both significantly lower than those for "Amakuma" and "Esi-Abaya".

Only "Amakuma" and "Afisiafi" showed ACMV disease incidence after subjecting the cuttings to heat treatment (Table 3). The highest incidence of symptoms (21.7) was in "Amakuma", followed by "Afisiafi" with 10% incidence. The incidence results reflected in the index of severity of symptoms (ISS_{AP}) of 1.25 and 1.15 and ISS_{DP} of 1 and 1.3 for "Amakuma" and "Afisiafi", respectively. Both "UCC-Bankye" and "Esi-Abaya" did not

show any incidence of the disease and therefore no severity index was recorded by visual inspection (Plate 5: e and h).

Table 4. Scores of the African cassava mosaic virus (ACMV) disease on heat-treated cassava shoots.

Cultivar	ISS_{AP}	ISS_{DP}	Incidence (%)
"Afisiafi" (AFH)	1.15 (1.283)	1.25(1.22)	10.0 (2.6)
"Amakuma" (AMH)	1.25 (1.319)	1.00(1.14)	21.7 (3.5)
"UCC-Bankye" (UCCH)	1.00 (1.225)	0.00(0.707)	0.0 (0.7)
"Esi-Abaya" (EAH)	1.00 (1.225)	0.00(0.707)	0.0 (0.7)
Grand mean	(1.26)	(0.94)	(1.89)
Lsd	(0.1)	(0.52)	(2.88)
Cv(%)	(4.8)	(34.2)	(95.2)

**ISS_{AP} = Index of severity of symptoms on all plants*
**ISS_{DP} = Index of severity of symptoms on diseased plants only*
**Parenthesis = Transformed data used for analysis.*

4.2. Effects of thermotherapy and meristem size on plant regeneration of cassava accessions

The three (3) different explant sizes excised from the four cassava accessions showed sharp differences when shoots from which they derived were heat-treated (Table 5). The large (2.0-3.0mm) explant size recorded the highest (58.3%) plant regeneration, obtaining 7 plants out of 12 explants cultured. The least (7.7%) plant regeneration was obtained by the small (0.2-1.0mm) explant sizes cultured, recording 1 plant out of 13 explants cultured. Three (3) of the accessions ("Afisiafi", "Amakuma" and "UCC-Bankye") produced 66.7% plant regeneration with the large explant size, but 0% regeneration with the small explants cultured. "Esi-Abaya" produced 33.3% plant regeneration, representing 1plant out of 3explants cultured, from the large explants cultured and the only cassava accession

that regenerated from the small explant size cultured. These grew into whole plants with well-differentiated shoot and root systems.

Table 5. Effect of thermotherapy and size of meristem on plant regeneration from four (4) cassava accessions

Variety	D1+1(0.2-1.0mm)				D2+2(1.0-2.0mm)				D3+3(2.0-3.0mm)			
	Treated		Control		Treated		Control		Treated		Control	
	Inoc.	**Surv**	**Inoc.**	**Surv**	**Inoc.**	**Surv**	**Inoc.**	**Surv**	**Inoc.**	**Surv**	**Inoc.**	**Surv**
"Afisiafi"	4	0	4	1	3	2	4	0	3	2	3	0
"Amakuma"	3	0	4	1	3	1	4	1	3	2	4	2
"UCC-Bankye"	3	0	4	2	3	1	3	0	3	2	3	1
"Esi-Abaya"	3	1	4	1	3	0	3	0	3	1	3	1
Total	13	1	13	5	12	4	14	1	12	7	13	4

* ***Inoc*** = Number of explants cultured

* ***Surv*** = Number of plants regenerated

The plant regeneration was high (31.3%) obtaining 5 plants out of 16 explants cultured with the small (0.2-1.0mm) explant size cultured when their donor shoots were not heat-treated (control). The large (2.0-3.0mm) explant size followed closely with 30.8% (4 plants out of 13 explants cultured) plant regeneration. The medium (1.0-2.0mm) explant size recorded the least (7.1%) plant regeneration. "Afisiafi" recorded 25% regeneration, obtaining 1 plant out of 4 explants of the small size cultured, but obtained 0% both the medium and the large explant sizes cultured. "Amakuma", "UCC-Bankye" and "Esi-Abaya" recorded 25, 50 and 25% plant regeneration from the small explant sizes and 50, 33.3% with the large explant sizes respectively.

