Efficacy of thermotherapy combined with chemotherapy and meristem tip culture in reducing Cassava brown streak virus in infected cassava

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Abstract: Thermotherapy, chemotherapy and meristem tip culture have been used either alone or in combination to eliminate viruses from plants. . The objective of this study was to determine the efficacy of combining thermotherapy with meristem tip culture and chemotherapy on the reduction of CBSV from infected cassava.Cassava Brown Streak Virus infected cuttings of Guzo variety collected from Coast region of Kenya, were established and maintained in a greenhouse at the Plant Quarantine Station, Kenya Plant Health Inspectorate Service (KEPHIS) in Muguga. Cassava leaves were sampled from eighteen plants and virus indexing done using RT-PCR with virus specific primers. Those confirmed to be positive for CBSV were used as initiation materials for the prospective test plants. From the initiated tissue culture materials, the 2nd subcultures were subjected to heat treatment at 38°C for 21 days. They were later subjected to ribavirin treatment at varying concentrations of (10mg/l, 20mg/l, and 30mgl) then left to establish for 14 days. Meristems of 1mm were excised from heat treated plantlets at 38°C for 21 days and cultured i*n vitro* in modified Murasgige and skoog media. Nodal plantlets of 10mm not subjected to any treatment were used as controls. There was a significant difference ($P<0.01$) in the number of plants that survived among the treatments. Thermotherapy combined with chemotherapy resulted in complete mortality of plants due to the high stress levels from the high temperatures combined with the phytotoxic effect of the ribavirin. Thermotherapy followed by excision of meristem tips (1mm) resulted in 68.8% shoot survival with 84% being virus-free. Thermotherapy combined with meristem culture was successfully employed to produce CBSV-free cassava plants.

Key words: Cassava Brown steak virus, Chemotherapy, meristem culture, Thermotherapy

Introduction

The most viable methods for obtaining virus-free stocks is eradication using tissue culture techniques, aided by thermo or chemotherapies (Mellor and Stace-Smith, 1970; Pannatoni *et al.,* 2013). Plant thermotherapy is described as achieving a cellular environment which is progressively less adequate for virus vitality (Pennazio, 1995). Similar interactions are also reported by Mink *et al.* (1998) who discussed the effects of heat treatment on the functionality of viral movement. In fact the different ability for the movement of viral particles in plant tissues influenced the choice of elimination (Pannatoni *et al.,* 2013) with thermotherapy as the most effective against viruses characterized by parechymatic localization (Panattoni *et al.,* 2013) compared to meristem culture which is more suitable for phloemetic viruses that are limited to vascular tissues (Grout,1990).

Developments in research over the last 20 years have suggested that heat treatment

triggers natural antiviral response produced by infected plant, particular reference being to virus induced gene silencing (VIGS) induced by the presence of viral RNA in infected plants (Ruitz *et al.,* 1998). Chellapan *et al.* (2005) studied the mechanisms that determine the influence of temperature on the antiviral silencing for *Geminiviruses* (ssDNA) by applying heat treatment (25 to 30◦C) to cassava (*Manihot esculenta)* and tobacco (*Nicotiana tabacum*) plants infected by cassava mosaic disease and they confirmed the close relationship between temperature and the VIGS.

Heat treatment of stock plants prior to meristem tip culture is often used to enhance virus elimination (Mink *et al.,* 1998). Temperatures ranging from 34 to 40◦C for periods ranging from days to weeks are efficient for viral eradication (Betti, 1991). Meristem tip culture has been used effectively in combination with thermotherapy to obtain clean nectarine at 35°C with meristems ranging from 1.8mm-2mm (Manganaris *et al*., 2003). Application of meristem culture combined with thermotherapy is reported to increase the survival rate of explants *in-vitro* (Manganaris *et al*., 2003); since larger tips can be obtained from heat-treated plants while ensuring virus-free plant production. 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 at least 30 days at 35°–38°C, and cultured meristems on modified MS (Murashige and Skoog, 1962) medium. Successful elimination of sweet potato featherly mottle virus (SPFMV) from Egyptian Abees sweet potato cultivar by heat therapy and meristem tip culture has also been reported (Mervet *et al.,* 2009).

Improved virus elimination can also occur if chemotherapy is combined with thermotherapy with subsequent meristem tip culture (Kartha and Garmborg, 1986; Spiegel *et al.,* 1993; Luciana *et al.,* 2007). Joint effects of thermotherapy at 37°C and ribavirin applied to *in vitro* plants were highly efficient in eliminating potato virus Y resulting in 83.3% of virus-free potato plants (Nascimiento *et al.,*2003). Further reports support the use of thermotherapy together with the addition of antiviral agents to the growth medium as the best treatments for virus elimination in potato (Fletcher *et al*., 1998, Griffith *et al*., 1990). The objective of this study was to determine the efficacy of combining thermotherapy with meristem tip culture and chemotherapy on the reduction of CBSV from infected cassava.

