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ELIMINATION OF AFRICAN CASSAVA MOSAIC VIRUS (ACMV) IN CASSAVA (*MANIHOT ESCULENTA CRANTZ*) USING MERISTEM CULTURE ASSOCIATED TO THERMOTHERAPY

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ABSTRACT

Cassava (*Manihot esculenta, crantz*), is an important root crop in the tropics and substropics of Africa and Latin American. It is vegetatively propagated crop. Due to the following cycles of propagation, viruses are accumulated, which contributes to the decline of cassava yield and quality. Stem cuttings of four cassava accessions Six-mois, Rendre, yalipé and M61/033 were collected in experimental field and have brought to laboratory. Infected plants were thermotherapy treated by incubation of plants at 42°C/day and 38/night for 2 weeks. The shoot sproot leaves from thermotherapy are symptoms free of ACMV, shoots apicals were taken for meristem tip culture and allowed to grow in vitro. Leaves from thermotherapy were also taken for PCR test to confirm the success of ACMV elimination on thermotherapy only compare to meristem tip culture associated to thermotherapy. Clean plants material were acclimated and taken to the experimental field. A total of 637 meristems were isolated in vitro, 512 (80,32 %) were regenerated. A regeneration percentage of 80 % for *Yalipe* accession, 82 % for *M61/033* accession, 85,2 % for *Rendre* accession and 92,3 % for *Six-mois* accession. A PCR test has confirmed 95 % of virus free plants from meristems tips culture associated to thermotherapy, otherwise 99 % percent of plants from thermotherapy treatment without meristems tips culture were ACMV virus infected. A total of 712 explants nodals virus free were submitted to subculture and accimated, 363 plants of African Cassava Mosaic Virus free were obtained and transfered to experimental field.

INTRODUCTION

Cassava (*Manihot esculenta Crantz*) is a perennial crop of the family Euphorbiaceae, grown mainly for its enlarged tuberous roots. Cassava is the most important cultivated species in the tropics and subtropics, with thousands of accessions and cultivars.

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Cassava roots are rich in carbohydrates and have been used mainly in the production of flour for humain consumption in developing countries, where calorific deficiencies and malnutrition are widespread (Olsen et Schaal, 1999). The crop is drought tolerant, can be grown in diverse edapho-climatic conditions, in depleted soils, and is able to recover from damage caused by pests, diseases and bush fires (IITA, 1990; IFAD, 2008). These advantages and the adaptability to indigenous farming systems explain the rapid spread of the crop on the African and Asian continents (Byrne, 1984). Cassava has potential yield of 90 tonnes per hectare per year (Cock, 1985). However, the average yield in Indonesia is 10.3 tonnes per hectares (Soenarjo *et al*., 1987), and in Africa it is even lower (Anonymous, 1993). This low yield has been attributed to the poor fertility of the soils where cassava is

grown, deterioration of roots and severe incidence of pests and diseases. African Cassava Mosaic Virus, for example, can cause yield losses of up to 95 % (Legg *et al*., 2011). Mosaic disease of cassava (*Manihot esculenta Crantz*) poses serious problems to the crop and is prevalent in most of the cassava-growing regions of the world (Legg *et al*., 2011). Cassava is the main importance crops in Central African Republic, with production dried, around 600 000 T/HA. Very far from maize 60 000T/HA, following by peanut; Cassava, remain the first crops, and the most revenue in CAR, (Malhouhi and Kafara, 2002). The cassava main disease in Central African Republic (CAR) is Cassava Mosaic Disease what causes yield loss. The incidence in the country is around 85 % for symptom severity 3, according to cours scales ZINGA *et al* 2012).

The Cassava Mosaic, Disease survey, done in 2005, shown cassava mosaic incidence in some areas of country such as Bangui, Boali, Sibut, Damara, Bossembélé and Bouar run from 67 to 97 % and caused lost crops of roots around 49 % (Zinga *et al*., 2008). Besides the lack of resistant cultivars, the use of stakes infected with diseases and pests enhances their persistence. Propagation through stem cuttings encourages the spread of many virus diseases, such as cassava mosaic virus, within a region and from one region to the others. Healthy planting material can be produced via meristem culture. This technique was used to free cassava from Cassava Mosaic Virus (Kartha and Gamborg, 1975). Healthy plants can be produced in large numbers by multiple shoot culture (Smith *et al*., 1986). However, this technique requires several tissue culture steps which make the procedure labour intensive. A method for propagating plants from apical meristem has not been available. In this work, an attempt was made to obtain Cassava free plant material through thermotherapy and meristem-tip culture of infected cassava shoots and test by PCR to confirm CMD elimination.