4.3. Incidence of African cassava mosaic geminivirus in shoots regenerated from *in vitro* thermotherapy

4.3.1 Symptoms severity index:

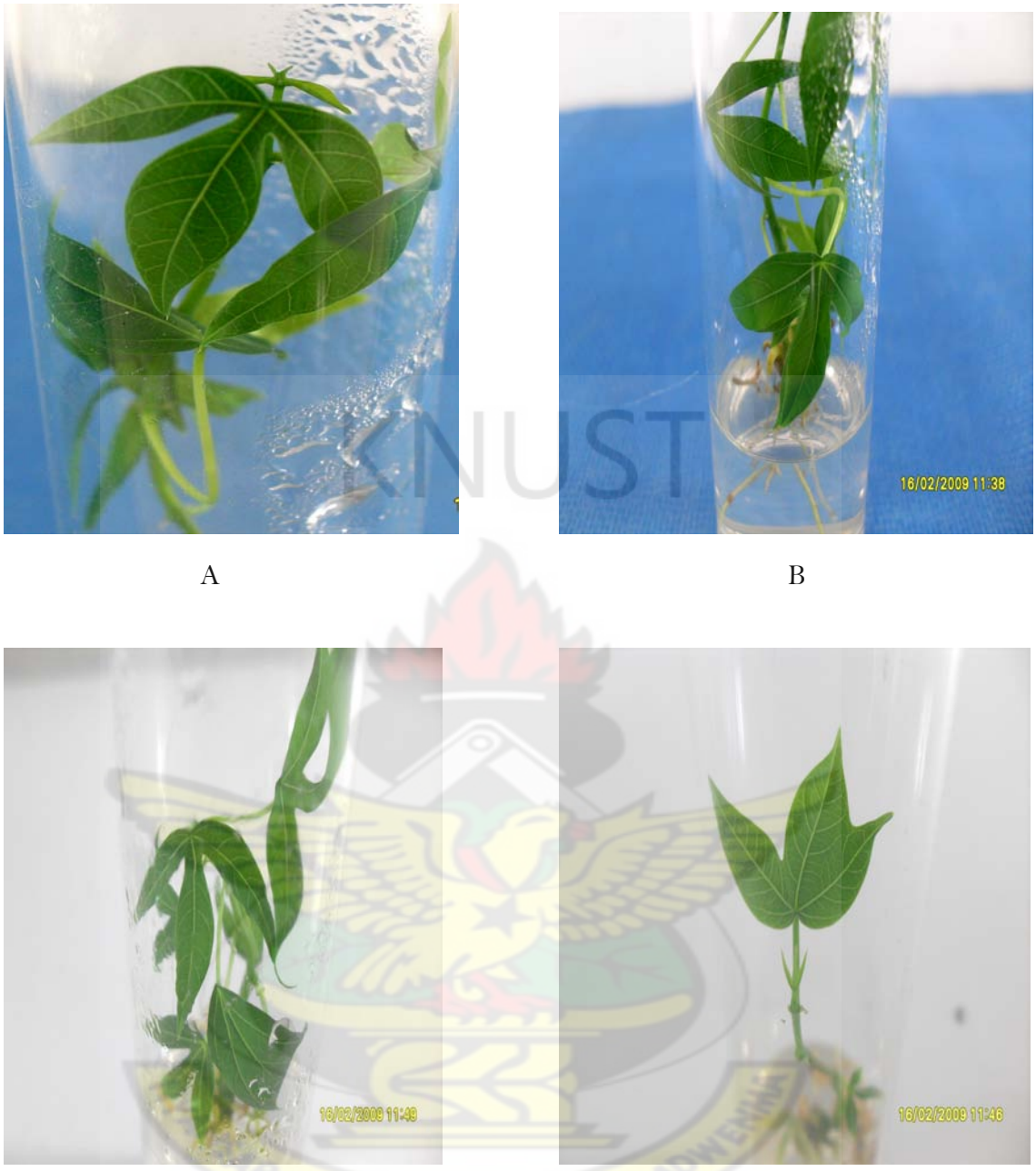
Scores of the presence and severity of symptoms on regenerated plantlets from the three explant sizes (0.2-1.0mm, 1.0-2.0mm and 2.0-3.0mm) recorded 0% incidence of the disease heat-treated and cultured (Table 6), with the derived plantlet from large and medium explant size scoring 1 on the scale of Index of severity of symptoms (ISS) on both diseased and all plants assessed for the four cassava accession regenerated. The small explant size for "Afisiafi", "Amakuma" and "UCC-Bankye" scored 0 for both ISS_{AP} and ISS_{DP} because there was no plant regenerated to assess for disease symptoms. "Esi-Abaya" scored 1 for index of disease symptoms on the regenerated plants from small and the large explant sizes.

