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In-vitro techniques for elimination of viruses causing cassava mosaic disease and cassava brown streak disease

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Abstract

Cassava brown streak disease (CBSD) and cassava mosaic disease (CMD) are the two most important viral diseases affecting cassava (*Manihot esculenta*) production in Uganda. The two diseases persist in the plant system causing yield losses and reduce quality of planting materials for the next planting. Cassava germplasm conservation efforts are still at its infancy and the current cassava seed systems is characterized by free exchange of cassava stakes among local communities, which promote the spread of CMD and CBSD. Farmer-preferred cassava varieties are particularly affected, and these need to be cleaned regularly. This paper highlights *in-vitro* techniques that have been developed and described for plant virus elimination including meristem tip culture, thermotherapy, and chemotherapy. The techniques may be used singly or in combination for effective virus elimination. The effectiveness of meristem tip culture, which is the most commonly used method for *in-vitro* virus elimination, critically depends on size of meristem. Nonetheless, virus type, position of meristem on the plant, and host plant species also determines efficiency of virus elimination and survival of explants. Thermotherapy and/or chemotherapy followed by meristem tip culture have been used to enhance virus elimination. The combination of thermotherapy followed by meristem tip culture minimizes difficulties associated with excision of small meristem size since thermotherapy allows for use of larger meristems. Further, chemotherapy followed by meristem tip culture increases efficiency of virus elimination but plant regeneration is low. High concentration of antiviral compounds may result in phytotoxicity and genetic mutations in plants. Together with application of sensitive virus detection and quantification methods before and after *in-vitr*o treatment, a cheap combination of *in-vitro* techniques could be optimized for elimination of CMD and CBSD causing viruses.

Key words: Cassava viruses, chemotherapy, *Manihot esculenta*, meristem tip culture, thermotherapy

Résumé

La maladie de la striure brune du manioc (CBSD) et la maladie de la mosaïque du manioc (CMD) sont les deux maladies virales les plus importantes qui affectent la production du manioc (Manihot esculenta) en Ouganda. Les deux maladies persistent dans le système cultural, provoquant des pertes de rendement et réduisant la qualité du matériel de plantation pour la prochaine plantation. Les efforts de conservation du germplasm de

manioc en sont encore à leurs débuts et les systèmes actuels de semences de manioc sont caractérisés par un libre échange de boutures de manioc entre les communautés locales qui favorisent la propagation de la CMD et de la CBSD. Les variétés de manioc préférées par les agriculteurs sont particulièrement touchées et doivent être assainies régulièrement. Cet article met en évidence les techniques in vitro qui ont été développées et décrites pour l'élimination du virus des végétaux, y compris la culture de méristème, la thermothérapie et la chimiothérapie. Les techniques peuvent être utilisées seules ou en combinaison pour une élimination efficace du virus. L'efficacité de la culture de méristème, qui est la méthode la plus couramment utilisée pour l'élimination in vitro du virus, dépend de façon critique de la taille du méristème. Néanmoins, le type de virus, la position du méristème sur la plante et l'espèce de la plante hôte déterminent également l'efficacité de l'élimination du virus et la survie des explants. La thermothérapie et / ou la chimiothérapie suivies d'une culture de méristème ont été utilisées pour améliorer l'élimination du virus. La combinaison de la thermothérapie suivie d'une culture de méristème minimise les difficultés associées à l'excision de la petite taille du méristème puisque la thermothérapie permet l'utilisation de large méristème. En outre, la chimiothérapie suivie de la culture de méristème augmente l'efficacité de l'élimination du virus, mais la régénération des plantes est faible. Une concentration élevée de composés antiviraux peut entraîner une phytotoxicité et des mutations génétiques chez les plantes. Conjointement à l'application de méthodes de détection et de quantification des virus sensibles avant et après le traitement in vitro, une combinaison moins couteuse de techniques in vitro pourrait être optimisée pour l'élimination des virus responsables du CMD et du CBSD.

Mots clés: Virus de manioc, chimiothérapie, Manihot esculenta, culture de méristème, thermothérapie

Background

Plant viruses are the major cause of many important plant diseases and are responsible for huge losses in crop production worldwide. Vegetatively propagated crops are particularly prone to viruses. Cassava for instance is vegetatively propagated through stem cuttings, and at least 16 viruses, have been found to affect it (Calvert and Thresh, 2002). The most important cassava infecting viruses in East Africa are cassava brown streak viruses (CBSVs) and Cassava mosaic geminiviruses (CMGs). The viruses are transmitted by whiteflies (*Bemisia tabaci*), but are also predominantly disseminated through planting infected stem cuttings (Legg *et al.,* 2011). The use of low quality disease affected stem cuttings as planting materials for next cropping cycle result in poor yields and further maintenance of the disease.

