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Comparative Elimination of Begomoviruses in Cassava Meristems and Axillary Buds

Adonise F. Valam Zango[1,2], Innocent Zinga[1*], Régis Dimitri Longué Soupké[1], Simplice Prosper Yandia[1,3], Brice Toko Marabana[1], Nicole Gado Yamba Kassa[1], Françoise R. Otabo[4], Emmanuel Kamba Mebourou[1], Kosh Komba Ephrem[1], Armand M'Villa[4], Guy Florent Ankogui[4], Semballa Silla[1] and Joseph Mabanza[2]

[1]Laboratoire des Sciences Biologiques et Agronomiques pour le Développement (LASBAD), Faculté des Sciences, Université de Bangui, Central African Republic.
[2]Faculté des Sciences et techniques, Université Marien Ngouabi de Brazzaville, Congo.
[3]Institut Supérieur de Développement Rural (ISDR), Université de Bangui, Central African Republic.
[4]Institut National de Recherche Agronomique (INRA), Brazzaville, Congo.

Authors' contributions

This work was carried out in collaboration between all authors. Authors AFVZ, IZ, RDLS, BTM, NGYK, SPY, FRO, EKM, AM, GFA and SS designed the study and wrote the protocol. Authors AFVZ, IZ, RDLS, NGYK and SPY managed the experiments. All authors read and approved the final manuscript.

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ABSTRACT

Aim: The production of healthy cuttings from a local cassava cultivar for cassava mosaic control.
Study design: The study was carried out at the In Vitro Culture Laboratory using the techniques of thermotherapy and culture of tissues and explants in a specific medium.
Place and duration: The study was carried out at the Laboratory of Biological and Agronomic Sciences for Development at the University of Bangui, Central African Republic from December 2017 to June 2018.
Methodology: A variety of cassava called *six-month* very susceptible to mosaic was used for this work. The cuttings used were infected by Cassava Mosaic Begomoviruses (CMBs) with high severity. It were subcultured in a room under the heat of 37 ° C to 40 ° C for two weeks. Explants and meristems were taken from the stems and the apices, respectively. These collected materials

**Corresponding author: E-mail: zinga.innocent37@googlemail.com, zinga.innicent37@googlemail.com;*

were treated and seeded on appropriate culture media. After the plants produced in vitro were acclimatized and the leaves were removed to check their phytosanitary state by the PCR technique.
Results: The rate of emergence of the acclimatized plants and the expression of the disease on the microplants were evaluated. The results show that 75% of the weaned vitro plants recovered under acclimatization. In addition, the acclimatized plants left growing in the greenhouse for four months remained asymptomatic. Molecular analysis by PCR showed that begomoviruses were not detected on meristem samples unlike samples from stem fragments.
Conclusion: The combination of thermotherapy technique associated with the culture of meristems constitutes an effective means useful for the sanitation of infected plant material.

Keywords: Manihot esculenta; mosaic; thermotherapy; in vitro culture; central African Republic.

1. INTRODUCTION

Cassava is an important food in the fight against food insecurity of populations in sub-Saharan Africa [1]. Cassava is the main food crop in the Central African Republic with a production of 2.4 million tons of fresh tubers or 714,042 tons of chips, ahead of yams at 476,674 tons and maize at 92,355 tons [2]. It constitutes the staple food of the population of the Central African Republic and represents a main source of income [3]. Its culture is practiced throughout the territory and feeds almost the entire Central African population. Cassava leaves are eaten in several producing countries and particularly in the CAR. The leaves are particularly rich in proteins, carotenes, minerals (K, Ca, Na,) and vitamins B1, B2, and C [4]. Amino acid in cassava leaves are also useful for wound healing in the skin, replacing damaged cells, bone health, helping us with good memories, and to help the body's metabolic system. Chlorophyll in the leaves serve as antioxidants and anticancer agent. Benefits of cassava leaves are very much; health can be supplemented with the intake of cassava leaves. Known cassava leaves have a number of important benefits such as suitable for those who are running the diet program. In addition, cassava leaves can also be used as medicine rheumatism, headaches, ulcers, cold medicine, diarrhea, overcome the worms, and increase stamina. It also used as a herbal medicine that can drink it with water directly. The active principle of cassava leaves have pesticide activity [4].