Similarly, derived plantlets from untreated shoots scored 1 on the scale of both ISS_{AP} and ISS_{DP} for regenerated plants of the small (0.2-1.0mm) explant sizes cultured from "Afisiafi", "Amakuma", "UCC-Bankye" and "Esi-Abaya". However, the ISS_{AP} and ISS_{DP} score of 2 for "Amakuma", and "UCC-Bankye" was observed on the regenerated plants of the large explant size cultured, with 33.3% incidence on the two accessions (Table 6), respectively.

Table 6. Scores of ACMV disease symptoms on derived plantlets from different sizes of heat-treated and meristem culture explants.

Explant size	Parameter	"Afisiafi"		"Amakuma"		"UCC-Bankye"		"Esi-Abaya"	
		H	C	H	C	H	C	H	C
D1+1 (Small)	ISS_{AP}	0	1	0	1	0	1	1	1
	ISS_{DP}	0	1	0	1	0	1	1	1
	Incidence	0	0	0	0	0	0	0	0
D2+2 (medium)	ISS_{AP}	1	0	1	1	1	0	0	0
	ISS_{DP}	1	0	1	1	1	0	0	0
	Incidence	0	0	0	0	0	0	0	0
D3+3 (large)	ISS_{AP}	1	0	1	2	1	2	1	1
	ISS_{DP}	1	0	1	2	1	2	1	1
	Incidence	0	0	0	33.3	0	33.3	0	0

*_H_= Thermotherapy
*_C_= Control treatment



A B

C D

Plate 3. Plantlets regenerated from meristems. A= Treated "Afisiafi" D3+3 , B= Treated "Amakuma" D2+2 , C = Treated Afisiafi" D2+2 and D = Treated "UCC- Bankye" D2+2.

4.3.2 Post culture virus indexing using PCR

Different amplification profiles were obtained from the genomic DNA using the primer pair's sequences F1/R1, F2/R2 and AL1/F/ARO/R relative to ACMV (Table 1). The cassava samples in lanes 1, 2, 3, 6, 7, 8, 9, and 10 did not reveal presence of ACMV within the generated amplification profiles, representing DNA samples from plantlet leaves obtained from the different sizes of explants of "Afisiafi", "Amakuma", "UCC-Bankye" and "Esi-Abaya" that received heat-treated and tissue culture.

In contrast, samples in lanes 4 and 5 revealed presence of ACMV showing bands across the specific primers used were obtained from DNA samples of *in vivo* untreated plants.