Materials and Methods

Selection of infected cassava plants

Cassava brown streak positive plants were collected from farms at the Coast of Kenya, initiated *in vitro* and used as prospective test plants for meristem tip culture.

Application of Thermotherapy combined with meristem tip culture

Single nodal cuttings from the second subcultures were cultured in modified Murashige and Skoog Media and incubated for 14 days at 24±1°C for establishment and later transferred to the thermotherapy chamber at 38°C and 80% relative humidity for 21 days. The temperature was maintained under a photoperiod cycle of 16/8 hr light /dark. *In vitro* meristems (1.0mm) were then excised and cultured in modified MS media supplemented with 30 g/litre of sucrose; 7.0 g/litre of agar; 0.002 mg/litre of GA3 0.1mg/l BAP and 0.15mg/l Naphthalene acetic acid After a period of three weeks the meristems were sub cultured onto MS without Benyzl-amminopurine and Naphthaline acetic acid hormones.

Application of thermotherapy combined with chemotherapy

Single nodal cuttings from the second subcultures were cultured in modified MS media and incubated for 14 days at 24±1°C for establishment and later taken to the thermotherapy chamber at 38°C and 80% relative humidity for 21 days. From the heat treated plants, nodal cuttings were cultured in modified MS media supplemented with 10, 20, and 30mg/l of ribavirin and incubated for two weeks. Nodal plantlets of 10mm size were used as controls and incubated at 24±1°C under photoperiod cycle of 16/8 hr. as light /dark.

Experimental design and data collection

A completely randomized design was used to evaluate the tissue cultured material in the different treatments. Each treatment had a total of 15 plants replicated three times to create a total sample size of 45 plants. The number of surviving plantlets, CBSV-positive and CBSV-negative plants after virus testing was recorded.

Data analysis

ANOVA using Genstat software was used to calculate the significance at ($P \leq 0.01$) for the plants that survived subjected to thermotherapy combined with meristem tip culture and chemotherapy.

Virus reduction was determined as the proportion of CBSV-negative plants obtained expressed as a percentage.

Results

Effects of thermotherapy combined with meristem tip culture and chemotherapy on reduction of CBSV from infected cassava

Plants that were subjected to meristem tip culture and thermotherapy at 38°C exhibited slight chlorosis due to the high temperatures compared to the controls left at room temperature of 24°C (Plate 1 a). Plants that were subjected to heat treatment in combination with chemotherapy dried up resulting in mortality of all plants subjected to this treatment (Plate 1 b)

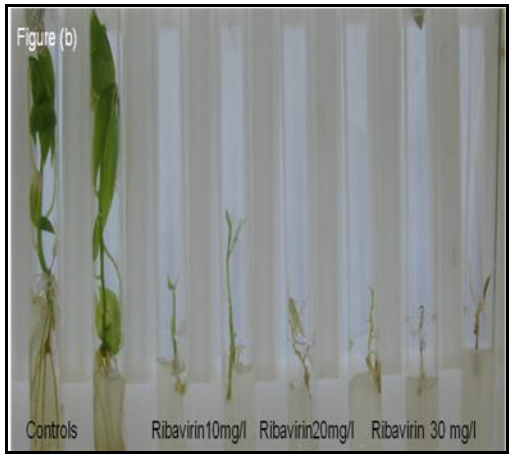

Plate 1: (a) Heat treated plants at 38°C (b) Plants subjected to thermotherapy in combination with chemotherapy

Detection of CBSV in plants established from meristems excised from heat treated plants

The PCR products of the survived putative virus-free material amplifications were visualized on agarose gel as illustrated in

Plate 2. Out of 31 plants tested, 27 were CBSV-free (Plate 2).