Cassava is vulnerable to around twenty viral diseases, the most important of which currently in Africa are cassava mosaic disease (CMD) and cassava brown streak disease (CBSD) [5,6]. Cassava mosaic has been reported in several sub-Saharan African countries [7,8,6] where it causes serious yield problems [9,10]. Surveys carried out in the Central African Republic between 2005 and 2012 showed that the main constraint of cassava is CMD with an average incidence of 85% and that 79% of cuttings used for replanting by producers were infected with CMBs [10,11]. The same surveys made it possible to determine two species of CMBs on Central African cassava: ACMV and EACMV-UG [12]. Surveys carried out by a regional project gave the same trends [12]. Cassava mosaic disease has become a persistent and most virulent threat, with the disease incidence of 85% and a severity of 3 on the IITA [13].

However, the selection of cuttings from asymptomatic plants has made it possible to obtain yields equivalent to those of resistant varieties [14,15,16]. The impact of CMDon the yield is more than 70% for plants originating from infected cuttings while it is only 10% on initially healthy plants when the infection occurs 120 days after planting [17]. Studies carried out in Ivory Coast have shown that plants contaminated by cuttings had a yield loss of over 75%. On the other hand, the plants that get inoculated with CMBs by the whitefly had had a less important loss, which decreased as the inoculation time increased until no longer having an effect if the inoculation took place 70 days after planting [14]. It is therefore necessary to limit the damage caused by theCMDin cassava fields. One way to control mosaic disease is in vitro culture. During the last 30 years, in vitro culture techniques have developed widely and have been applied to more than 1000 different species [18,19]. These tissue culture techniques are of great interest on the one hand for the collection, multiplication and conservation of genetic material [14]; on the other hand for the production of healthy planting material. Tissue culture systems allow plant material to be propagated with high multiplication rates in an aseptic environment. In vitro culture combined with heat therapy has been effective in previous studies because heat therapy eliminates the mosaic virus [20,21]. According to Zinga, the treatment of cassava cuttings, infected

with CMBs at 49 ° C for 30 minutes in a water bath, generates 43% of the plants free from symptoms of the disease one month after planting with a harvest yield equivalent to that of the initially healthy plants [10].

A recent survey carried out in the Central African Republic shows that 90% of consumers have culinary preferences for leaves with mosaic symptoms (data not yet published). This attitude is worrying, because it could favor the spread of the disease. This further supports the need for this work. This work combines the technique of thermotherapy with that of tissue culture in order to sanitize plant material to serve as seeds for producers in order to improve cassava production in the Central African Republic. It was carried out in several stages: - Transplanting of the infected cuttings was carried out in a chamber under the heat of 37°C to 40°C for two weeks - Tissue culture was performed for the production of vitro plants. - The vitro plants were weaned and placed in the greenhouse for 4 months for their development. - The leaves of vitro plants were analyzed by PCR to check their phytosanitary state.

2. MATERIALS AND METHODS

2.1 Experimental Place

The study was carried out at the Laboratory of Biological and Agronomic Sciences for Development at the University of Bangui, Central African Republic from December 2017 to June 2018.

2.2 Plant Material

Cassava cuttings of the Six months variety, very susceptible to cassava mosaic disease, were used as plant material. It is a local variety appreciated by the rural population because of its early maturing despite its susceptibility to mosaic disease.

2.3 Culture Media for the Production of Vitro Plants

Show in Table 1.

2.4 Sampling

200 Cuttings from a local "six-month" cassava cultivar very susceptible to mosaic disease but much preferred by cassava growers in the Central African Republic are used as material. The cuttings were taken from 12-month-old cassava stems whose leaves showed mosaic symptoms with an average severity of 3.5 on the IITA scale. These cuttings were collected in Pissa, 74 km from Bangui and transferred to the Laboratory of Biological and Agronomic Sciences for Development (LASBAD) to be sanitized.