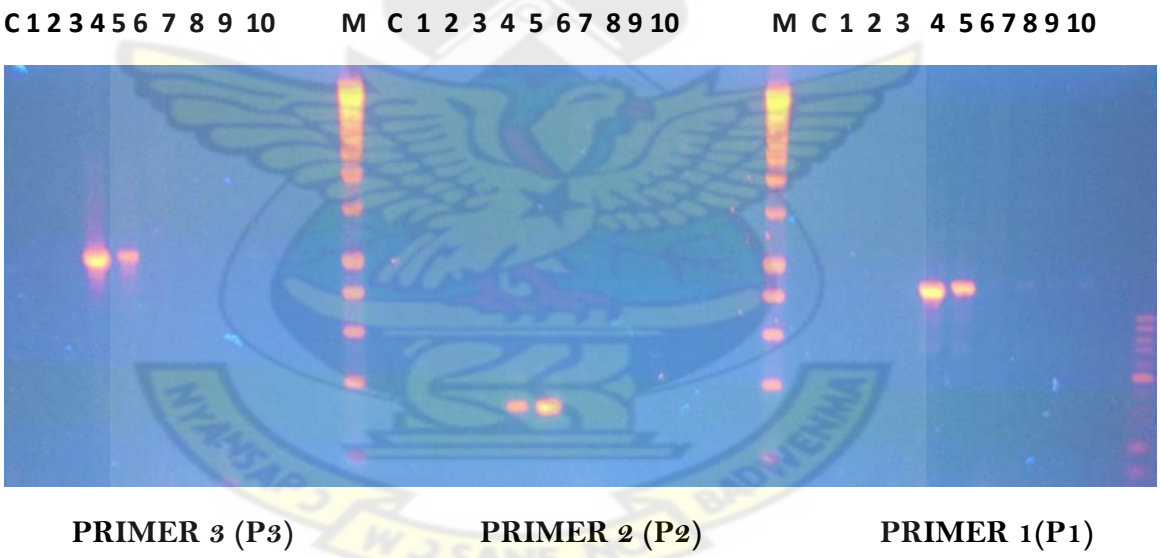


Figure 4. Amplification profile of genomic DNA of cassava samples treated with heat and meristem-tip culture using primers F1/R1 (P1), F2/R2 (P2) and AL1/F / ARO/R (P3). Lanes M, 100bp DNA ladder, lane c, water negative control, lanes 1 (heat-treated "UCC-Bankye"), 2 (heat-treated "Esi-Abaya"), 3 (heat-treated "Afisiafi"), 4 ("UCC-Bankye" no treatment), 5 ("Esi-Abaya" no treatment), 6 ("Amakuma" cultured only), 7 ("UCC-Bankye" cultured only), 8 ("Esi-Abaya" cultured only), 9 ("Afisiafi" cultured only) and 10 ("Afisiafi heat-treated plus culture).

A

B

C

D

Plate 4. Disease status of "Afisiafi" and "Amakuma" (A and C, respectively) and replica (B and D, respectively) submitted to thermotherapy, produced seemingly clean plants.

E

F

G

H

Plate 5. Disease status of "UCC-Bankye" and Esi-Abaya" (E and G, respectively) and replica submitted to thermotherapy(F and H, respectively), produced seemingly clean plants.

CHAPTER FIVE

DISCUSSIONS

5.1. Virus disease status of cassava accessions

The cuttings of the four cassava accessions showed varying degrees of foliar symptom severity typical of the cassava mosaic disease. The disease is characterized by leaf chlorotic blotches, distortion and a reduction of the leaf area, depending on the degree of severity. "Esi-Abaya" (ISS_{DP}= 3.30) and "Amakuma" (ISS_{DP}= 2.80) were the most severely affected by the disease among the four cultivars studied recording a 100% incidence, respectively. Unlike "Esi-Abaya" and "Amakuma", "UCC-Bankye" and "Afisiafi" exhibited mosaic symptoms with reduced chlorotic pattern over the entire leaf while the leaf appeared green as these two accessions recorded the ISS_{DP} of 2.0 and 2.30, with disease incidence of 80 and 85% respectively. This result agrees with what was observed by Hahn *et. al.*, (1980); Jennings, (1994); Thresh *et. al.*, (1994) which stated that differences in disease severity symptoms expressed depended on the level of resistance of the cultivar.

Shoots that was subjected to pre-culture thermotherapy alone at 35-37°C appeared to be disease-free of the cassava mosaic symptoms (Plates 4 and 5) when the leaves were assessed for incidence and severity of symptoms, after three to four weeks of treatment. Similar effect of thermotherapy on disease suppression has been reported to produce clean cassava planting materials (Garcia *et al.*, 1993; Zok, 1993; Delgado and Rojas, 1993), suggesting that thermotherapy alone might be sufficient to produce virus-free plants. This is because of the ability of heat to inhibit the multiplication of the virus during its application in many plants, including fruit trees (Manganaris *et al.*, 2003).

5.2. Response of explant size to thermotherapy and *in vitro* culture

The technique of isolating meristem tips is an art, which can only be perfected with practice over time. Initial attempts failed because of damage to the fine epidome making the cultures turn brown after 3- 4 days. However, after culture initiation, meristem-tips became green within 1-2 weeks, with the larger explants turning green first. Thereafter, the bases of the meristem-tips developed into compact calli in which the tips grew into plantlets that later produced their own roots. Of the 12 explants each of the small, medium and large sizes cultured from thermotherapy shoots only the large explant (D3+3) size recorded 7 survival, representing 58.3%. This was followed closely by the medium sized (D2+2) explants cultured recording 4 survival representing 33.3%. The lowest survival of explants was the small (D1+1) size excised and cultured, obtaining 1 plantlet out of 13 explants cultured, representing 7.7%. This result shows that efficiency of viability and regeneration was directly proportional to the size of meristem. According to Walkey and Web, (1968), the smaller the size of the meristem explant, the lower the viability as well as the regeneration potential. Adejare and Coutts (1981) also observed that the survival rate of meristem tips was highly correlated with size of cultured tip when cassava explants were obtained from shoots that were subjected to thermotherapy, thus the larger the tip the higher the survival rate. However, among the explant sizes excised and cultured from untreated shoots, the small sized (D1+1) of explants excised recorded the highest (5) number of regenerated plantlets, representing 31.3% survival, close to the number of regenerated plantlets (4) from the large (D3+3) size of explants cultured (30.8%), regenerating into virus-free plantlets. The results presented in this study show that the survival rate of the explants is high even though they were not heat-treated, especially in the large explant sizes that were more likely to transmit disease pathogens and the smallest meristem that may be having a reduced chances of regeneration into

