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Research Paper

In vitro meristem-tip culture and regeneration approaches in Congolese cassava accessions (*Manihot esculenta* Crantz cv. Boma and cv. Mpelo-Nlongi)

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Abstract

Shiny dome-like structures measuring less than 1mm in length were excised aseptically from shoot tip buds of infected of two cassava (*Manihot esculenta* Crantz) local cultivars (Boma and Mpelo Nlongi) and cultivated *in vitro* in two types of media with different combination of growth hormone: Murashige and Skoog supplemented of sucrose (20 g/l), Naphtalenacetic acid (NAA, 10 µM), Benzylaminopurine (BAP, 0.66 µM) as well as Gibberellic acid (GA3, 0.1 µM) with 80 mg/l of Adenine sulphate and MS-free growth regulators. After four weeks, data were scored: 29.5% responding explant with callus formation and 20.5% responding explants to shoot development in the medium with growth regulators for the cultivar Boma whereas the cultivar Mpelo-Nlongi presented 5.7% and 25.7% respectively of callus formation and shoot development. The cultivar Boma presented a tendency more pronounced for the callus formation rather than with the shoot development contrary to the cultivar Mpelo-Nlongi. In regards of this experiment, it was shown that the media composition and genotype are essential factors, which influence in vitro growth, mainly the shoot development, in the culture of meristems for cassava local accessions.

Keywords: *Vitroplant; Phytohormone; Mpelo-Nlongi; Boma; Callus; Naphtalenacetic Acid; Benzylaminopurine; Gibberellic Acid.*

1. Introduction

Cassava (*Manihot esculenta* Crantz) remains the principal agricultural crop and the most significant food and energy resource in several tropical areas of the world (Soyode & Oyetunji 2009), in fact, in Central Kongo Province, South-west part of Democratic Republic of the Congo. Although it can adapt to a broad range of ecological conditions and its capability to tolerate long drought periods, the cassava has an unlimited growth, and its tuberoses roots can be left in the ground during several months until the need for their consumption or their transformation could be felt (Tresh & Cooter 2005). Considering the yield loss mainly caused by the African Cassava Viral Mosaic Disease (CMVD) and the Cassava Brown Streak Viral Disease (CBSVD), several programs diffused some varieties resistant or tolerant of these diseases in the region. These programs obtained a great success and had like negotiable instrument control of these pandemics and the maintenance of their severity on a moderate level.

However, certain lately introduced varieties characteristics (bitter savour, bad leaves or tuberoses roots texture and flavour, weak tubers conservation in field, early flowering,…) led many farmers to return to the local varieties culture, however sensitive to CMVD (Kawuki et al. 2011). Facing this new challenge, Mallowa et al. (2006, 2011) support that it is necessary to implement other alternative methods of the CMVD control by using local varieties available in agricultural areas post-epidemic situation.

The use of healthy vegetable material and the adequate implementation of the phytosanitation techniques can lead to the maintenance of these pandemics on a moderated level so that it cannot cause considerable losses any more. Indeed, Bock (1988) noted a weak rate of recontamination by the African Cassava Mosaic Viral Disease (CMVD) in the lots without mosaic and affirmed that, in Kenya, the disease could have been effectively controlled by using local vegetable material free of mosaic. Thus, the techniques of Biotechnology, namely the apical meristems culture associated with thermotherapy, can be used in the elimination of the viruses and the regeneration of the plantlets on which the viruses were excluded (Kartha and Gamborg 1975; Thresh and Cooter 2005; Wasswa et al. 2010). Indeed, the apical meristems of the infected plants are generally either free of viruses, or they only contain them in very small quantities. The reasons of this phenomenon can be charged, according to Manosh et al. (2007) and Wasswa et al. (2010), to the fact that : (i) certain viruses circulate more easily in the plant through the vascular system which lack in the meristems, (ii) a great metabolic activity in the meristematic cells in intense splitting does not allow the viruses replication because of the competition and (iii) a high level of endogenous auxins in the stems apexes can also inhibit the viruses replication.

This study aims to obtain cassava vitroplants free viruses from infected plants materials of two cassava local varieties (sensitive to the CMVD) by the culture of their apical meristems.