MATERIALS AND METHODS

Plant material

Stems of four cassava cultivars: *Six-mois*, *M61/061*, *Yalipe* and *Rendre*, were collected from the experimental field (Kapou) 45 Km of Bangui.

Thermotherapy

Stems were cut in 20 cm fragments; each cutting contained around 5 to 8 nods. Stems are disinfected in 70 % alcohol for 20 mn duration and washed in tap water three times and planted in polybacks filled with soils.

Plants were subjected to the thermotherapy treatment as follows:

Plants were placed in a thermotherapy chamber under the conditions of 16 hours day photoperiod at 31°C and 8 hours dark period at 27°C. Temperature were raised 2°C/2 days till final setting of 42°C day and 38°C night was obtained. Plants were maintained under these conditions for additional 2 weeks. Shoot tip explants were collected from about two week old vigorously sprouting shoots from stake grown in the heat chamber. The shoot tips were decapitated and trim off all older leaves. Explants are brought to the laboratory (kitchen) and left under running tap for about 30 mn, and then transferred into a sterile honey jar bottle and wash with sterile distilled water (SDW). The bottle was shaked with the explants for about five minutes, this step is repeated two times or three if necessary, surfaced sterilized by immersing in 70 % ethanol for 10 seconds followed by 2% sodium hypochlorite solution containing, 0,05 % tween-20 for 5 minutes and rinsing for three times, 2 minutes each, with a sterile distilled water.

Meristem Tip culture

Following thermotherapy, axillary shoot tips with a long of 2-3 cm and nodal of long of 3-6 cm were cut. A part of the apex of approximately 0,25 to 0,4 mm long with two leaf primordial were excised and nodal around 2 cm for culture on tube containing 15 ml of MS salt medium (Murashige and Skoog, 1962) supplemented with NAA 0,02 mg/l, BAP 0,05 mg/l, MS 4,4 g/l, Sugar 30 g/l, Myo-inositol 100 mg/l, CuSO4 1ml, Thyamin 1mg/l, pyridoxine 1,5 mg/l, nicotinic acid 1,5 mg/l, Glycin 2mg/l.

In vitro plant multiplication

After 2 months of subculture, plant regenerated from meristem tip culture were transferred on proliferation medium. For 8 weeks.

ACM detection by polymerase chain reaction (PCR)

Plants from thermotherapy and thermotherapy followed by meristem tip culture, were tested by PCR using primers ACMV-F1, sequence 5' TTC AGT TAT CAG GGC TCG TAA, position in DNA 271-29[b] and ACMV-F2, sequences (5'au 3') GTG AGA AAG ACA TTC TTG GC position in DNA 2558 -2577 [b] that could detect ACMV. Total DNA extraction from the samples was done as reported by Dellaporta *et al*. (1983).

The reaction mixture per tube contained 2, 5 µl each of thermo buffer (10 X concentration), $MgCl_2$ (2.5 mM) and Tween-20 (55 mg ml^{-1}); 2.0 µl of dNTPs (2.5 mM), 1.0 µl each of forward and reverse primers (5.0pM); 0,4 µl (2 units) of Tap DNA polymerase (Promega product); 9,5 µl sterilized distilled water and 5µl(1-2ng μl^{-1}) of DNA sample. These add up to 26.4 µl/reation tube. The reaction cycles in the Perkin Elmer Gene Amp PCR system, model 9600, were as reported by Zhou *et al*. (1997). The PCR products were separated by electrophoresis in a 1% agarose gel at 100 volts for about 1.5 H. The gel was attained in ethidium bromide (10 µg ml^{-1}) for about 60 min and the DNA bands were observed under the UV light. The photograph was taken by gel documentation and analysis system computer software.