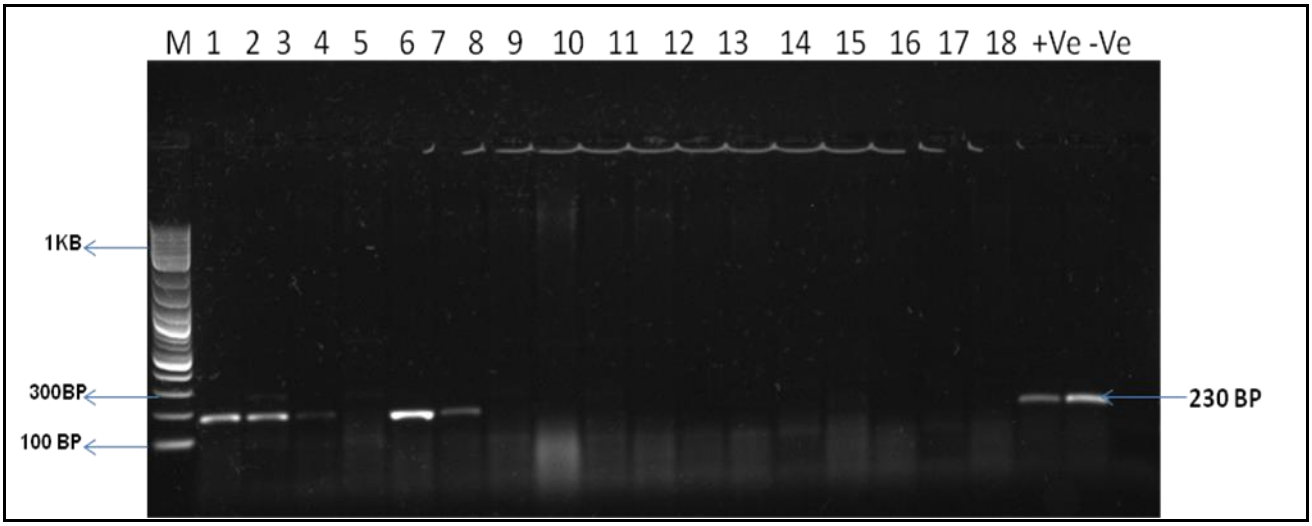

Plate 2: Agarose gel electrophoresis of CBSV detection of *in vitro* meristem excised from heat treated plants at 38°C at 1% (w/v) agarose gel; lane 1 1kb ladder lane; 2-18 *in vitro* meristems; lane19 (positive control); lane 20 (negative control)

Survival and virus reduction of regenerated plants

Thermotherapy combined with meristem tip culture resulted in 68% of regenerated plants with 84% being virus-free (Table 1). Thermotherapy combined with chemotherapy resulted to no survival. Controls obtained from nodal plantlets resulted into positive plants.

Table 1: Survival and virus reduction (%) of plants subjected to thermotherapy combined with meristem tip culture and chemotherapy

Treatment	**Initiated explants (No)**	**Regenerated plants (No)**	**Positive plants (%)**	**Negative plants (%)**
Meristem Tip Culture+ Thermotherapy	45	31	15.6	84.4
Chemotherapy+ Thermotherapy Ribavirin 10mg/L	45	0	(0) 0	(0) 0
Chemotherapy+ Thermotherapy Ribavirin 20mg/L	45	0	(0) 0	(0) 0
Chemotherapy+ Thermotherapy Ribavirin 30mg/L	45	0	(0) 0	(0) 0
Control	45	45	(45) 100	(0) 0

Analysis based on survival **(**P≤0.01) (S.E-standard error) 0.0368

Data in parenthesis are actual numbers of plants

Comparison of the means at P≤0.05 by LSD indicates significant differences between the means (Table 2).

Table 2: Means of survivals from plants subjected to thermotherapy combined with chemotherapy or meristem tip culture.

Treatment	**Mean survival (%)**
Control	100.0
Thermo+ meristem (1 mm)	68.9
Thermo+ ribavirin 10 mg/l	0.0
Thermo+ ribavirin 20 mg/l	0.0
Thermo+ ribavirin 30 mg/l	0.0
$LSD_{0.05}$	8.6

Discussion

Joint effect of thermo and chemotherapies did not give the expected results. These plants completely dried up turning brown and total mortality was recorded. These results differ with Nascimiento *et al.* (2003) and Fletcher *et al.* (1998) who found the combination of thermo and ribavirin applied to *in vitro* potato being highly efficient in elimination of potato virus Y.

The combination of chemotherapy with thermotherapy caused high stress levels to the plants subjected to the treatments. This can be explained by the fact that concentrations of many antiviral chemicals required during chemotherapy to inhibit virus multiplication are very close to the toxic concentration for the host plant (Panattoni *et al.,* 2013) which can be lethal to the plants under virus elimination. It is also noteworthy that the complex interaction between the host and biological characteristics of a virus strongly interfere with the outcome and effects of virus elimination (Panattoni *et al.,* 2013). Meristem excised from plants subjected to thermotherapy treatment enhanced CBSV eradication compared to the control resulting in 68.8% of plant survival with 84% being virus-free. These results are not surprising since thermotherapy treatment to *in vitro* plants prior to meristem excision has been found to yield fewer virus infected plants in various vegetatively propagated species (Acedo, 2006). The combination of meristem tip culture and thermotherapy to efficiently eliminate sweet potato featherly mottle in sweet potato has been reported by Mervet *et al.* (2009). To improve survival, application of meristem culture of 1.8-2mm combined with thermotherapy at 35°C is reported to increase the survival rate of *in vitro* explants (Manganaris *et al*., 2003). This is because larger tips can be obtained from heat-treated plants while ensuring virus-free plant production. The use of meristem tips measuring 1mm and subjecting them to thermotherapy at 38°C *in vitro* was efficiently used in the reduction of CBSV from infected cassava *in vitro.*