2.5 Heat Therapy Treatment

25 cm cuttings (200 cuttings) were soaked in 10% bleach solution for 10 min, then rinsed with tap water, then transplanted into black polyethylene bags containing sterilized soil. The transplanted cuttings were taken to the thermotherapy room to be placed in culture trays, they were watered every 48 h. The temperature in this chamber fluctuated between 37 °C to 40 ° C during the day. The photo periodicity was 16/8. These plants were kept under these conditions for two weeks before being subjected to experimental testing (Fig. 1A).

2.6 Preparation of Culture Media

Murashige and Skoog's medium (4.4g) were used to perform this work. The volume of the solution was 1 liter to which were added 100 mg of myoinositol, 30 g of Sucrose, 1 mg of thiamine, 1.5 mg of Pyridoxine. And phytohormones such as 0.02 mg of NAA; 0.05 mg of BAP; 0.02 mg GA3 (Table 1). The pH of the medium was adjusted to 5.7 (METROHM pH meter, MettlerToledo AG. 8603 Schwerzenbach. China) using 0.1N HCl or 1N NaOH solution. 3.5 g of phytagel was added to the Murashige solution described below. The resulting mixture was placed in a microwave for homogenization for 4 min. The solution taken out of the microwave was mixed with the rest of the solution in the Erlenmeyer flask on the magnetic stirrer. The solution resulting from the mixture was poured into test tubes and sterilized in an autoclave (SULZER autoclave, manufactured TRADE, Raypa . R. Espinar S.L. Espana) at 121 ° C for 30 min. After sterilization, the media was cooled and stored in a refrigerator for use in inoculation.

2.7 Culture of Meristems and Stem Fragments

One day after the preparation of the culture media, the explants taken from the tips of the cassava stems were washed with tap water for 5 min, then they were transferred to the laminar flow hood. Bleach (10%) (fresh chorox Bleach. Fresh Organic Herbal (FOH), Douala) was added to the explants introduced into jars, the whole was stirred for 5 min. The explants were then placed in 70% alcohol for 5 minutes while stirring

slowly (Bleach and ethanol are used to disinfect plant material). Finally, they were rinsed with sterile water 3 times for 5 minutes while vigorously shaking the jars to completely get rid of the reagents used (Fig. 1B). In this study, we used two cultivation methods: seeding from meristems and seeding from stem fragments. These explants were removed using a scalpel blade under a horizontal laminar flame hood and placed in the culture medium or initiation medium. The meristems were collected using a stereoscopic microscope to rid the leaf envelope of blanks. On the other hand, the stem fragments were taken in such a way that each fragment comprises a node. The seeded tissues were transferred to the acclimatization room at a temperature of 18 ° C and a light illumination of 3000 Lux with a photo periodicity of 16 H / 8.

2.8 Micro Propagation

After three weeks, the shoots were extracted from the tubes under the hood and cut into micro-cuttings and then subcultured onto a proliferation medium. The multiplication of micro-plants was carried out for four (4) months, systematically removing the apical bud, as well as the base nodes showing the yellowed leaves (Fig. 1C).

2.9 Acclimatization of Vitro Plants

After multiplication, vitro plants were weaned according to the OTABO protocol, [25]. The vitro plants having at least 3 leaves, 3 roots and a size of 5 cm were chosen to carry out this operation. The black soil was used as a subtract and was autoclaved for 30 min at 160°C. The microplants extracted from the tubes were rinsed with water to remove the agar and then they were introduced directly into the substrate in the weaning tank previously covered with two plastic films. The microplants were put into regeneration in a mini-greenhouse. One week after weaning, the first plastic film was removed and two weeks after the second plastic film and removed (Fig. 1E). The microplants were then removed and immediately transplanted into a new substrate for hardening (Fig. 1F).

2.10 Detection of Mosaic Viruses in Weaned Cassava Plants

To check the phytosanitary state of these microplants, samples of the leaves were taken for analysis by PCR. Samples were collected from the leaves of microplants from stem fragments and also from those of microplants from meristems. All samples were screened against positive control samples.