Micropropagation and acclimatization of ACMV free vitro-plants

Vitro-plants ACMV free tested by PCR were micrpropagated in MS: 4,4 mg/l, Morel vitamin: 2ml, and solidified by 3,5 g/l of phytagel. In-vitros Plants from 8 weeks old were transferred in soil, with 50 % humidity; relative, were adjusted from 96 % to 99 % for the first week and 85 % for second week acclimatization.

RESULTS

Thermotherapy treatment

A total of 200 cassava stems were treated by thermotherapy. Nearly all (100 %) of the heatherapy treated plants survived. The gradual regime used for raining the temperature allowed plants to survive. Shoots plants after thermotherapy raised with virus free symptoms (Fig.1).

Meristems Culture

A total of 637 meristems have been isolated out of 512 (80.32 %) vitro-plants were regenerated (Table 1). Pourcentages regenerations were 80 % for accession *Yalipe* 82 % for accession *M61/033*. 82,5 % for accession *Rendre* and 92.5 % for accession *Six-mois*. Around 113 vitro-plants were regenerated without root and were brought in root medium subculture (IBA, 0, 05 mg/l) (Fig. 2)

accessions from thermotherapy treatment without meristem culture were found ACMV-positive. (Fig. 3)

Micro propagation and acclimatization of vitro-plants free of Cassava Mosaic Virus

A number of 869 nodals cutting from in vitro-plants tested virus free were culture, 215 for accession M61/033, 238 for Yalipe accession, 181 for Rendre accession et 235 for Six-mois accession. A total of 794 in vitro plants were regenerated, 186 in vitro-plants for M61/033, 221 in-vitro-plants for Yalipe accession, 168 for Rendre accession and 219 vitro-plants for Six-mois accession, a regeneration percentage of 91, 36 % of vitro-plants free of virus has been obtained on 15 ml of tube medium, each containing 4.4 mg/l of MS (Murashige and Skoog), 30 g/l of Sugar and 2ml/L of morel vitamine, 0.05 mg/l IBA. We noticed that *M61/033* were resistants accession and were noted tested by PCR. (Table2).

Table 1. Meristems culture, plants regeneration and pourcentage of vitro-plants regenerated

Accessions	Nomber of meristems culture	Nomber of meristems regenerated	Percentage of vitro-plants regenerated
M61/033	187	153	82 %
Yalipé	145	113	78,62 %
Rendre	122	103	85,2 %
Six--mois	183	168	92,35 %

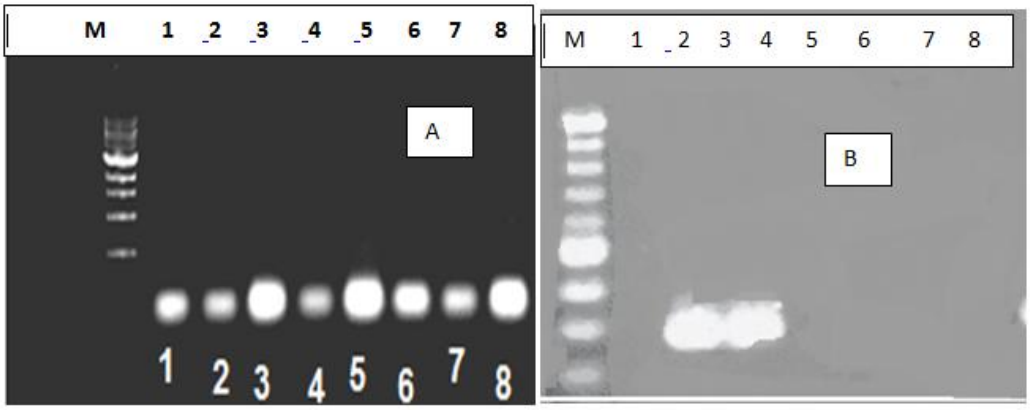

Fig 3: PCR test for detecting cassava virus on meristem-tip and nodal culture

A. PCR test on in-vitro plantlets from thermotherapy and nodal culture, N° 1, 2, 3: *Six-mois* accessions, N° 4, 5, 6: *Yalipe* accession; 7, 8 : *Rendre* accession.

