Using Thermotherapy And Meristem Tip Culture For Producing Virus-Free Cassava Planting Material From Six Varieties Cultivated In Côte d'Ivoire

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Abstract: Cassava cultivation in Côte d'Ivoire is constrained by cassava mosaic disease (CMD) caused by African cassava mosaic virus (ACMV) and East African cassava mosaic Cameroon virus (EACMV-CM). The disease is transmitted principally through infected cuttings. This study combined thermotherapy and meristem culture to produce virus-free in vitro plantlets. These sanitation techniques were applied to six highly valued cassava varieties. Cuttings taken from plants showing CMD symptoms were grown in greenhouses as mother plants. At 2 weeks old, PCR of these plants showed presence of ACMV and EACMV-CM in single and double infections respectively. Heat treatment was then applied of day/night with 40/36°C and a photoperiod of 16/8 h. Meristem tips were used to produce in vitro plantlets, which were re-checked for viruses using PCR, and virus-free plantlets were multiplied through several sub-cultures. Using 60 isolated meristematic explants, we regenerated 44 in vitro plantlets. We achieved a sanitation success rate of 100 % for four varieties (Agba blé 3, IM89, Koko Soclo 5 and Bayéré), 80 % for Yacé and 62.5 % for Yacé 2. A second indexing of viruses showed that 88.33 % of regenerated in vitro plantlets were free from both ACMV and EACMV-CM. After three consecutive sub-cultures of the virus-free plantlets from all six varieties, we obtained 800 in vitro plantlets. This study showed that the thermotherapy technique combined with meristem culture was effective in eliminating these viruses. The use of virus-free planting material will reduce the impact of CMD and boost cassava production in Côte d'Ivoire.

Keywords: Cassava, ACMV, EACMV-CM, Heat treatment, Meristem, Virus-free plantlets, Côte d'Ivoire

1 INTRODUCTION

In Côte d'Ivoire, cassava-derived products such as attiéké, attoukpou, foutou and placali, are the staple foods of most of the population [1]. Cassava cultivation is therefore essential for food security in the country but is threatened by many diseases. Among them, cassava mosaic disease (CMD) is the most damaging, resulting in a 90 % of yield loss for susceptible varieties [2]. It is caused by begomoviruses: African cassava mosaic virus (ACMV) and East African cassava mosaic virus Cameroon variant (EACMV-CM). These viruses are spread by the insect vector Bemisia tabacci and infected cuttings. However, in East and West Africa, their dissemination is done preferentially through the use of infected cuttings when establishing new plantations [3, WAVE's unpublished data]. Providing farmers with healthy cassava cuttings is therefore an effective way to control the disease.

Thermotherapy consists of the use of high temperatures, either by exposing plant material to hot air or by immersing it in hot water baths [4], [5]. In conjunction with meristem culture, this technique can remove viruses and generate healthy seedlings from virus-infected mother plants [4], because the meristem of an infected plant is generally virus-free or contains a very low viral load. Additionally, high temperatures inhibit virus replication and contribute to lowering viral load and promoting virus elimination through in vitro culture of meristems [6]. The culture of meristems for sanitation is based on the fact that the distribution of viruses in meristems of infected stems shows a progressive decrease in the number of viral particles toward the apical parts, with the apex being devoid of them. Undifferentiated cells present at this level can be used to reproduce healthy clones [7]. These combined techniques have been used to produce healthy planting material for several years [8], demonstrating that it is possible to obtain virus-free cassava seedlings from mother plants infected with Begomovirus species. An established thermotherapy method is the exposure of mother plants to hot air followed by cultivation of meristems [9], [10]. However, the success of virus elimination depends on the viral species, the cultivar and whether the plant is infected with a single virus or several viruses [11]. The objective of this study was therefore to determine the effectiveness of this sanitation technique on selected cassava varieties grown in Côte d'Ivoire with the overarching goal of producing healthy plant material on a large scale.