Total DNA was extractedusing the Qiagen Kit. In this study degenerate primers were used to detect begomoviruses in the material analyzed [26]. The reaction mixture was carried out under the following conditions: 14.87 µl water; 2.5 µl 10X PCR buffer; 2µl of dNTPs; 1.5µl MgCl2; 1µl primer1; 1 µl primer2 and 0.13 µl of Taq polymerase. DNA extract (2 µl) was then added to the reaction mixture to carry out the amplification of the viral genome, which gave a total volume of 25 µl. The primer sequencies are provided in Table 2.

Table 1. The components of culture media and their concentration

Media components	Con. / Initiation medium	Con. / Proliferation medium
Naphthalene acetic acid (NAA)	0,02mg/ml	0
Benzalaminopurin (BAP)	0,05mg/ml	0
CuSO4	1ml/l	0
Muruschige and Shoog (MS)	4,4g/l	4,4g/l
Sucrose	30g/l	30g/l
Phytagel	3,5g/l	3,5g/l
Myoinositol	100mg/l	100mg/l
Thiamine	1mg/l	0
Pyridoxine	1,5mg/l	0
Nicotinic acid	1,5mg/l	0
Glycine	2mg/l	0
Gibberellic acid (GA3)	0,02mg/ml	0

Source: Ng [22]; Mabanza et Jonard [23]; Murashige et Skoog [24]

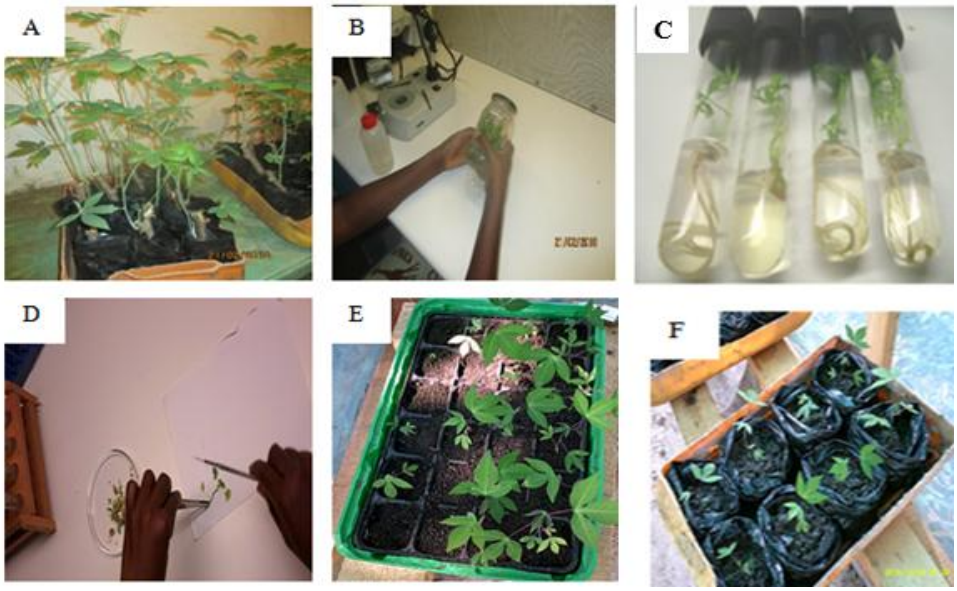

Fig. 1. Stages of in vitro culture and weaning

A: Regeneration of cassava cuttings in the thermotherapy room. B: Sanitation of the explants under the hood. C: Production of tissue culture plants. D: Multiplication of in vitro plants. E: Development of vitro plants two weeks after weaning. F: Curing of in vitro plants.

Table 2. Primer sequences used to detect CMD begomovirus in cassava leaves samples

Specificity	Names/sequences of primers	Expected size of amplicon (bp)
CMD virus	VD1863/TCRTCAATGACGTTGTACCA C12D2391/TTTCCAYCCVAACATTCARGG	520

2.11 Amplification Program of the Viral DNA by the PCR with Universal Primers

The amplification of viral DNA was carried out according to the following program : denaturation at 94 °C for 5 min; followed by 30 cycles of : Denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 1 min and a last elongation at 72 °C for 7 min.