plantlets (Appiano and Pennazio, 1972). However, this observation is contrary to the findings reported by Hu and Wang (1983) and Kartha (1986). With this result, smaller explants survived well without heat treatment to mother plant.

Among the four cultivars studied under the three categories of explant sizes, "Afisiafi" recorded 2 plantlets each for the medium (D2+2) and the large (D3+3) explant sizes cultured after shoots were submitted to thermotherapy, representing 66.7% survival respectively. Similarly, "Amakuma" and "UCC-Bankye" recorded 2 (66.7%) regenerated plantlets each from the large (D3+3) explant size, but recorded 1 plantlet (33.3%) each from the medium (D2+2) explant size cultured and 0 from the small (D1+1) size. "Esi-Abaya" was the poor performing variety in terms of number of survival, though it recorded one survival each in both the smallest and largest explant sizes. "Afisiafi" which recorded highest number of survival of treated explants performed badly when the shoots were not treated, trailing behind "Amakuma", "UCC-Bankye" and "Esi-Abaya", which yielded higher numbers of explant survival.

5.3. Thermotherapy and virus elimination in cassava

Virus elimination through meristem-tip is possibly because the meristematic dome of infected plants are generally either free or carry very low titre of the virus (Bhojwani and Razdan, 1983; Hu and Wang, 1983; Kartha, 1986). Thermotherapy plays an important role in this process by slowing down the replication of the virus and facilitates the excision of larger explants to generate successful plantlets from culture.

However, the success of this method depends on an effective method of virus detection. Therefore, a rigorous test is needed to ensure that plantlets regenerated are disease-free. In this study, a test by Polymerase Chain Reaction (PCR) was carried out to detect the presence of virus titres in the regenerated plantlet samples analyzed. The test of the amplified DNA samples from the regenerated plantlets of "Afisiafi", "Amakuma", "UCC-Bankye" and "Esi-Abaya" under the 3 explant sizes excised after receiving thermotherapy and meristem tip culture revealed no presence of the disease. The PCR result of this study corroborated the observed scores of the visual inspection analyzed, an indication of truly disease-free regenerated plantlets. However, when samples of the amplified DNA from leaves of untreated shoots were analyzed the disease presence was shown, as indicated in the presence of bands on the gel electrophoresis visualized (Figure 4). Because PCR has proven to be 100-1000 fold more sensitive (Henson and French, 1993; Hadidi *et. al.*, 1995; Candresse *et. al.*, 1998), the technique is able to produce accurate results. Virus particles can appear latent to escape detectable levels by visual symptom inspection because of the titre load (Ogbe *et. al.*, 2003). The PCR is capable of distinguishing between plants with low viral titre and those that are totally virus-free. It is therefore, adequate to confirm that the thermotherapy-meristem culture applied has been effective in eliminating the virus. Of the six primers used, only three amplified the disease virus in the genome DNA, inferring the absence of that virus strains in the cassava cultivars.

CONCLUSION

1. Thermotherapy treated meristem showed a better regeneration potential in larger explant size than smaller ones in the cassava accessions studied. There were varietal differences in the response to both thermotherapy and meristem tip culture.

2. Donor explants subjected to thermotherapy showed no amplification of the virus in the cassava genome while non-treated explants showed amplification band. Thus, the results presented in this study clearly showed that virus-free planting materials could be produced using thermotherapy-meristem culture for farmers to increase their yield.

RECOMMENDATIONS

In spite of the positive results produced by thermotherapy, there are some challenges, and the following are therefore recommended:

1. In view of the fact that the cultivars used grew fast with slim stems torching the roof of the chamber, meristem tips excised from these plants resulted in tinny explant thereby failing to grow in the nutrient medium. It is therefore recommended that a proper glasshouse with heat chamber be used to provide heat treatment to plants.

2. Detailed studies on the response of different diseased cassava cultivars to heat treatment should be carried out since thermotherapy is very efficient in the control of viral diseases.

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