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2. MATERIALS AND METHODS

2.1 Plant material

The plant material consisted of cuttings from six cassava varieties that are extensively cultivated in Côte d'Ivoire: Koko Soclo 5, Agba blé 3, Yacé 2, IM89, Bayéré and Yacé. All these varieties expressed CMD symptoms and were collected in Bouaké (central Côte d'Ivoire) from the cassava collection of the Centre National de Recherche Agronomique (CNRA) and in Cassava fields in Aboisso (south Côte d'Ivoire).

2.2 Phytosanitary evaluation and collection of cuttings from the six cassava varieties

The six varieties were subject to a visual phytosanitary evaluation prior to collection. The purpose of this was to assess their response to CMD in their usual growing environment. Thus, for the varieties collected in Bouaké, in particular Koko Soclo 5, Agba blé 3, Yacé 2 and IM89, 10 plants were scored according to the CMD symptom scale of 1–5 (Table 1) indicating the severity of the CMD symptoms [12]. For varieties Bayéré and Yacé collected in the field, 30 plants were evaluated, 15 on each field-diagonal using the same CMD symptom scale.

2.3. Production of mother plants and first virus-indexing

Production of mother plants. Stems of the six varieties were divided into cuttings of approximately 30 cm long for production of mother plants in greenhouses at West African Virus Epidemiology (WAVE) site in Bingerville. The cuttings were first disinfected with 80 % Mancozeb (fungicide) and 18 % Abamectine (insecticide). They were then cultivated for 2 weeks on a substrate composed of lowland soil and compost in equal proportions and regularly watered. First indexing. This step aimed to confirm the presence of viruses in mother plants and detect the viral species causing the CMD symptoms, using visual phytosanitary evaluation and PCR. The phytosanitary evaluation used the same scale adopted for the collection of stems as described earlier and leaves from these plants were used for molecular analysis. The DNA was extracted from these leaves according to the CTAB modified protocol [13]. The PCR was carried out to detect viruses using several pair of primers: JSP001 (5'ATGTCGAAGCGACCAGGAGAT-3') and JSP002 (3'-TGTTTATTAATTGCCAATACT-5') for ACMV, JSP001 and JSP003 (3'-CCTTTATTAATTTGTCACTGC-5') for EACMV, and VNF031 (5'-GGATACAGATAGGGTTCCCAC-3') and VNF032 (3'-GACGAGGACAAGAATTCCATT-5') for the Cameroon variant of EACMV (EACMV-CM). The PCR products were electrophoresed on a 1 % agarose gel.

Table 1: *The CMD symptom scale of 1–5*

Scale	Symptoms description
1	Unaffected shoots (no symptoms)
2	Mild chlorosis, mild distortions at bases of most leaves while the remaining parts of the leaves and leaflets appear green and healthy (symptoms on about 25% of the leaves)
3	Pronounced mosaic pattern on most leaves, narrowing and distortion of the lower one-third of the leaflets (symptoms on about 50% of the leaves)
4	Severe mosaic distortion of two-thirds of most leaves and a general reduction of leaf size and stunting of shoots (symptoms on about 75% of the leaves)
5	Very severe mosaic symptoms on all leaves, distortion, twisting and severe reduction of most leaves, accompanied by severe stunting of plants (symptoms on about 100% of the leaves)

Fig.1*. Thermotherapy chamber containing 3 Weeks old mother plants*

2.4. Heat treatment and phytosanitary evaluation of heat-treated mother plants

After two weeks of cultivation, the mother plants were placed into thermotherapy chambers for 4 weeks with day/night conditions of 16/8 h and 40/36°C [4]. Relative humidity levels were 70 % during the day and 90 % at night. Twenty 60-Watts incandescent lamps mounted on bracket were used as a heat source. A large 40-Watts white light lamp was used to simulate daylight in the room. The lamps were automatically switched on and off and temperature in the room was homogenized by two ceiling fans (Fig. 1).

Water containers placed in the chamber maintained humidity. The combination of heat, humidity and air generated by the fans had created hot air in the thermotherapy room. Temperature and relative humidity in the room were monitored using a thermo hygrometer. At the end of the 4 weeks in the thermotherapy chambers, the plants were re-evaluated for virus symptoms using the same scale used for the first indexing. Leaf Samples were collected from each heat-treated mother plants for checking the presence of the viruses using the same primers used earlier.