Conclusion

Thermotherapy combined with meristem tip culture (1mm) resulted in 68.8% of

regenerated plants with 84% being virus-free. Based on efficiency of virus reduction and survival, meristem tip culture combined with thermotherapy was found optimum for reduction of CBSV from infected cassava *in vitro.*

Recommendation

Farmers are encouraged to use *in vitro* raised cassava materials that have been adequately diagnosed to be free from CBSV. This will ultimately reduce the risk of spreading CBSV to uninfected cassava fields.

There is need for studies to evaluate the effects of these cleaning methods on reduction of Ugandan cassava brown streak virus (UCBSV) from infected cassava plants (Guzo variety)

Studies should also be done to evaluate the effect of chemotherapy on CBSV reduction by subjecting CBSV infected plants to lower antiviral concentrations and increasing duration of the treatment to evaluate the effect of CBSV reduction in Guzo variety.

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References

Acedo VZ. (2006). Improvement of *in vitro* techniques for rapid meristem development and mass propagation of Philippine cassava. *J. Foods. Agric. Dev*, 4,220-224.

Adejare GO and RH Coutts. (1981). Eradication of cassava mosaic disease from Nigerian cassava clones by meristem-tip culture. *J. Plant Cell, tissue & organ culture,* 1,25-32.

Betti JA. (1991). Obtencao de material Propagative vegetal testado livre de virus. In: Crocomo OJ, Sharp WR and Melo M. *Biotechnologia para producao vegetal Piracicaba* Cebtec Falq, 145-172.

Chellappan P, R Vanitharani and CM Fauquet. (2005). Effects of temperatures on geminiviruses induced RNA silencing in plants .*Plant physiol*, 138,1828-1841.

Fletcher PJ, JD Fletcher and SL Lewthwaite. (1998). *In vitro* elimination of onion yellow dwarf and shallot latent viruses in shallot (*Allium cepa* var. *ascalonicum* L.). *New Zeal J Crop Hort,* 26, 23-26.

Griffiths HM, S Slack and J Dodds. (1990). Effect of chemical and heat therapy on virus concentration in *in vitro potato* plantlets. *Canadian. Journal. of Botany*, 68,1515-1521.

Grout BW. (1990). Meristem tip culture for propagation and virus elimination. In plant cell culture protocols (Hall RD, eds) Human Press inc Totowa (USA), 115-125.

Kartha KK and Gamborg OL. (1986) .Elimination of cassava mosaic disease by meristem tip culture. *Phytopathology*, 65,826-828.

Luciana CN, Gilvan P, Lilia W and Genira PA. (2007). Stock indexing and potato virus y elimination from potato plants cultivated *in vitro* .*Scientia Agricola*. 60, 525–530.

Manganaris GA, Economou AS, Boubourakas IN and Katis NI. (2003). Elimination of PPV and PNRSV trough thermotherapy and meristem tip culture. *Plant cell rep,* 22,195-200.

Mellor FC and Stace Smith R. (1970) .Virus differences in virus eradication of potato x and s. *Phtopathology* 60:1587-1590.

Mervat MM, EL Far and A Ashoud. (2009) .Utility of Thermotherapy and

Meristem tip culture for freeing Sweetpotato from Viral Infection. *Australian Journal of Basic and Applied Sciences,* 3:153-159.

Mink GI, Wample R and Hoel WE. (1998) .Heat treatment of perennial plants to eliminate phytoplasmas viruses and viriods while maintaining plant survival. In Hadidi (Eds).The American phytopathology society, pp 332-345.

Murashige and skoog. (1962) .A revised medium for rapid growth and bio assays with tobacco cellcultures. *Physiologia Plantarum,* 15:473-497.

Nascimiento LC, Pio Ribeiro G, Willadino L and Andrade GP. (2003). Stock indexing and potato virus Y elimination from potato plants cultivated *in vitro.* Sci Agri, 60,525-530.

Panattoni A, Luvisi A and Triolo E. (2013) .Review: Elimination of viruses in plants: twenty years of progress. *Spanish J. of Agric. Research, pp* 11,173-188.

Pennazio S. (1995) .Effect dell energia termica sui virus delle piante superiori, informatore Fitopatologico, 9,46-45.

Ruitz MT, Voinnet O and Baulvombe D. (1998). Initiation and maintenance of virus induced gene silencing. *Plant cell*, 10, 937-746.

Spiegel S, Frison EA, Converse RH. (1993). Recent development in therapy and virus-detection procedures for international movements of clonal plant germplasm. *Plant Dis*, 77, 176-1180.