2. Materials and methods

2.1. Plant materials

Cuttings of two cassava local cultivars (Boma and Mpelo-Nlongi) sensitive to CMVD had been selected in Nkamba village (5°27'37.9'' Lat.S and 14°53,5'0''Long.E), close to M'vuazi INERA Research Centre (Democratic Republic of the Congo). The plants from which these cuttings had been collected presented a CMVD severity score ranging between 2 and 3 (Bansimba Mukiese 2015). These cuttings were then planted by constituting a flower-bed at the Regional Nuclear Research Centre of Kinshasa (CREN-K) (geographical references: 4°24'58.90'' Lat.S and 15°18'32.14'' Long. E) during the rainy season from Octobre 2008.

2.2. Culture media preparation

Two types of solid media with different composition were prepared for this experiment. The first culture medium contained a basic medium of Murashige and Skoog of salts and vitamins supplemented of sucrose (20 $g.l^{-1}$) to which were added growth regulators: Naphtalenacetic acid (NAA, 10 µM), Benzylaminopurine (BAP, 0.66 µM) as well as Gibberellic acid (GA_3, 0.1 µM). With the precondition, 80 mg of Adenine sulphate had been added to 1 liter of the solution, and the pH of the culture medium had been adjusted to 5,7 by addition of some drops of NaOH 0.1N. 7g of Agar-agar was mixed for one liter of solution. The prepared medium set out again in various pyrex test tubes (25 mm diameter, 10 cm long) at a rate of five ml per tube. The tubes containing the culture medium had been then closed using a plastic cap and autoclaved during 15 minutes at 121°C under a pressure of 2 atm. The second culture medium without phytohormones was prepared in the same conditions (Table 1).

Table 1: Experimental Plots Arrangement of Cassava (*Manihot esculenta* Crantz) Meristem-Tip According To the Cultivar and the Culture Medium. Data are expressed in the form of N (%).

Presence of Phytohormones	Cultivar		Total
	Boma	Mpelo-Nlongi	
With Phytohormones	78 (78.0)	35 (67.3)	113 (74.3)
Without Phytohormones	22 (22.0)	17 (32.7)	39 (25.7)
Total	100 (100.0)	52 (100.0)	152 (100.0)

2.3. Meristems extraction and culture conditions

Buds stem apexes (± 3cm) of each infected accession had been extracted and dipped immediately in sterile distilled water. The apexes had been disinfected in an alcohol solution (70%) during 5 minutes, then in a mercuric chloride solution ($HgCl_2$ 0.125%) supplemented with a drop of tween 20 during 3 minutes and finally rinsed five times in a sterile distilled water .

The buds stem apexes had been placed on a sterilized filter paper and dissected under sterile conditions using binocular microscope under the laminar hood flow bench. The shiny dome apical meristem-tip was extracted (0.3mm< t < 0.5mm) and immediately introduced into test tubes containing the culture medium. The cultures were then incubated at 26°C under a light of 2000 lux and a 16 hours photoperiod by a relative humidity of 70%.

2.4. Data analysis

The considered parameters were the presence of the growth regulators and the type of medium used. The variables of study taken into account were the % of callus formation (Callogenesis) and the % of the stem development (Caulogenesis). An independence Chi-square test had been used in order to evaluate their degree of relationship. In all the cases, the analysis had been carried out using SPSS Statistics 17 software.

3. Results

The presence of growth regulators in the culture media strongly influenced the cassava meristems responses. In table 2, one can note that 50% of Boma cultivar placed in medium with growth regulators presented a real initiation meristem growth. Among them, 29.5% presented a simple callogenesis whereas 20.5% of them evolved to plantlet (caulogenesis). In the medium without growth regulators 77.3% of the cultures underwent a development and, among them, we noticed 59.1% of simple tip swelling and the remainder (18.2%), stopped at the simple stage of callus development.

The situation appears rather reversed for Mpelo-Nlongi cultivar at which the caulogenesis tendency was much more marked as well in the medium with growth regulators as in the free hormone medium (Table 3).

Table 2: Effect of Growth Regulators on the Development of Cassava (*Manihot esculenta* Crantz cv. Boma) Meristems after 30 Days of Culture. The Results are expressed in the Form of N (%)

Development level	Presence of Phytohormones		Total
	With	Without	
None :	39 (50.0)	5 (22.7)	44 (44.0)
Simple tip swelling :	0 (0.0)	13 (59.1)	13 (13.0)
Callus formation :	23 (29.5)	4 (18.2)	27 (27.0)
Plantlet :	16 (20.5)	0 (0.0)	16 (16.0)
Total	78 (100.0)	22 (100.0)	100 (100.0)

Source: Computed on the basis of laboratory data, 2008. The dependence is highly significant $\chi^2 = 54.317^{**}$ (DF=3); 1-p ≥ 99%

Table 3: Effect of Growth Regulators on the Development of Cassava (*Manihot esculenta* Crantz cv. Mpelo-Nlongi) Meristems at 30 Days Of Incubation. The Results are expressed in the Form of N (%).