2.5. Meristem tip culture

Meristem tip culture was carried out according to the International Institute of Tropical Agriculture (IITA) and Wasswa protocols [4], [14]. The shoot tips of 2–3 cm from plants apexes that had undergone 4 weeks of heat treatment were excised and stored in sterile distilled water. After disinfection of the shoot tips in a solution of 70 % alcohol and 3 % sodium hypochlorite, followed by three rinses with sterile distilled water, the meristematic apexes bearing 1 or 2 leaves were taken and cultured on MS medium [15] + 0.1 mg /l NAA + 0.5 mg/l BAP + 0.2 mg/l GA3+ 80 mg/l Adenine sulfate with 3% of sucrose. The shoots obtained after two weeks were transferred to a propagation medium composed of MS + 0.01 mg/l NAA + 0.05 mg/l BAP (Fig. 2). Ten replicates per variety (60 meristems) were grown in total.

2.6. Second indexing and multiplication of in vitro plantlets from meristem culture

Leaf and stem samples were collected from in vitro plantlets derived from meristems during the first sub-culture on MS + 0.01 mg/l NAA + 0.05 mg/l BAP. Total DNA of each in vitro plantlet was extracted from these samples and tested with the same primers as used during the first indexing. The free-virus in vitro plantlets underwent two additional sub-cultures.

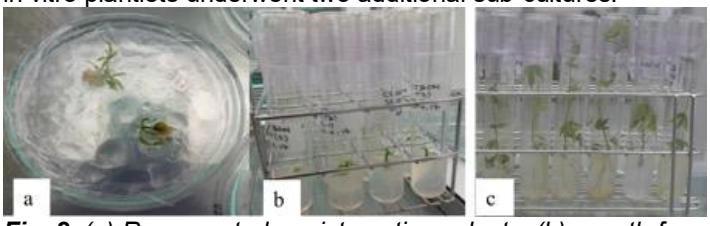

Fig. 2. *(a) Regenerated meristematic explants, (b) growth from meristems transferred to propagation media and (c) plantlets from sub-culture*

3. RESULTS

3.1. Assessment of CMD infection in field and greenhouse

All six cassava varieties expressed CMD symptoms both in the field and the greenhouse. In the field, CMD severity in varieties Agba blé, Yacé and Bayéré was higher than in Yacé 2, IM89 and Koko Soclo (Fig. 3). In the greenhouse, CMD symptoms on mother plants were moderate with severity scores in the range of 2–3. In the first indexing, we detected ACMV in both single and double infection with EACMV-CM (Table 2).

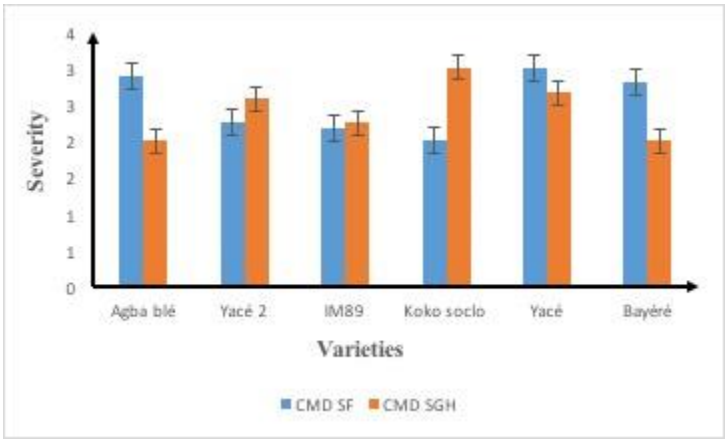

Fig.1*. CMD severity in the field (CMD SF) and greenhouse (CMD SGH)*

Table 2: *Viruses diagnosed on mother plants in the greenhouse*

Variety name	Origin	Virus(es) found
Agba blé 3	CNRA Bouaké	ACMV
Yacé 2	CNRA Bouaké	ACMV
IM89	CNRA Bouaké	ACMV
Koko Soclo 5	CNRA Bouaké	ACMV
Yacé	Aboisso	ACMV and EACMV-CM
Bayéré	Aboisso	ACMV and EACMV-CM