In comparison to bacteria and fungi, which can be controlled by use of chemical, no chemical has been developed to control plant viruses (Lal *et al.,* 2015). The use of resistant varieties has been effective in management of viruses, nonetheless breeding for virus resistance is generally difficult and ineffective to improve the health status of already affected varieties with valuable agronomic characteristics. Moreover, stems of high yielding clones could

be rejected when infected by viruses. The use of clean planting materials therefore, is important for improvement of cassava productivity, international germplasm exchange, and germplasm maintenance. A number of tissue culture techniques such as meristem tip culture, thermotherapy, and chemotherapy have been employed in the production of virus free stocks (Mellor and Smith, 1970). Details of some of these virus elimination techniques are discussed.

Meristem tip culture

Meristem tip culture is the most widely used method in the production of virus free plants from several plant species. It is well known that distribution of viruses in the plants is uneven. In infected plants, the meristems tips are either free or carry very low virus concentration (Wang and Hu, 1980). Failure of viruses to invade the meristem tips are attributed to absence of vascular system in meristematic cells through which the virus move, high metabolic activity in the actively dividing meristematic cells which does not allow virus multiplication, and high level of endogenous auxin in the shoot apices which may inhibit virus multiplication (Wang and Hu, 1980). Success in obtaining virus free plants by meristem tip culture depends on virus type, host plant species, position of meristem on plant, and size of meristem used for culture (Maruthi *et al.,* 2013). The meristem tip size is most critical factor for effective virus elimination, and also determines survival of explants on specific medium and time required for regeneration of new plants (Panattoni, 2013). The meristem tip consists of actively dividing cells and is often less than 1 mm in length, comprising of the apical dome and limited number of youngest leaf primordia (Grout, 1999). For successful virus elimination, balancing the size of the meristem tip with the effect on regeneration, and efficiency in virus elimination is important. Therefore, relatively small meristem tip which results in efficient virus elimination and high regeneration of plants should be used instead of larger explants which results in low virus elimination but with high plant regeneration rates (Facciolo and Marani, 1998). For the case of cassava mosaic virus, for instance, out of the 90-95% of cassava plants regenerated when explants of ~ 0.4 mm was used, 60% were virus-free (Kartha and Gamborg, 1975). For clonally propagated plants this method is easy to apply and can be used directly or with slight modification. Meristem tip culture also has ability to regenerate plants that are true-to-type with minimal genetic variation. The challenge with this method however, is the difficulty in excision of the small meristem tip, which is time consuming, requires sophisticated equipment and plant regeneration rate is low. And, although meristem tip culture alone can be used to eliminate plant viruses, using it in combination with thermotherapy and chemotherapy increases success of virus elimination.

Thermotherapy

The use of thermotherapy alone has not been successful for virus elimination, but it has been used to reduce the level of virus in infected plants. The effect of heat on plant viruses is not well understood but it is believed to be effective in inhibiting viral replication and synthesis of movement proteins by blocking transcription (Mink *et al.,* 1998). Conventionally, thermotherapy involves growing the whole plant or subjecting

tissue cultured plantlets at temperatures between 35-40^{0}C for few minutes or weeks before excision of the meristem tips (Kartha and Gamborg, 1986). Hot water treatment of plant parts at temperatures between 30-70^{0}C has also been used, but has been largely unsuccessful for virus elimination (Nyland and Goheen, 1969; Kaiser and Louie,1979). For example, two successive hot water treatments at 50 and 55^{0}C of African cassava mosaic virus (ACMV)-infected cuttings for several minutes, was nontherapeutic as all the stem cuttings upon sprouting expressed mosaic symptom (Kaiser and Louie, 1979). In contrast, Zinga *et al.,* 2014 reported that 40% of cassava mosaic disease free cassava was obtained at one month after planting when cassava mosaic disease affected cuttings were treated at 49^{0}C for 30 minutes with hot water. Similarly, Aqleem *et al.* (2016) reported reduction of Potato leaf roll virus (PLRV) in Potato using hot water treatment. Hot air treatment at 37^{0}C for 87-105 days resulted in 33-44% virus free plants (Kaiser and Louie, 1979).