B. PCR test on in vitro plantlets from meristems tips and thermotherapy culture; N°1: Six-mois accession; N° 2, 3: Positives controls, N° 4, 5, 6: Yalipe accession, N° 7, 8: Rendre accession

Table 2. Micropropagation of cassava in-vitro-plants free from Cassava Mosaic Disease

Accessions	Nomber of nodals culture	Nomber of in vitro plants regenerated	Cost of regeneration (%)
M61/033	215	184	86,51
Yalipé	238	214	92,85
Rendre	181	166	92,82
Six-mois	235	218	93,2

Indexing of plants for CMD

Rooted-plantlets eight (8) weeks old subjected to PCR analysis using specifically designed primers based on the conserved regions present in all viral strains obtained from cassava CMD. 95 % of detected samples from meristems tip-culture and thermotherapy were found to be ACMV-free, A band n° 2 and 3 are positives controls. All in-vitro cassava

Plantlet establishment

Young cassava plantlets, produced from microcuttings of virus-free cultures, were established and acclimatized successfully and grew satisfactorily in a screen house (Fig.2C). Shoots from these plantlets tested “negative” for the presence of ACMV 6 month after the initial virus testings, confirming the earlier tests for virus (data not shown) were allowed to experimental field (Fig. 2E).

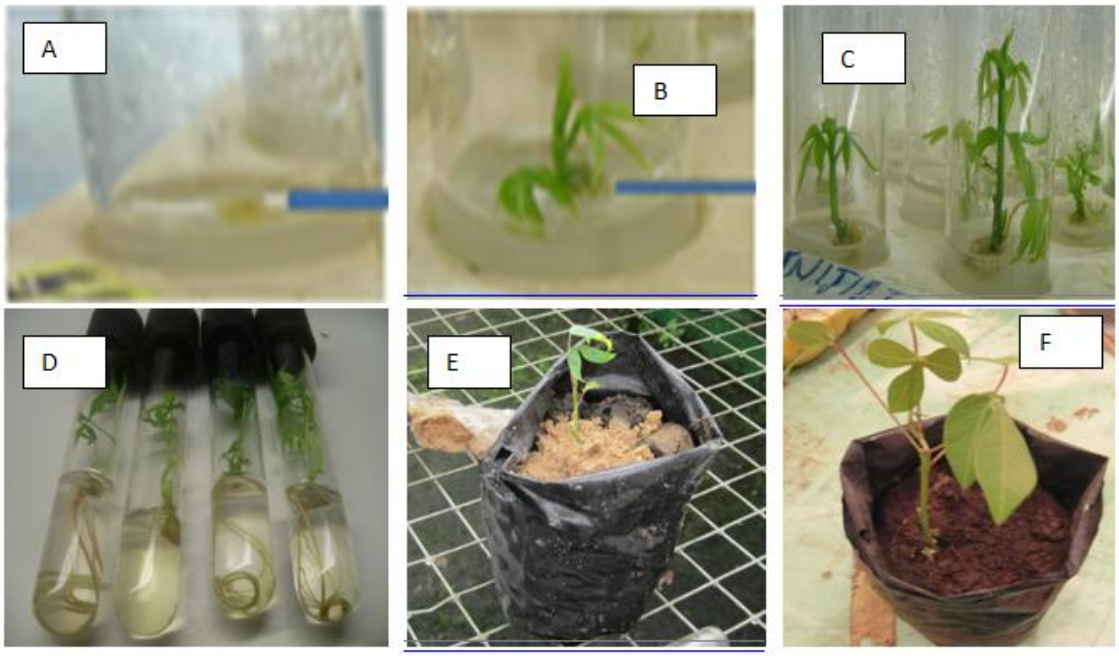

Fig. 2. Procedure of plant free of ACMV production through meristem-tip culture
A. Meristem-tip explant on medium
B. Regeneration of meristem tip on the medium
C. In-vitro-plant regenerated from meristem explant
D. In-vitro explants regenerate from meristem (one month)
E. In-vitro-plants Acclimatization from meristems culture
F. In-vitro plants Acclimatization

DISCUSSION

Out of 763 meristems isolated from cassava CMD infected materials, 512 CMD virus-free vitro-plants become established. After PCR test, 95 % of in vitro plant have shown virus free CMD disease from cutting materials from meristem culture associated to thermotherapy otherwise 90 % of plants from thermotherapy alone have shown CMD. This study show that CMD can be very efficiently eliminated from cassava infected plants using thermotherapy in a combination with meristem tip culture (Cooper and walky, 1978; Mink., 1998). While elimination of viruses by meristem culture is size dependent, it was recommended that the smaller the tip that is taken, the better chance for virus exclusion (data not shown), it comes with the findings of (Michael, 1996), because in the meristematic zone the rate of plant growth is increasing compring with the rate of virus multiplication.