3.2. Thermotherapy treatment

All 60 plants that underwent thermotherapy (all of which contained viruses) survived the treatment, i.e. a survival rate of 100%. However, CMD symptoms, including leaf distortions and mosaics, were reduced or eliminated by heat treatment. After the thermotherapy treatment period, the mother plants that had expressed medium severity symptoms (score 2) produced new leaves that were asymptomatic, whereas mother plants with original severe to very severe symptoms (score 3–5) produced new leaves with only moderate (score 2) symptoms (Fig. 4). The average severity of CMD before heat treatment was higher than that obtained afterward (Table 3). Our results clearly showed that thermotherapy eradicated or reduced CMD symptoms in some plants. We detected the same viruses found from the first indexing (Before heat-treatment).

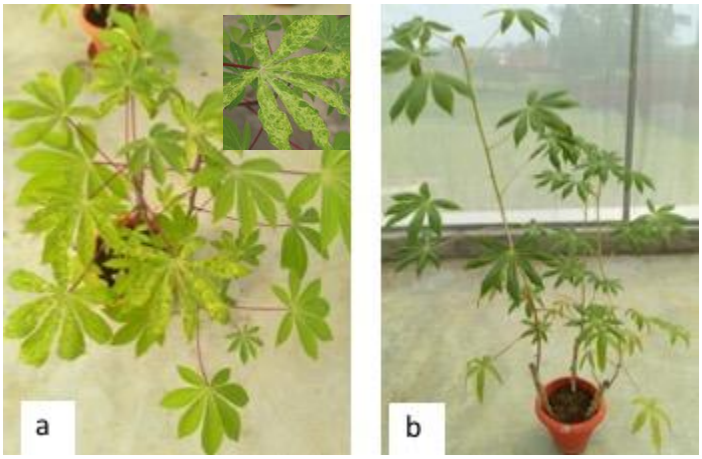

Fig. 4*. (a) Mother plant before heat treatment expressing slight mosaic symptoms and (b) mother plants free of symptoms of CMD, following the heat treatment*

Table 3: *CMD severity in the greenhouse (CMD SGH) and after heat treatment (CMD SHT)*

Varieties	CMD SGH	CMD SHT
Agba blé	2.0 ± 0.11	1.0 ± 0
Yacé 2	2.6 ± 0.16	1.0 ± 0
IM89	2.3 ± 0.16	2.0 ± 0
Koko Soclo	3.0 ± 0	2.0 ± 0
Yacé	2.6 ± 0.2	2.0 ± 0
Bayéré	2.0 ± 0.1	1.0 ± 0

3.3. Meristem tip culture

Of the total of 60 meristematic explants cultured, 44 in vitro plantlets were regenerated (73.33%). There were significant differences in the regeneration rate of meristems among the varieties ($P < 0.01$). Three homogeneous groups were obtained, based on the regeneration rate of meristems. In the first group, varieties Yacé and Koko Soclo 5 had regeneration rates of 100%.

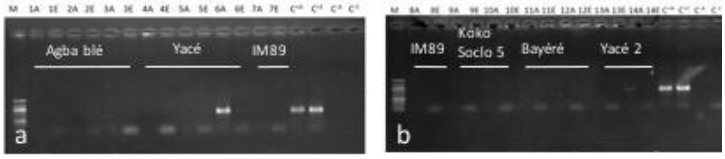

Fig. 5*. PCR test for the detection of begomoviruses (A, ACMV; and E, EACMV-CM) on plantlets from in vitro culture of meristems: positive control, C^{+A}, C^{+E} and negative control C^{-A}, C^{-E}; Absence or presence of bands in the sample line means plantlets were successfully cleaned or cleaning failed, respectively*

Varieties Yacé 2, Agba blé 3 and Bayéré constituted the second group with regeneration rates of 80 %, 70 % and 50 %, respectively. Variety IM89 represented the third group with the lowest regeneration rate of 40 %. With regard to viral disease, ACMV in single and double infection with EACMV-CM was eliminated (Fig. 5). A 100 % sanitation rate was obtained for four varieties: Agba blé 3, IM89, Koko Soclo 5 and Bayéré. Varieties Yacé and Yacé 2 had the lowest sanitation rates, with 80 % and 62.5 % respectively. The sub-culture of the free-virus in vitro plantlets allowed obtaining 800 plantlets.