Development level	Presence of Phytohormones		Total
	With	Without	
None :	17 (48.6)	0 (0.0)	17 (32.7)
Simple tip swelling :	7 (20.0)	10 (58.8)	17 (32.7)
Callus formation :	2 (5.7)	1 (5.9)	3 (5.8)
Plantlet :	9 (25.7)	6 (35.3)	15 (28.8)
Total	35 (100.0)	17 (100.0)	52 (100.0)

Source: Computed on the basis of laboratory data, 2008. The dependence is highly significant $\chi^2 = 13.897^{**}$ (DF=3); 1-p ≥ 99%

Table 4: Effect of Type of Accession on the Development of *Manihot esculenta* Crantz cv. Boma and cv. Mpelo-Nlongi Meristems at 30 Days of Culture. The Results are expressed in the form of N (%).

Development level	Cultivar		Total
	Boma	Mpelo-Nlongi	
None :	44 (44.0)	17 (32.7)	61 (40.1)
Simple tip swelling :	13 (13.0)	17 (32.7)	30 (19.7)
Callus formation :	27 (27.0)	3 (5.8)	30 (19.7)
Plantlet :	16 (16.0)	15 (28.8)	31 (20.4)
Total	100 (100.0)	52 (100.0)	152 (100.0)

Source: Computed on the basis of laboratory data, 2008. The dependence is highly significant $\chi^2 = 18.393^{**}$ (DF=3); 1-p ≥ 99%.

Among the cultivars studied, one can also note a difference in behaviour in the culture medium. Indeed, an aptitude of callus induction was much more significant at the cultivar Boma compared to the cultivar Mpelo-Nlongi which presented, on the contrary, a more noticed capacity of shoot development (Table 4).

From this analysis, it is advisable to retain that the hormonal composition of the culture media and the genotype are essential factors which influence the *in vitro* growth, mainly the caulogenesis, in meristem-tip culture for local cultivars of Cassava.

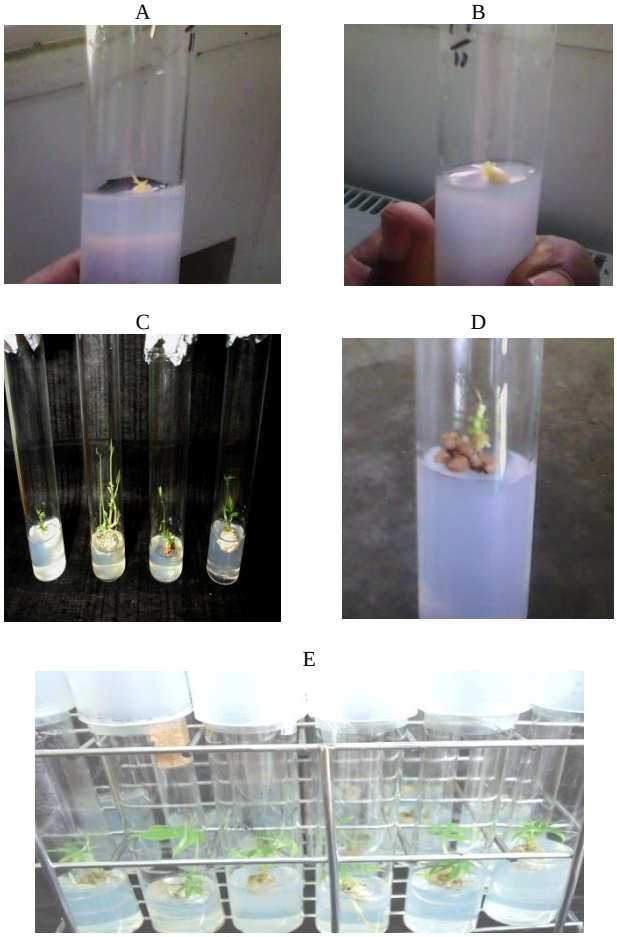

Fig. 1: Various Aspects of Cassava Development in Meristem-Tip Culture. A. Simple Tip Swelling Initiation; B. Callogenesis Initiation Step after 7 Days of Culture; C.Whitenish Calli and Shoots Developments; D. Brownish Calli and Small Shoot Development; E. *In vitro* Plantlets Development.