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2.13 Revelation of PCR Amplifiers Amplified

DNA sequences were revealed by 1% agarose gel electrophoresis stained with ethidium bromide under UV illumination. Preparation of the gel Agarose (1 g) was dissolved in 100 ml of TAE 1x. The solution was homogenized by boiling using a microwave for few minutes. The solution was cooled by running cold water over the walls of the erlenmeyer. Ethidium bromide (4 µl) was deposited on the gel after cooling at 65 °C. The gel is then poured into a tray and a comb is placed at one end to delineate wells in the gel. The agarose was left for polymerization at ambient temperature for 30 min. The TAE 1x buffer was then added to the electrophoresis tank until the gel was completely immersed.

2.14 Gel Loading and Electrophoretic Migration

Two microliters of loading buffer (blue color) were mixed with 10 µl of PCR products. A volume of 10 µl of the mixture obtained was deposited in a well which has been delimited by the comb. Ten microliters of molecular weight marker were deposited in the first and last wells. The electrophoretic migration was then carried out under a constant electric potential of 100 volts for 1 h. The gel was deposited in a BET solution for 15 min and rinsed by immersing in

water for 1h before the revelation of PCR products under UV illumination.

2.15 Parameters Studied and Data Analysis

The parameters studied are the effect of thermotherapy on the mosaic, the rate of regeneration of the microplants after weaning, the expression of the mosaic on the microplants and the phytosanitary evaluation of the microplants. The data collected was entered on the Excel version 2010 spreadsheet and analyzed by the XLSTAT software. Student's t-test was used to determine the effect of heat therapy on the incidence of mosaic disease and the success of vitro plants at acclimatization.

3. RESULTS

3.1 Cassava Mosaic Disease Incidence

The cuttings taken for sanitation were all diseased (100%) with an average severity of 3.5, aaccording to visual observation based on the IITA scale. After thermotherapy treatment of these cuttings 65%were free of mosaic symptoms. Thermotherapy reduced the incidence highly significantly (p< 0.01%).
In this study, it should be noted that out of 240 weaned vitroplants, 180 regenerated, i.e. 75% success rate with a very significant difference of p<0.05%.

3.2 Acclimatisation Success Rate of In vitro Plants

In this study, it should be noted that out of 240 weaned vitroplants, 180 regenerated, i.e. 75% success rate with a very significant difference of p<0.05%.

3.3 Mosaic Expression on Microplants

After four (4) months of curing the microplants in the greenhouse, no symptoms were recorded on all the plants.

3.4 Detection of Begomoviruses

Testing for virus strains in leaf samples from acclimatised glasshouse plants showed positive bands with the universal primers on all samples from stem fragments (samples 1, 2, 3, 5, 8, 9) and controls, while no viral DNA bands were observed on samples from meristems (samples 4, 6, 7) (Fig. 2).

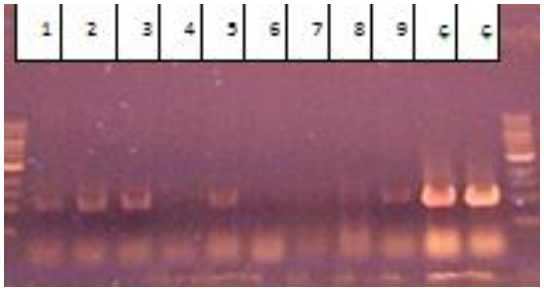

Fig. 2. Detection of begomoviruses in tissue culture plant leaf samples
Samples 1, 2, 3, 5, 8, 9 are from axillary buds (explants) Samples 4, 6, 7 are meristem samples C: control. Size of the ladder: 1kb

The PCR results show 100% of tissue culture plants from meristems (75 samples) are free from the virus, whereas those from axillary buds (72 samples) although being asymptomatic carried 92% of the viral particles. What shows that the best plant material for sanitizing infected cassava plant material is the meristem.