4. DISCUSSION

The plant visual evaluation in the greenhouse confirmed the sensitivity of the six tested varieties to CMD. The first indexing showed the presence of two CMD viruses, in particular ACMV in single and double infection with EACMV-CM. Previous research [16], [17] showed that these two viruses are the cause of CMD in Côte d'Ivoire. Some varieties like TMS 30001 and TMS 30395 are resistance or tolerant to these viruses because they suffer little or no damage if they are infected [12]. However, they do not always have the taste qualities sought by consumers and this could explain the continuing large-scale use of susceptible varieties [18]. Sanitation, which involves producing virus-free planting material in the laboratory from infected plants, produces healthy plant material. The two traditional methods of sanitation (thermotherapy and meristem culture) have made it possible to obtain free-virus plant from infected plant, while maintaining their agronomic qualities [7]. The success of heat treatments applied to target plants depends mainly on the type of virus and plant species, as well as the virus–host combination. Thus, the temperature regime applied is 35–42°C for 4–6 weeks [19], [20]. For cassava, previous studies showed that adequate temperatures are within 36–42°C with a photoperiod of 8/16 h for 4 weeks [9], [10], [21]. In this study, the thermotherapy method applied was the exposure of plant material to a temperature varying between 36 to 40°C. This temperature regime was adequate, allowing a 100 % survival rate for donor plants subjected to thermotherapy. Choosing the right thermotherapy regime should allow the treated plant to survive while inactivating the virus. The heat treatment applied in this work reduced or eliminated the CMD symptoms. This is attributable to the thermal masking observed in several studies using thermotherapy [10], [21], [22], in which the high temperatures inhibit virus replication and movement. Indeed, the links between the subunits of protein that protect the nucleic acids of the virus becomes weak. This leads to appearance of cracks over time and attack of the virus' nucleic acids by nucleases, which inactivate the virus and decrease its concentration [23]. Our work differed from previous studies [9], [21], because in our case the CMD symptoms persisted for some donor plants after heat treatment. This difference may be related to the different cassava genotypes used in this study compared to those in previous studies. Indeed, the effectiveness of thermotherapy depends largely on the type of virus and the plant genotype [20]. All of the plants that underwent heat treatment still had the CMD virus, although symptoms were absent in some cases, showing that thermotherapy alone was insufficient to eliminate viruses. This study also showed that thermotherapy associated with meristem culture was effective in eliminating ACMV in single and double infections with EACMV-CM. However, the main problem is the ability to regenerate meristems, which differs among varieties. This makes it difficult to use a generic protocol [4]. It would therefore be interesting to use other hormonal combinations adapted for each variety in order to have a maximum regeneration rate. The sub-cultures of the virus-free seedlings obtained make it possi*ble to obtain an exponential number of in vitro plantlets as suggested by Acedo [24], who calculated that 159,432–3,774,873 cassava seedlings could be produced from one nodal explant per year.

5. CONCLUSION

This study was carried out with the aim of improving and applying the technique of thermotherapy using hot air combined with meristem culture to produce healthy in vitro plantlets from various cassava varieties cultivated in Côte d'Ivoire. This study showed that this technique was reliable for all six tested varieties. However, the major difficulty encountered was the ability to regenerate plants from meristems, which varied among the varieties. In the future this problem may be resolved by testing different hormonal combinations in the media to determine optimal combinations for each variety. If this combined approach (thermotherapy and meristem culture) was put into large-scale practice, cassava cultivation in Côte d'Ivoire could be improved. The production and use of virus-free planting material would limit the spread of CMD viruses and the resulting increase in yield could improve the living conditions of producers.

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