Moreover, we were interested to know if the addition of the Phytohormones could have an effect on the color and the size of the callus from which the plantlets developed. Table 5 shows that the presence of Phytohormones in the culture medium have no effect on the callus color. On the other hand, through Table 6, one can see that the presence of the Phytohormones had a very significant effect to induce callus of bigger size, independently to induction of the caulogenesis.

Table 5: Effect of Phytohormones on the Callus Color in the Culture of Meristem-Tip of Cassava (*Manihot esculenta* Crantz cv. Boma and cv. Mpelo-Nlongi) at 30 Days of Incubation. The Results are expressed in the form of N (%).

	Presence of Phytohormones		
Callus color	With	Without	Total
Yellow	36 (72.0)	10 (90.9)	46 (75.4)
Brown	14 (28.0)	1 (9.1)	15 (24.6)
Total	50 (100.0)	11 (100.0)	61 (100.0)

Source: Computed on the basis of laboratory data, 2008. The dependence is not significant $\chi^2 = 1.739^{NS}$ (DF=1); 1-p ≤ 95%.

Table 6: Effect of Growth Regulators on the Callus Size in the Culture of Meristem-Tip of Cassava (*Manihot esculenta* Crantz cv. Boma and cv. Mpelo-Nlongi) at 30 Days of Incubation. The Results are expressed in the Form of N (%). Callus Size: (+): ≤ 0.3cm; (++): (0.4 – 0.7) Cm and (+++): ≥ 0.8 Cm.

	Presence of Phytohormones		
Callus size	With	Without	Total
+	20(40,0)	10(90,9)	30(49.2)
++	16(32.0)	1(9.1)	17(27.9)
+++	14(28.0)	0(0.0)	14(23.0)
Total	50(100.0)	11(100.0)	61(100.0)

Source: Computed on the basis of laboratory data, 2008. The dependence is highly significant. $\chi^2 = 9{,}529^{**}$ (DL=2); 1-p ≥ 99%

4. Discussion

This study about Cassava regeneration by meristem-tip culture was undertaken while varying two essential factors: the culture medium composition and the accession. The results were evaluated by analyzing some variables such as the rates of the callogenesis and the caulogenesis responses, the color and the size of calli. With regard to the callogenesis, the simultaneous presence of the auxins and cytokinins had a very significant effect on the size of the callus like underlined by Nitsch & Lance-Nougarede (1967) on the tobacco.

As for caulogenesis, the variety as well as the hormonal composition of the culture medium also seemed to play a considerable role. After one month of culture, shoot developed and roots appeared after of forty five on culture media (Figure 1E). Indeed, Kartha et al. (1981) as well as Malaurie et al. (1995) had also noted a simple formation of the stem in the culture of the cassava meristems in the presence of BA (6-benzyladenine) and GA_3.

In caulogenesis, the genotype factor is essential and determining. The results of our study made it possible to highlight a great genotypic variability with respect to the caulogenesis. The best aptitudes to caulogenesis are expressed by the cultivar Mpelo-Nlongi with a plantlet rate convertion of 28.8% per comparison with a rate of 16% at the cultivar Boma. The genotype factor was also reported by other authors in the meristem culture, namely Malaurie et al. (1995) for the yam.

The strong presence of cytokinines in the culture medium surely had a stimulative effect on the caullogenesis (Figure 1C). And the balance of auxin/Cytokinin had a big influence in the callogenesis (Figure 1C) Although the unique culture medium used, recommended by the IITA(IITA,1990), is favourable in the development of the shoots and roots for the meristem-tip culture of cassava varieties (Figure 1E). Through our investigation, the above mentioned medium can be also recommended for all cassava accession in Africa.

5. Conclusion

This study enabled us to achieve the goal to obtain vitroplants starting from cassava stems of some local varieties sensitive to the CMVD by the culture of the apical meristems. It represents a contribution of our share, for the regeneration of whole plants at the local cultivars of cassava in order to obtain healthy and free from virus plants.

However, taking into account the very restricted number of the local cultivars subjected to the experiment, the widening of the experiments to others cultivars thus proves necessary. This work thus deserves to be continued for better controlling *in vitro* regeneration at the local cultivars of cassava by the culture of meristem-tips.

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