Meristem culture technique has regenerates over 35 genera (Quak, 1987). In contrast (Lankes, 1995; karesove, *et al*., 2002; Theilter-Hedtrich, and Baumann, 1989) reported that meristem tip culture alone obtained a few virus-free plants. However thermotherapy followed by meristem culture resulted in CMD free plants. These findings raised the question of the mechanism by which thermotherapy enhanced eradication of CMD. Early studies indicated that thermotherapy inhibits viral replication while virus degradation continues, which results in subsequent elimination of the virus from meristem tips (Cooper, and Walkey, 1978; Kassassin, 1975), later (Szittya, *et al*., 2003; Qu, *et al*., 2005) found that, the efficiency of virus induced RNA silencing found to be significantly enhanced at the high temperature, Indeed, since, the first studies that aimed to produce virus-free plants from infected individuals, it has been known that the efficiency of virus eradication in a given host species differs depending on the virus and the host genotype. With some plants, thermotherapy followed by meristem tip culture did not result in any virus free plantlet. Recently, a novel approach based on cryotherapy was found to solve this problem, (Wang, Q.C. and J.P.T. Valkomen, 2008).

The In-vitro-plants derived from meristem-tip culture gave more in vitro necrosis and callus formation than in-vitro plants from nodal subculture. Some meristem-tip explants formed undesired callus and were discarded in order to avoid somaclonal variation. The reason of callus formation may be due to the fact that, meristem-tip is tissus and organogenesis processus may fail and the tissue turned brown and failed to grown. Others meristems explants culture formed necrosis. The most likely cause of the necrosis is phenolic oxidation, as it is seems that cassava is prone to phenolic problems like other woody species (Skirvin *et al*. 1986; Shu and Timon 1993).

Contaminated explants percentage of meristem culture ranged from 4,2 to 13,32 % where as contaminated explants percentage from nodal culture ranged from 18,8 to 29,36 %, this results is due to the fact that the size of nodal explants used of in-vitro culture is bigger(2 to 20 mm) than meristem-tip size culture (0,4 à 0,8) and could be associated with infection such as funger or bacterial. However the regrowth ability of the meristem tip decreased according to meristem size used as explants, the smaller the size of meristem, poor ability to regenerate. These results are in agreement with Faccioli, G. and F. Marani, 1998; Fuglie, KO. *Et al*., 1999. They reported that meristem tips of 0,2 and 0,3 mm with two

leaf primordial are commonly used for virus elimination in various plant species, and the survival and growth are greatly improved if the tissue taken includes slightly explanded leaf primordial. Meristem tips and nodal culture that were established successfully in vitro on basal medium produced explanded shoots (Fig. 2D) and multiplied (3- 6 per explants) in the proliferation medium. Those cultures that proved virus-free by PCR were used in subcultures for microcuting production. Microcuttings, harvested from virus-free cultures, rooted readily in the rooting medium, at a rate of nearly 80 % with one month.

A total of 3 63 plants free of ACV from meristem tip and thermotherapy culture were acclimated in the green house and have taken to experimental field (data not shown). In conclusion, the acquisition of virus-free plant material in cassava from meristem-tip explants derived from stem plants grown plants showed satisfactory results regarding explants survival and therefore, it is recommended. On the other hand, the application of thermotherapy, followed by meristem-tip culture (allowing a larger meristem to be excised) gave encouraging results indicating that the acquisition of plant propagation material free from ACMV is possible even in the case of virus-infected cassava plant with high symptom severity. The research should also be extended to create resistance varieties by resistance gene engineer or somatic hybridation.

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