Because of the very low or even no success of virus elimination, thermotherapy has been combined with meristem tip culture to regenerate virus free plants. African cassava mosaic virus (ACMV) and Cassava brown streak virus (CBSV) were eliminated from five cultivars when meristems tips of size 0.2-0.4 mm from plants heat treated at 37^{0}C for 30-36 days and cultured (Kaiser and Teemba, 1979). Similarly, Wasswa *et al.* (2010) obtained 40% of CBSV free cassava, when meristem tips of ~0.5 mm in length from infected plantlets were heat treated at 36^{0}C for 8 hours darkness/16 hours of light (Table 1). Likewise, ACMV was 100% eliminated after meristem tips were taken from infected cassava plants grown at temperatures ranging 35-39^{0}C for 4-6 weeks in heat chamber (Acheremu *et al.*, 2015). Thus, thermotherapy followed by meristem tip culture is efficient in elimination of plant viruses, though careful decision regarding duration of thermotherapy is critical, since continuous exposure to high temperature is harmful to the plant. Efficiency of virus elimination depends on virus type and sensitivity of the plant to high temperatures.

Chemotherapy

This involves the use of chemicals such as antibiotics, plant growth regulators, amino acids, purines and pyrimidine analogues for inactivation of virus replication or for inhibition of virus multiplication. The chemicals are either sprayed on virus-infected plant before meristems are excised or can be supplemented in the culture medium. Among these chemicals, ribavirin compound (1-β-D-ribofuranosyl-1, 2, 4-triazole-3-carboxamide) commonly known as virazole is the most widely used. Ribavirin is a broad range antiviral compound (Walkey, 1985). It has been used effectively to eradicate large number of plant viruses, such as Potato viruses (X, Y, and S) from potato (Nascimento, 2003), and Sugarcane mosaic virus (SCMV) from sugarcane (Balamuralikrishnan *et al.,* 2002). In cassava, 88.9% and 100% of CBSV free plants were regenerated CBSV when 30 mg/l of ribavirin and salicylic acid (30mg/l) were combined with meristem tip culture, respectively (Mwangangi *et al*., 2014) (Table 1). The combination of chemotherapy and thermotherapy was however, found to be unfavorable to the plants. Similarly, Maruthi *et al.* (2013) compared the efficiency of thermotherapy or chemotherapy (ribavirin, 25mg/l) combined

with meristem tip culture, and combination of all three techniques for elimination of CBSVs from three cassava cultivars with different level of resistance. The maximum efficiency of 48% CBSVs free tested plants were obtained for Kaleso (CBSD resistant) and Kiroba (CBSD tolerant) with thermotherapy followed by meristem culture. There was no significant difference between thermotherapy followed by meristem culture and a combination of all three methods for Kaleso and Kiroba. However, there was significant difference in efficiency of CBSVs elimination from cultivar Albert (CBSD susceptible). Chemotherapy followed by meristem culture produced 38%, while thermotherapy followed by meristem culture and combination of all three methods produced 27% and 35% respectively. Thus, antiviral compounds are effective for virus elimination but plant regeneration is low. Also, high concentration of antiviral compounds results in phytotoxicity, and a possibility of causing genetic mutations in plants. Besides, these compounds are expensive.

Table1: Effect of different treatments on regeneration and production virus free cassava plants

Virus	Treatment	Plant regenerated %	Percentage (%) of virus plants	**References**
EACMV, CBSV	thermotherapy + meristem culture	26.3	100	Kaiser and Teemba, 1975
ACMV	Meristem tip culture	93	60	Kartha and Gamborg, 1979
CBSV	Thermotherapy + meristem tip culture	49	40	Wasswa *et al.,* 2010
CBSV	Meristem tip culture	37.7	82.2	Mwangangi *et al.,* 2014
CBSV	Meristem culture	80.1	66.7	Mwangangi *et al.,* 2014
CBSV	Chemotherapy (Ribavirin)	13.3 - 46.7	68.8 - 88.9	Mwangangi *et al.,* 2014
CBSV	Chemotherapy (Salicylic acid)	2.2 - 31.1	78.6 - 100	Mwangangi *et al.,* 2014
CBSV	Chemotherapy (Ribavirin)+ thermotherapy+ meristem tip culture	68.9	84.4	Mwangangi *et al.,* 2014

Conclusion

Meristem tip culture, thermotherapy or chemotherapy followed by meristem culture has been successfully applied in the production of virus free plants. This has helped to increase the supply of quality planting materials to meet the demand for increased productivity, through virus diseases management. However, because of increased demand for clean plantings materials, which is a requirement for increasing productivity of cassava and other vegetatively propagated crops, there is need to popularized use of combination of thermotherapy and

meristem tip culture as this has potential to permit use of larger meristem size, leading to high efficiency in virus elimination and improved regeneration rates. The main limitation of thermotherapy is that it requires sophiscated and expensive equipment such as thermal chamber. Further research is needed to improve the technique so as to make it suitable for routine use in tissue culture, *in-vitro* and on-farm conservation. Chemotherapy is effective against a wide array of plant viruses, except that antiviral compounds are expensive and high concentration results in phytotoxicity and a possibility of causing genetic mutations.

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