4. DISCUSSION

The use of stem cuttings of poor sanitary quality could be explained both by the unavailability of healthy material and by the farmers' lack of knowledge about CMD. A recent study showed that the incidence of CMD was 85% and that 79% of cuttings used by growers were infected with mosaic [11]. In all cases, our results emphasized the need to put in place a control strategy based on both the use of resistant varieties and the sanitation of plant material. This work showed that thermotherapy had a significant effect on the incidence and severity of mosaic disease [20,21,10], when the cuttings used were taken from cassava plants infected with mosaic. We found that after heat treatment, most cassava plants did not show symptoms of mosaic disease. This could explain why heat therapy has played a very important role in the elimination of cassava mosaic disease. These results corroborate with previous studies on thermotherapy which have shown that it has this capacity to inhibit the multiplication of the virus during its application in many plants [27,20,28,29,30,31,21,12]. The regeneration of the explants evaluated after one week of culture in the acclimatization room showed that 48% of the meristems and 50% of the stem fragments had recovered. Infections due to microorganisms were also noted with losses of 52% and 50% respectively for meristems and stem fragments.

In this study it was found that the infection rate is high, this could be due to the use of contaminated materials. It is therefore necessary to use sterile materials. Some studies have shown that the surface disinfection usually done during in vitro culture does not effectively eliminate infections, especially bacterial infections which are endogenous and appear gradually during culture [32,33,34,35]. Thus, antimicrobial agents (bactericides and / or fungicides) are sometimes added to the culture medium in order to eliminate germs or prevent their expression [36,35]. During this study, weaning took into account several parameters: the size of the tissue culture plants, the number of leaves, the number of roots, the number of days after in vitro multiplication and the physical aspect of the tissue plant to avoid losses. This has led to a significant reduction in the number of tissue culture plants ready for weaning. This work gave a higher success rate of tissue culture plants at weaning than loss with a significant difference. The number of leaves of each weaned in vitro plant increased after two weeks. This is consistent with the work done by Otabo and Mabanza in 1996 on weaning and transfer of tissue culture plants to the field. At the end of weaning, no mosaic symptoms were observed on the leaves of in vitro plants, this does not justify the absence of viruses in the microplants, the virus can be expressed directly when the microplants are transferred to the field. As Féreol has suggested, however, it is far from asserting that tissue culture plants are free from the virus because in in vitro culture the symptoms can be masked [20]. The viral load may be low, which may not allow the disease to express itself. It is therefore necessary to use molecular techniques to verify the phytosanitary status of these microplants before confirming that they are healthy. The study showed that all samples from the meristem were negative while samples from the stems were positive with faint bands compared to controls, supporting the low viral load hypothesis. OTABO team has proven that growing meristems is the most effective way to sanitize cuttings infected with cassava mosaic. Early studies confirmed this finding by indicating that heat therapy inhibits replications as virus degradation continues, resulting in subsequent virus elimination from meristems [27]. Another hypothesis, it can be thought that the speed of growth of seedlings under heat is greater than the speed of upward migration of the virus. This means that the meristems have remained free from the virus unlike the explants. Other study on diseased cassava cuttings were treated in a heated water bath at temperatures ranging from 43ºC to 51ºC for 30 min before being grown for 12 months in the field. The data clearly demonstrate, the highest yield was obtained from plants grown from diseased cuttings treated at 49ºC (4.7kg / plant), a yield statistically similar to that of untreated symptomless cuttings (4.6kg / plant). Although plants grown from diseased cuttings treated at 47ºC, 49ºC and 51ºC had a similar CMD incidence at one MAP (61%, 59% and 57%, respectively), the diseased cuttings treated at 49ºC showed significantly lower CMD incidence between two and five MAP compared to plants in the two other treatments [12].

5. CONCLUSION

Sanitation of the plant material by culture of the meristem combined with heat therapy produced apparently healthy tissue culture plants in which no viral DNA were detected and no disease symptoms were observed after weaning. PCR testing for viruses in leaf samples confirmed the complete absence of viruses on samples from meristems unlike those from stem fragments. It is therefore important to check the health status of tissue culture plants using molecular techniques before disseminating cuttings from tissue culture plants to producers. This control strategy is very useful in the Central African context where the best culinary varieties are sensitive to mosaic and the majority of producers use the contaminated cuttings for replanting. Appropriation of these techniques by agricultural extension services is essential to help producers have healthy cuttings to improve cassava yields.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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